General Information

GENERAL INFORMATION

The General Information describes reference information and reference test methods necessary to assure the quality of medicines, which is attached to the JP. Therefore, the General Information is positioned as important information supplementing the JP although it should not be taken as indicating standards for conformity of drugs, except in the case specified when the drugs are granted approval based on the Law on Securing Quality, Efficacy and Safety of Products Including Pharmaceuticals and Medical Devices. Combination use of the General Information and the JP can contribute to improve quality of the JP and user's convenience.

The general information is classified into the following categories according to their contents, and each general information is individually numbered.

G0. Basic Concepts on Pharmaceutical Quality
G1. Physics and Chemistry
G2. Solid-state Properties
G3. Biotechnological/Biological Products
G4. Microorganisms
G5. Crude Drugs
G6. Drug Formulation
G7. Containers and Package
G8. Reference Standards
GZ. Others

The salient points of the revision in this volume are as follows:

1. Each general information was individually numbered according to the following rule.

An individual number consists of three blocks. The left block indicates the category number and the central block indicates the number in the category. The figures in right block consist of the first two digits from the left indicating the JP at the recent revision (or new preparation, if not revised) and the third digit indicating as follows: 0 for major revision, 1 for supplement I, 2 for supplement II, and 3 for partial revision. For citation between the general information, the number corresponding to the general information is indicated in angle brackets < >.

2. Categories were reviewed.

(1) As basic concepts on pharmaceutical quality, ''G0 Basic Concepts on Pharmaceutical Quality'' was newly added at the opening.

(2) ''Others'' was located at the end as ''GZ'', considering that any new categories would possibly be added after ''G9''.

(3) Water-related categories were abolished and included in ''GZ Others''.

3. The following were newly prepared.

(1) Basic Concept of the Quality Assurance on Biotechnological Products (Biopharmaceuticals) <*G3-1-180*>

(2) Control of Culture Media and Strains of Microorganisms Used for Microbial Tests <*G4-2-180*>

(3) Bacterial Endotoxins Test and Alternative Methods using Recombinant Protein-reagents for Endotoxin Assay <*G4-4-180*>

(4) Radioactivity Measurements Method for Crude Drugs <*G5-8-180*>

(5) Tablet Hardness Determinations <*G6-4-180*>

(6) Packaging Integrity Evaluation of Sterile Products <*G7-4-180*>

(7) Leak Tests for Packaging of Sterile Products <*G7-5-180*>

4. The following were revised.

(1) Capillary Electrophoresis <*G3-7-180*>

(2) On the Scientific Names of Crude Drugs listed in the JP <*G5-1-180*>

(3) International Harmonization Implemented in the Japanese Pharmacopoeia Eighteenth Edition <*GZ-3-180*>

5. The following was deleted.

(1) Control of Elemental Impurities in Drug Products

G0 Basic Concepts on Pharmaceutical Quality

Basic Concepts for Quality Assurance of Drug Substances and Drug Products <*G0-1-172*>

Introduction

Quality of drug substances and products are generally assured through manufacturing and testing under appropriate Good Manufacturing Practice (GMP) conditions reflecting knowledge obtained from designing and developmental stages and manufacturing stage on management of raw materials and other materials, control of manufacturing process, specifications, etc. As shown in the General Notice 5, JP listed drugs are to be tested according to the provisions given in the pertinent monographs, General Notices, General Rules for Crude Drugs, General Rules for Preparations, and General Tests for their conformity to the *Japanese Pharmacopoeia*. In addition to these, compliance with GMP, management of raw materials and other materials, and management of manufacturing process are fundamental factors required to assure the quality of JP listed products in actual production.

The present chapter summarizes general concepts concerning measures for quality assurance of drug substances and products mainly aimed at chemicals, including chemically synthesized antibiotics and semisynthetic antibiotics, synthetic peptides, oligonucleotides, and biotechnological/biological products, and shows the principle idea of quality assurance in the process listing a drug as an individual monograph in the JP. Although radiopharmaceuticals, crude drugs, herbal products, and crude products of animal or plant origin are excluded from the subjects of the concepts, these concepts are useful for the management of any type of drugs.

Basic Concept

In recent years, the mainstream concept for quality control of drugs has been implemented according to a control strategy that their quality is assured by control of manufac-

turing process, including management of raw material and other materials, and quality testing of final products (drug substances or drug products) that are conducted mutually complementary. The control strategy is implemented based on Quality Risk Management (QRM). The first and most important step is identifying Critical Quality Attributes (CQAs) which are the attributes or properties required to ensure the desired product quality, and it is necessary to specify physical, chemical, biological, microbiological characteristics or properties of the product which should be within the appropriate limits, ranges and distributions. The next step is to guarantee that the CQA falls within the defined range, limit, and distribution by using specification tests, in-process tests and various measures, for that the quality of the drug will eventually be realized.

The specification is one of the elements of control strategy and not all the CQA need to be included in the specifications. CQA is (1) included in specifications and confirmed by testing final products (including periodical or skip testing, described later), (2) included in specifications and confirmed by process controls (e.g., real time release testing, described later), or (3) not included in specifications but can be ensured by controlling starting materials, raw materials and manufacturing process. As an example of (3), effective control over robust manufacturing processes can assure that certain impurities are controlled at an acceptable risk level or are efficiently removed below an acceptable level, and sometimes the purity testing for the final product may not be required and omitted from specifications. However, in the case of a drug listed in the JP monograph, regarding the manufacturing process control related to CQA, if necessary, the control method and control value are indicated in the Manufacture in individual monograph.

What kind of control strategy should be applied to a certain CQA is individually determined by QRM according to the understanding and risk of the manufacturing process.

1. Management of manufacturing process

1.1. Considerations of manufacturing process

Adequate design of manufacturing processes and knowledge of their capacity are important to establish manufacturing processes yielding drug substances or drug products that meet specifications and fulfill CQA, and to perform consistent manufacturing control, quality control, etc. appropriately.

From this standpoint, the limits for control of manufacturing processes should be based on information obtained from the entire process spanning the period from the early development through commercial scale production. The appropriateness of the limits also needs to be confirmed by evaluation, verification, review, and other examinations of manufacturing processes based on QRM.

In-process tests are tests that may be performed during the manufacture of either the drug substance or drug product, rather than specification tests for the final product. In-process tests are performed for quality verification during manufacturing processes that are likely to influence drug substance or drug product quality, or for confirmation of proper functioning of the manufacturing process. In-process tests may also be used for the evaluation of CQA.

Usually an in-process test is properly designed according to the risk on quality, however, the use of internal action limits by the manufacturer to assess the consistency of the process at less critical steps is also important. Provisional action limits should be set for the manufacturing process based on data obtained during development of the drug and during evaluation and verification of the manufacturing process, and should be further refined based on additional manufacturing experience and data accumulated after product approval for marketing.

1.2. Considerations of raw materials and other materials (starting materials, excipients, packaging materials, etc.)

The raw materials and other materials used in the production of drug substances (or drug products) should meet quality standards, appropriate for their intended use, and appropriate setting of specifications and test methods assuring CQA are required. Especially, biological raw/source materials may require careful evaluation to establish the presence or the absence of deleterious endogenous or adventitious agents. Procedures that make use of affinity chromatography (for example, employing monoclonal antibodies), should be accompanied by appropriate risk management to ensure that such process-related impurities or potential contaminants arising from their production and use do not compromise the quality and safety of the drug substance or drug product.

The quality of the excipients used in the drug product formulation (and in some cases, in the production of drug substance), as well as the primary packaging materials, should be controlled with specifications established based on the characteristics of the drug. If specifications and test procedures for a material are described by the JP, as a rule, at least the JP criteria should be satisfied. Concerning excipients and other materials not listed in the JP, appropriate specifications and test procedures should be established individually.

2. Quality tests of products (specifications)

A specification is defined as a list of tests, references to analytical procedures, and appropriate acceptance criteria which are numerical limits, ranges, or other criteria for the tests described. Specifications and test methods of the JP monograph are defined sets of quality characteristics needed for determination of whether the use of a drug substance or a drug product is appropriate for the intended purpose. ''Conformance to the specifications of the JP monograph'' means that the JP-listed drug substances and drug products, when tested according to the procedures described in general tests and drug monographs, will meet the all acceptance criteria except criteria of ''Description'', ''Containers and storage (for drug products)'' and ''Shelf life'' in the JP monographs.

However, as described in ''Basic Concept'' specifications of monographs and test procedures for drug substance and drug product are one part of a total control strategy for assurance of the quality and consistency of the substances/products. Other parts of control strategies include thorough characterization of the drug in developmental stage (specifications and test procedures are established based on the characterization), and management of manufacturing process and products' quality, such as evaluation, verification and review of manufacturing process, and management of raw materials, other materials and manufacturing process, that is to say, compliance with the GMP.

3. Periodic or Skip Testing

Periodic or skip testing is the performance of specified tests at release on preselected batches and/or at predetermined intervals, rather than on a batch-to-batch basis with the understanding that those batches not being tested still must meet all acceptance criteria established for that product. When this concept is applied, it is necessary to show its appropriateness and be approved previously by regulatory authority. This concept may be applicable to, for example, residual solvents and microbiological testing for

solid oral dosage forms. It is recognized that only limited data may be available at the time of submission of an application for marketing approval. Implementation of this concept should therefore generally be considered post-approval. When tested, any failure to meet acceptance criteria established for the periodic test should be handled by proper notification of the appropriate regulatory authorities. If these data demonstrate a need of testing for all lots, then batch-by-batch release testing should be reinstated.

4. Real-time release testing (RTRT) and parametric release

RTRT is a type of tests to evaluate the quality of in-process or final products based on process data (including results of in-process testing and data on process parameters) and to assure that the quality is acceptable. RTRT is a kind of specifications and consists of a valid combination of materials attribute (intermediate products) pre-evaluated and process control. RTRT is used for judgement of products release instead of the release testing of final products when the application containing RTRT is approved by a regulatory authority.

The usage of RTRT does not mean unnecessity of setting tests of a final product directly. Even if the decision of release is made by RTRT, the tests for final products need to be set as specifications. It is because final product testing may be requested for some reasons such as failure of data acquisition due to troubles of equipments used for RTRT and evaluation of stability of final products. The final products, of course, need to meet their specifications, when tested.

Likewise, in the case that the drugs that was approved for marketing with the RTRT is listed in the JP monograph, the RTRT can be continued to use for release judgement. However specification and test procedure that assure the quality as same as the RTRT for final products should be set in the monograph. Even for drugs whose specifications are listed in the monographs, when a new application (or application for partial change) containing RTRT is approved by the regulatory authority, the products release can be judged based on the results of the RTRT instead of the tests prescribed in the monograph. In addition, it is necessary to comply with the specification in the case of conducting the compendial tests. In either case, it is unnecessary to set specification for RTRT in ''Manufacture'' of the monograph since the control criteria for the target CQA is already shown for the RTRT.

If RTRT results fail or trend toward failure, RTRT should not easily be substituted by final product testing. In this case, it is important to investigate the cause properly and need to take corrective action. Also, if RTRT results fail, the products cannot be released unless they were caused by analysis failure such as equipment failure. If RTRT results are trending toward failure, the products release should be made carefully based on the results of the investigation.

Parametric release can be considered a type of real time release. One example of parametric release is to determine the suitability for release of terminally sterilized drug products based on the data on sterilizing process instead of the results of sterility testing. In this case, the release of each batch is based on satisfactory results from monitoring specific parameters, e.g., temperature, pressure, and time during the terminal sterilization phase(s) of drug product manufacturing. Parametric release based on above parameters is more reliable in predicting sterility assurance than determination of suitability for release based on sterility testing using limited number of final products. Besides, even if parametric release is applied, the final product testing need to be set because the testing is necessary in stability testing and post-marketing surveillance. If in-process data used for parametric release are not acceptable, the products cannot be released. The parametric release differs from RTRT in the case, for example where the data of monitoring specific parameters in terminally sterilized process is failed to obtain by a certain reason such as analysis failure by equipment failure and so on. The incomplete data acquisition means no assurance on sterilization process, it is impossible to substitute parametric release by sterility testing of final products in principle.

Basic Concept of Quality Risk Management <*G0-2-170*>

Introduction

Quality Risk Management (QRM) is a crucial constituent of Pharmaceutical Quality System (PQS). PQS is a kind of the Quality System to control pharmaceutical quality in industries. Quality System is a basic concept of International Standards such as ISO 9001, ISO 14001, and ISO 27001. With its framework of maintenance and continuous improvement of business operation based on PDCA cycle (Plan →Do→Check→Act), PQS has been incorporated in ICH Q10 guideline as the basic philosophy. QRM is applicable to secure quality of every pharmaceuticals including drug substances, drug (medicinal) products, and biological and biotechnological products. Cooperating with a control strategy reflecting latest knowledge and understandings on products and manufacturing process, QRM contributes to realization and maintenance with consistent quality by responding flexibly and securely to risk regarding qualities.

Risks associated with the quality of pharmaceutical products are evaluated in the process of listing in the Japanese Pharmacopoeia and the results are reflected in specifications of the individual monograph. However, the pharmaceuticals specified in the same monograph may each have different quality risk derived from difference in their manufacturing methods. Therefore, appropriate assessment and management is required for such risk to manufacturing quality in the course of actual drug development and manufacturing. Further, quality risk of pharmaceuticals should be re-evaluated on a regular basis during their lifecycle, i.e. from their initial development through commercialization to the end of manufacturing and sales, and it is required to take appropriate measures based on the results.

About a relationship between QRM and the Japanese Pharmacopoeia, it may be said additionally as follows. In addition to conduction of the standard tests of Japanese Pharmacopoeia, it is important to plan and carry out measures to properly control elusive risk, which derived from alterations of manufacturing and quality management such as changes of raw materials and resources, in order to properly hold the pharmaceutical quality. Besides, depending on the results of risk re-evaluation, it may become necessary to revise specification tests specified by the Japanese Pharmacopoeia.

1. Significance of QRM

It is commonly understood that risk is defined as the combination of the probability of occurrence of harm and the severity of that harm. However, achieving a shared understanding of the application of risk management among diverse stakeholders is difficult because of a large gap between stakeholders in type and size of risk recognized. In re-

lation to pharmaceuticals, although there are a variety of stakeholders, including patients and medical practitioners as well as government and industry, the protection of the patient by applying QRM should be considered of prime importance.

The manufacturing and use of a drug (medicinal) product, including its components, necessarily entail some degree of risk. The risk to its quality is just one component of the overall risk. The product quality should be maintained throughout the product lifecycle such that the attributes that are important to the quality of the drug (medicinal) product remain consistent with those used in the clinical studies. An effective QRM approach can further ensure the high quality of the drug (medicinal) product to the patient by providing a proactive means to identify and control potential quality issues during development and manufacturing. Additionally, use of QRM can improve the quality of measures and the speed of decision making if a quality problem arises. Effective QRM can provide regulators with greater assurance of a company's ability to deal with potential risks and can beneficially affect the extent and level of direct regulatory oversight.

As for QRM, it is neither always appropriate nor always necessary to use a formal risk management process. The use of informal risk management processes can also be considered acceptable. Appropriate use of QRM can facilitate but does not obviate industry's obligation to comply with regulatory requirements and does not replace appropriate communications between industry and regulators.

2. Scope of Application

QRM can be applied to every aspects of pharmaceutical quality. These aspects include development, manufacturing, distribution, and the inspection and submission/review processes throughout the lifecycle of drug substances, drug (medicinal) products, and biological and biotechnological products (including the use of raw materials, solvents, excipients, packaging and labeling materials in drug (medicinal) products, biological and biotechnological products).

3. Principle of QRM

Two primary principles of QRM are:

- Evaluation of the risk to quality should be based on scientific knowledge and ultimately link to the protection of the patient.
- Level of effort, formality and documentation of the QRM process should be commensurate with the level of risk.

4. General QRM Process

QRM is a systematic process for the assessment, control, communication and review of risks to the quality of the drug (medicinal) product across the product lifecycle. A model for QRM is outlined in the diagram (Figure 1). The emphasis on each component of the framework might differ from case to case but a robust process will incorporate consideration of all the elements at a level of detail that is commensurate with the specific risk. Decision nodes are not shown in the diagram because decisions can occur at any point in the process. These decisions might be to return to the previous step and seek further information, to adjust the risk models or even to terminate the risk management process based upon information that supports such a decision.

4.1. Initiation of QRM Process

QRM should include systematic processes designed to coordinate, facilitate and improve science-based decision making with respect to risk. Possible steps used to initiate and plan a QRM process might include the following:

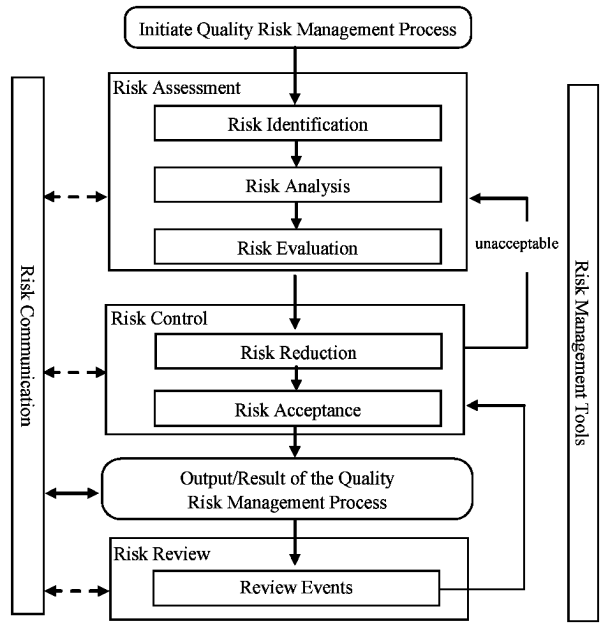


Fig. 1 Overview of a typical quality risk management process

- Define the problem and/or risk question, including pertinent assumptions identifying the potential for risk;
- Assemble background information and/or data on the potential hazard, harm or human health impact relevant to the risk assessment;
- Identify a leader and necessary resources;
- Specify a timeline, deliverables and appropriate level of decision making for the risk management process.

In the process described above, persons in charge (decision makers) should take responsibility for coordinating QRM across various functions and departments of their organization; and assure that a QRM process is defined, deployed and reviewed and that adequate resources are available.

4.2. Risk Assessment

Risk assessment consists of the identification of hazards and the analysis and evaluation of risks associated with exposure to those hazards. The step includes ''risk identification'', ''risk analysis'' and ''risk evaluation'',

As an assist to define risk clearly for purposes of risk assessment, the following three basic questions are often helpful.

1. What might go wrong?
2. What is the likelihood (probability) it will go wrong?
3. What are the consequences (severity)?

Risk identification is a systematic use of information to identify hazards referring to the risk question or problem description. Information can include historical data, theoretical analysis, informed opinions, and the concerns of stakeholders. Risk identification addresses the ''What might go wrong?'' question, including identifying the possible consequences. This provides the basis for further steps in the quality risk management process.

Risk analysis is the estimation of the risk associated with the identified hazards. It is the qualitative or quantitative process of linking the likelihood of occurrence and severity of harms. In some risk management tools, the ability to detect the harm (detectability) also factors in the estimation of risk.

Risk evaluation compares the identified and analyzed risk against given risk criteria. Risk evaluations consider the strength of evidence for all three of the fundamental ques-

tions.

The output of a risk assessment is either a quantitative estimate of risk or a qualitative description of a range of risk. When risk is expressed quantitatively, a numerical probability is used. Alternatively, risk can be expressed using qualitative descriptors, such as ''high'', ''medium'', or ''low'', which should be defined in as much detail as possible. Sometimes a ''risk score'' is used to further define descriptors in risk ranking. In quantitative risk assessments, a risk estimate provides the likelihood of a specific consequence, given a set of risk-generating circumstances. Thus, quantitative risk estimation is useful for one particular consequence at a time. Alternatively, some risk management tools use a relative risk measure to combine multiple levels of severity and probability into an overall estimate of relative risk. The intermediate steps within a scoring process can sometimes employ quantitative risk estimation.

4.3. Risk Control

Risk control includes decision making to reduce and/or accept risks. The purpose of risk control is to reduce the risk to an acceptable level. The amount of effort used for risk control should be proportional to the significance of the risk. Decision makers might use different processes, including benefit-cost analysis, for understanding the optimal level of risk control.

Risk control might focus on the following questions:

- Is the risk above an acceptable level?
- What can be done to reduce or eliminate risks?
- What is the appropriate balance among benefits, risks and resources?
- Are new risks introduced as a result of the identified risks being controlled?

Risk reduction focuses on processes for mitigation or avoidance of quality risk when it exceeds a specified (acceptable) level (see Fig. 1). Risk reduction might include actions taken to mitigate the severity and probability of harm. Processes that improve the detectability of hazards and quality risks might also be used as part of a risk control strategy. The implementation of risk reduction measures can introduce new risks into the system or increase the significance of other existing risks. Hence, it might be appropriate to revisit the risk assessment to identify and evaluate any possible change in risk after implementing a risk reduction process.

Risk acceptance is a decision to accept risk. Risk acceptance can be a formal decision to accept the residual risk or it can be a passive decision in which residual risks are not specified. For some types of harms, even the best quality risk management practices might not entirely eliminate risk. In these circumstances, it might be agreed that an appropriate quality risk management strategy has been applied and that quality risk is reduced to a specified (acceptable) level. This (specified) acceptable level will depend on many parameters and should be decided on a case-by-case basis.

4.4. Risk Communication

Risk communication is the sharing of information about risk and risk management between the decision makers and others. Parties can communicate at any stage of the risk management process (see Fig. 1: dashed arrows). The output/result of the quality risk management process should be appropriately communicated and documented (see Fig. 1: solid arrows). Communications might include those among interested parties; e.g., regulators and industry, industry and the patient, within a company, industry or regulatory authority, etc. The included information might relate to the existence, nature, form, probability, severity, acceptability, control, treatment, detectability or other aspects of risks to quality. Communication need not be carried out for each and every risk acceptance. Between the industry and regulatory authorities, communication concerning quality risk management decisions might be effected through existing channels as specified in regulations and guidances.

4.5. Risk Review

Risk management should be an ongoing part of the quality management process. A mechanism to review or monitor events should be implemented.

The output/results of the risk management process should be reviewed to take into account new knowledge and experience. Once a quality risk management process has been initiated, that process should continue to be utilized for events that might impact the original quality risk management decision, whether these events are planned (e.g., results of product review, inspections, audits, change control) or unplanned (e.g., root cause from failure investigations, recall). The frequency of any review should be based upon the level of risk. Risk review might include reconsideration of risk acceptance decisions (section 4.3).

5. Summary

The degree of rigor and formality of quality risk management should reflect available knowledge and be commensurate with the complexity and/ or criticality of the issue to be addressed.

Quality risk management is a process that supports science-based and practical decisions when integrated into quality systems. Appropriate use of QRM, however, does not obviate industry's obligation to comply with regulatory requirements.

6. Definitions

Decision Maker(s): Person(s) with the competence and authority to make appropriate and timely quality risk management decisions.

Detectability: The ability to discover or determine the existence, presence, or fact of a hazard.

Harm: Damage to health, including the damage that can occur from loss of product quality or availability.

Hazard: The potential source of harm (ISO/IEC Guide 51).

Product Lifecycle: All phases in the life of the product from the initial development through marketing until the product's discontinuation.

Quality: The degree to which a set of inherent properties of a product, system or process fulfills requirements. The suitability of either a drug substance or drug product for its intended use. This term includes such attributes as identity, strength, and purity.

Quality Risk Management: A systematic process for the assessment, control, communication and review of risks to the quality of the drug (medicinal) product across the product lifecycle.

Quality System: The sum of all aspects of a system that implements quality policy and ensures that quality objectives are met.

Requirements: The explicit or implicit needs or expectations of the patients or their surrogates (e.g., health-care professionals, regulators and legislators). In this document, ''requirements'' refers not only to statutory, legislative, or regulatory requirements, but also to such needs and expectations.

Risk: The combination of the probability of occurrence of harm and the severity of that harm (ISO/IEC Guide 51).

Risk Acceptance: The decision to accept risk (ISO Guide 73).

Risk Analysis: The estimation of the risk associated with the identified hazards.

Risk Assessment: A systematic process of organizing information to support a risk decision to be made within a risk management process. It consists of the identification of hazards and the analysis and evaluation of risks associated with exposure to those hazards.

Risk Communication: The sharing of information about risk and risk management between the decision maker and other stakeholders.

Risk Control: Actions implementing risk management decisions (ISO Guide 73).

Risk Evaluation: The comparison of the estimated risk to given risk criteria using a quantitative or qualitative scale to determine the significance of the risk.

Risk Identification: The systematic use of information to identify potential sources of harm (hazards) referring to the risk question or problem description.

Risk Management: The systematic application of quality management policies, procedures, and practices to the tasks of assessing, controlling, communicating and reviewing risk.

Risk Reduction: Actions taken to lessen the probability of occurrence of harm and the severity of that harm.

Risk Review: Review or monitoring of output/results of the risk management process considering (if appropriate) new knowledge and experience about the risk.

Severity: A measure of the possible consequences of a hazard.

Stakeholder: Any individual, group or organization that can affect, be affected by, or perceive itself to be affected by a risk. Decision makers might also be stakeholders. For the purposes of this guideline, the primary stakeholders are the patient, healthcare professional, regulatory authority, and industry.

Concept on Impurities in Chemically synthesized Drug Substances and Drug Products

〈G0-3-172〉

1. Classification of impurities found in chemically synthesized pharmaceuticals and the guidance to comply with their control

Impurities found in chemically synthesized pharmaceuticals are roughly classified into organic impurities, inorganic impurities and residual solvents. Those impurities in the new drug substances and the products are controlled by the following guidelines agreed upon at the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (hereinafter referred to as ''ICH''). More specifically, ''Impurities in New Drugs Substances (PAB/PCD Notification No. 877 dated September 25, 1995)'' (hereinafter referred to as ''ICH Q3A Guideline'')[1] on specifications for organic impurities in drug substances applies to applications for marketing approval after April 1, 1997, while ''Impurities in New Drug Products (PAB/PCD Notification No. 539 dated June 23, 1997'' (hereinafter referred to as ''ICH Q3B Guideline'')[2] on specifications for organic impurities in drug products applies to applications for marketing approval after April 1, 1999. Meanwhile, specifications for inorganic impurities were specified by Japanese pharmacopoeial standards and known safety data. Now ''Guidelines for Elemental Impurities (PFSB/ELD Notification No. 4 dated September 30, 2015)'' apply to applications for marketing approval after April 1, 2017. In regard to residual solvents, ''Impurities: Guidelines for Residual Solvents (PAB/ELD Notification No. 307 dated March 30, 1998)'' (hereinafter referred to as ''ICH Q3C Guideline'') applies to applications for marketing approval after April 1, 2000. Especially in regard to DNA-reactive impurities, ''Assessment and control of DNA reactive (mutagenic) impurities in pharmaceuticals to limit potential carcinogenic risk (PSEHB/ELD Notification No. 3 dated November 10, 2015)'' applies to applications for marketing approval after January 15, 2016. Although ICH Q3A guideline does not cover optical enantiomers, a type of organic impurities, ''Specifications: Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances (PMSB/ELD Notification No. 568 dated May 1, 2001)'' (hereinafter referred to as ''ICH Q6A Guideline''), which was published subsequently, provides that enantiomers are impurities that should be controlled and, if measurable, should be controlled in accordance with the principle of ICH Q3A guideline.

Control of impurities in accordance with the guidelines mentioned above is expected also for pharmaceuticals other than new drug substances and new drug products. Their applications for marketing (or applications for partial changes) are subject to those guidelines when necessary. The General Notices of the JP 17th Edition states that residual solvents of all JP-listed drugs, in principle, have to be controlled in accordance with specification ''Residual Solvents'' in General Tests unless otherwise specified in the individual monograph. In regard to elemental impurities, it has been decided in the basic principles for the preparation of the JP 18th Edition to create a roadmap for their incorporation into the JP for listing and to address its implementation.

2. The concept of ICH Q3A and Q3B guidelines for the control of organic impurities

ICH Q3A and Q3B guidelines require setting acceptance criteria for organic impurities based on the information gained from development stages for new drugs. Concerning impurities in drug substances, ICH Q3A guideline refers to the items to be examined from chemical and safety perspectives. ICH Q3B guideline complements Q3A guideline, and have the same basic concept as Q3A. Chemical aspects to be examined include classification and identification of impurities, their reporting method, specification settings and analytical methods. Safety aspects include specific guidelines for qualifying the safety of impurities that were not present, or were present at substantially lower levels, in batches of a drug substance used in safety and clinical studies.

Qualification of the safety is the process of acquiring and evaluating data that establishes the biological safety of an individual impurity or a given impurity profile at the level(s) specified. The applicant should describe a rationale for establishing impurity acceptance criteria that includes safety considerations in attachments when applicated for approval. The level of any impurities present in a new drug substance

that has been adequately tested in safety and/or clinical studies would be considered qualified.

Identified impurities, unidentified impurities and total impurities are specified based on the data obtained according to the guidelines. The threshold of unspecified impurities in a drug substance is determined depending on the daily intake of the drug substance. When the maximum daily dosage is not more than 2 g, it is set at 0.10%. The establishment of individual specifications is required for impurities at a level greater than 0.10%.

In regard to drug products, ICH Q3B guideline cover the degradation products of drug substances or reaction products between the drug substance and additive/primary packaging. Therefore, even if organic impurities other than degradation products (e.g., by-products and synthetic intermediates) in the drug substance are found as impurities in the drug product, they need not be monitored or specified since they have already been controlled as the drug substance specifications. However, degradation products elevated in the drug product need to be monitored and specified.

3. Principles for controlling organic impurities in the articles listed in the JP

Conventionally in the JP, specified impurities, unspecified impurities and total impurities are specified in accordance with ICH Q3A and Q3B guidelines for pharmaceutical products, whose impurities have been controlled by those guidelines, in the process listing in the JP. (However, this shall not apply to the long-term listed pharmaceutical products which had existed in the JP before these guidelines were applicable. However, when a new application is filed for those JP-listed pharmaceutical products, control of impurities in accordance with ICH Q3A and Q3B guidelines may be required, if necessary.) In order to specify the impurities, analysis data during development submitted from the drafting company and impurity analysis data from commercial production batches after consistent manufacturing is achieved should be assessed. Safety evaluation is not required again for the process listing in the JP since it has been performed at the time of approval.

ICH Q3A and Q3B guidelines cover impurities in the drug substances manufactured by chemical syntheses and the drug products manufactured with those drug substances. Similarly, the following types of products are not covered in the JP: biological/biotechnological products, peptides, oligonucleotides, radiopharmaceuticals, fermentation products and semi-synthetic products derived therefrom, herbal products and crude products of animal or plant origin.

When organic impurities assessed in accordance with the principles of ICH Q3A and Q3B guidelines are listed as JP tests of purity, the operational rationality of the JP is considered and its own modification is added. (i) Except in exceptional circumstances, impurity reference standards are not established. In order to identify an impurity using liquid chromatography, the relative retention time of the impurity to the drug substance is used for identification. (ii) When only unidentified impurities in highly pure pharmaceutical products (not more than 0.1%) are specified, it is generally exempted to set acceptance criteria for total impurities. (iii) When acceptance criteria set based only on actual measured values result in many impurities with slightly different acceptance criteria, consideration can be given so that the purity test consists of a small number of representative acceptance criteria, if possible. (iv) Chemical structural information and the chemical name of the impurities are not disclosed. Those measures enable impurity control without impurity reference standards, and can simplify system suitability test for highly pure pharmaceutical products.

Meanwhile, the method to identify impurities by use of relative retention time is column-dependent and analysis becomes difficult when appropriate columns are not available. Therefore, the JP 17th Edition also allows the use of the analysis method with impurity reference standards when designing purity tests for a drug substance. In addition, the JP adopted a policy to disclose chemical names and structure formulas as the information on impurities including, in principle, optical enantiomers.

The JP-specific consideration may be given to purity tests for organic impurities in drug products in the process listing in the JP. Also in the JP, impurities derived from the products of the reaction between the drug substance and additive/primary packaging are specified as impurities in the drug product. Those impurities are formulation-dependent and may not be formed in different formulations. Since the JP is an official compendium that allows a wide variety of formulations, when it is not appropriate to specify impurities uniformly in the individual monograph, they are subject to the specifications at the time of approval, along with the statement ''Being specified separately when the drug is granted approval based on the Law.''

When the specifications for impurities are reviewed for a new entry of a pharmaceutical product in an individual monograph of the JP, acceptance criteria for impurities may be included in the review according to the following concepts. ICH Q6A guideline point out: Data available upon the marketing application are limited and it has to be taken into consideration that the limited data may influence the design of acceptance criteria. Regarding impurities, since impurity profiles gained during the manufacturing stages may sometimes be different from that gained from development stage, it is stated that changes in impurity profiles at the manufacturing stage should be considered as appropriate. According to this concept, for impurities which should be specified in the process listing in the JP, not only information from development stage but also information about impurity profiles if there are changes at the manufacturing stage, and information at the stage after the product manufacturing becomes stable (hereinafter referred to as the ''stable production stage'') should be taken into consideration.

However, it is undesirable to remove impurities that are present at substantially lower levels, or become undetectable at the stable production stage indiscriminately from the list of candidate compounds to be specified. JP-listed drugs are accepted as drugs by conformance to the specifications in the individual monograph. However, generic drugs, whose manufacturing methods are not necessarily the same as that of the drug substance used for JP monograph, may have different impurity profiles and contain such impurities. Providing information in the process listing in the JP based on the detection results during development stage may result in encompassing impurities found in drug substances and drug products distributed as JP drugs.

Therefore, before the removal of impurities that are present at substantially lower levels or become undetectable at the stable production stage from the JP specification list, the need to establish specifications should be fully examined based on ICH Q3A and Q3B guidelines with respect to safety.

For a drug substance that was approved by the method to identify its impurities with impurity reference materials, it is desirable also in the individual JP monograph, in principle, to establish specifications and test methods appropriately so that the specified impurity becomes identifiable. In regard to impurity control during the manufacturing process, impuri-

ties can be controlled by establishing an appropriate control strategy including release testing, in-process tests and process parameters control.

4. References

1) ICH: Guideline for Q3A, Impurities in New Drug Substances.
2) ICH: Guideline for Q3B, Impurities in New Drug Products.

Stability Testing of Drug Substances and Drug Products
〈G0-4-171〉

1. Introduction

It is essential that the quality of a drug is maintained during the period from being manufactured to being administered in a patient. Stability testing is performed in order to ensure that the quality is maintained during the period. The purpose of stability testing is to provide evidence on how the quality of a drug substance or drug product varies with time under the influence of a variety of environmental factors such as temperature, humidity and light, and to establish a re-test period for the drug substance or a shelf life for the drug product and recommended storage conditions.

The re-test period of a drug substance is the period of time during which the drug substance is expected to remain within its specification and, therefore, can be used in the manufacture of a given drug product, provided that the drug substance has been stored under the defined conditions. After this period, a batch of drug substance destined for use in the manufacture of a drug product should be re-tested for compliance with the specification and then used immediately. A batch of drug substance can be re-tested multiple times. For certain antibiotics known to be labile, it is more appropriate to establish a shelf life than a re-test period. The shelf life of a drug product is the period in which a batch of the product is expected to remain within the approved shelf life specification if stored under defined conditions.

This general information mainly illustrates a standard implementation that can be set when we perform stability tests of a chemical drug substance and the associated drug product, and it is also helpful in stability tests of pharmaceuticals other than chemical drugs. Also, this leaves sufficient flexibility to encompass the variety of different practical situations that may be encountered due to specific scientific considerations and characteristics of the materials being evaluated. Alternative approaches can be used when there are scientifically justifiable reasons.

2. Conditions of stability testing

Stress testing, long term testing, accelerated testing and if necessary intermediate testing are performed as stability testing for drugs.

2.1 Stress testing

Stress testing of the drug substance can help identify the likely degradation products, which can in turn help establish the degradation pathways and the intrinsic stability of the molecule and validate the stability indicating power of the analytical procedures used. Stress testing should include the effect of temperatures (in 10°C increments (e.g., 50°C, 60°C, etc.) above that for accelerated testing), humidity (e.g., 75% RH or greater) where appropriate, oxidation, and photolysis on the drug substance. The testing should also evaluate the susceptibility of the drug substance to hydrolysis across a wide range of pH values when in solution or suspension.

Stress testing of the drug product is undertaken to assess the effect of severe conditions on the drug product. Such studies include photostability testing and specific testing on certain products, (e.g., metered dose inhalers, creams, emulsions, refrigerated aqueous liquid products).

2.2 Long term testing, accelerated testing and intermediate testing

Long term testing is undertaken on batches of a drug substance or drug product according to a prescribed stability protocol to establish the re-test period of the drug substance or the shelf life of the drug product.

Accelerated testing is a stability study designed to increase the rate of chemical degradation or physical change of a drug substance or drug product by using exaggerated storage conditions. Data from these studies, in addition to long term stability studies, can be used to assess longer term chemical effects at non-accelerated conditions and to evaluate the effect of short term excursions outside the label storage conditions such as might occur during shipping.

Intermediate testing is conducted at 30°C/65% RH and designed to moderately increase the rate of chemical degradation or physical changes for a drug substance or drug product intended to be stored long term at 25°C. Intermediate testing is implemented only when a significant change occurs in the accelerated testing.

Long term and accelerated testing, also if needed intermediate testing should be performed on at least three primary batches. The primary batches of the drug substance should be manufactured to a minimum of pilot scale by the same synthetic route as, and using a method of manufacture and procedure that simulates the final process to be used for, production batches. The overall quality of the batches of drug substance placed on the stability studies should be representative of the quality of the material to be made on a production scale. The stability studies should be conducted on the drug substance packaged in a container closure system that is the same as or simulates the packaging proposed for storage and distribution. The primary batches of the drug product should be of the same formulation and packaged in the same container closure system as proposed for marketing (including, as appropriate, any secondary packaging and container label). The manufacturing process used for primary batches should simulate that to be applied to production batches and should provide the product of the same quality and meeting the same specification as that intended for marketing. Two of the three batches should be at least pilot scale batches and the third one can be smaller, if justified. The primary batch may be a production batch. Where possible, batches of the drug product should be manufactured by using different batches of the drug substance. The pilot scale batch is a batch of a drug substance or drug product manufactured by a procedure fully representative of and simulating that to be applied to a full production scale batch. For solid oral dosage forms, a pilot scale is generally, at a minimum, one-tenth that of a full production scale or 100,000 tablets or capsules, whichever is the larger.

The storage conditions used for stability testing are shown in Table 1.

3. Testing attributes and testing frequency

Stability studies should include testing of those attributes of the drug substance or the product that are susceptible to change during storage and are likely to influence quality, safety, and/or efficacy. Validated stability-indicating analytical procedures should be applied. Whether and to what

Table 1 Storage condition

Storage condition and package	Long term	Accelerated	Intermediate
General case (drug substance and product)	25 ± 2°C/ 60 ± 5%RH or 30 ± 2°C/ 65 ± 5%RH[1)]	40 ± 2°C/ 75 ± 5%RH	30 ± 2°C/ 65 ± 5%RH[2)]
Storage in a refrigerator (drug substance and product)[3)]	5 ± 3°C	25 ± 2°C/ 60 ± 5%RH	—
Storage in a freezer (drug substance and product)[4)]	− 20 ± 5°C	—	—
Storage below − 20°C (drug substance and product)	case-by-case basis		
Drug products packaged in impermeable containers	Study can be conducted under any controlled or ambient humidity condition		
Drug products packaged in semi-permeable containers[5)]	25 ± 2°C/ 40 ± 5%RH or 30 ± 2°C/ 35 ± 5%RH[6)]	40 ± 2°C/ not more than (NMT) 25%RH	30 ± 2°C/ 65 ± 5%RH[7)]

1) It is up to the applicant to decide whether long term stability studies are performed at 25 ± 2°C/60 ± 5%RH or 30 ± 2°C/65 ± 5%RH.

2) If ''significant change'' occurs at the accelerated storage condition, additional testing at the intermediate storage condition should be conducted. However, if 30 ± 2° C/65 ± 5%RH is the long term condition, there is no intermediate condition. ''Significant change'' for a drug substance is defined as failure to meet its specification. In general, ''significant change'' for a drug product is defined as:

1. A 5% change in assay from its initial value; or failure to meet the acceptance criteria for potency when using biological or immunological procedures;
2. Any degradation product's exceeding its acceptance criterion;
3. Failure to meet the acceptance criteria for appearance, physical attributes, and functionality test (e.g., color, phase separation, resuspendibility, caking, hardness, dose delivery per actuation); however, some changes in physical attributes (e.g., softening of suppositories, melting of creams) may be expected under accelerated conditions;

and, as appropriate for the dosage form:

4. Failure to meet the acceptance criterion for pH; or
5. Failure to meet the acceptance criteria for dissolution for 12 dosage units.
6. Physical changes shown in the following may be observed in accelerated testing, but the changes are not considered as ''significant change'' which needs intermediate testing, when there is no ''significant change'' in other attributes.

• Softening of suppositories designed to melt at 37°C, when its melting point is shown clearly.

• When it is clear that ''significant change'' is due to crosslinking, the dissolution of gelatin capsules and gel coating tablets do not conform to the acceptance criteria for 12 dosage units.

When confirming that there is no ''significant change'' in other attributes, consider the possibility that these physical changes affect the other attributes.

3) The drug product is packaged in a semi-permeable container, appropriate information should be provided to assess the extent of water loss. In the accelerated testing of drug substances or products intended for storage in a refrigerator, if significant change occurs within the first 3 months, it is considered unnecessary to continue to test a product through 6 months.

4) Testing on a single batch at an elevated temperature (e.g., 5 ± 3°C or 25 ± 2°C) for an appropriate time period should be conducted to address the effect of short term excursions outside the label storage condition, e.g., during shipment and handling.

5) Aqueous-based products packaged in semi-permeable containers should be evaluated for potential water loss under conditions of low relative humidity. Other comparable approaches can be developed and used for non-aqueous, solvent-based products.

6) It is up to the applicant to decide whether long term stability studies are performed at 25 ± 2°C/40 ± 5% RH or 30 ± 2°C/35 ± 5%RH.

7) If ''significant change'' other than water loss occurs during the 6 months' testing at the accelerated storage condition. Additional testing at the intermediate storage condition should be performed. A significant change in water loss alone at the accelerated storage condition does not necessitate testing at the intermediate storage condition. However, data should be provided to demonstrate that the drug product will not have significant water loss throughout the proposed shelf life if stored at 25°C and the reference relative humidity of 40% RH. If 30 ± 2° C/35 ± 5% RH is the long term condition, there is no intermediate condition. A 5% loss in water from its initial value is considered a significant change for a product packaged in a semi-permeable container after an equivalent of 3 months' storage at 40°C/NMT 25% RH. However, for small containers (1 mL or less) or unit-dose products, a water loss of 5% or more after an equivalent of 3 months' storage at 40°C/NMT 25% RH may be appropriate, if justified.

extent replication should be performed will depend on the results from validation studies.

For long term studies, frequency of testing should be sufficient to establish the stability profile of the drug substance and product. For drug substances or products with a proposed re-test period or shelf life of at least 12 months, the frequency of testing at the long term storage condition should normally be every 3 months over the first year, every 6 months over the second year, and annually thereafter through the proposed re-test period or shelf life. At the accelerated storage condition, a minimum of three time points, including the initial and final time points (e.g., 0, 3, and 6 months), from a 6-month study is recommended. When testing at the intermediate storage condition is called for as a result of significant change at the accelerated storage condition, a minimum of four time points, including the initial and final time points (e.g., 0, 6, 9, 12 months), from a 12-month study is recommended.

A reduced design, i.e., matrixing or bracketing, where the testing frequency is reduced or certain factor combinations are not tested at all, can be applied, if justified, for the testing of combination of drug products having multiple design factors (e.g., strength, container size and/or fill). A bracketing design assumes that the stability of any intermediate levels is represented by the stability of the extremes tested. This is the design of a stability schedule such that only samples on the extremes of certain design factors (e.g., strength, container size and/or fill). Bracketing can be applied to studies with multiple strengths of identical or closely related formu-

lations. Examples include but are not limited to (1) capsules of different strengths made with different fill plug sizes from the same powder blend, (2) tablets of different strengths manufactured by compressing varying amounts of the same granulation, and (3) oral solutions of different strengths with formulations that differ only in minor excipients (e.g., colorants, flavorings). Bracketing can be applied to studies of the same container closure system where either container size or fill varies while the other remains constant. The use of a bracketing design would not be applicable if it cannot be demonstrated that the strengths or container sizes and/or fills selected for testing are indeed the extremes. An example of a bracketing design is given in Table 2. This design is provided for illustrative purpose, and should not be considered the only, or the most appropriate, design in all cases.

A matrixing design assumes that the stability of each subset of samples tested represents the stability of all samples at a given time point. This is the design of a stability schedule such that a selected subset of the total number of possible samples for all factor combinations would be tested at a specified time point. At a subsequent time point, another subset of samples for all factor combinations would be tested. Matrixing designs can be applied to strengths with identical or closely related formulations. Examples include but are not limited to (1) capsules of different strengths made with different fill plug sizes from the same powder blend, (2) tablets of different strengths manufactured by compressing varying amounts of the same granulation, and (3) oral solutions of different strengths with formulations that differ only in minor excipients (e.g., colorants or flavorings). Other examples of design factors that can be matrixed include batches made by using the same process and equipment, and container sizes and/or different fills in the same container closure system. An example of a matrixing design is given in Table 3. This design is provided for illustrative purpose, and should not be considered the only, or the most appropriate, design in all cases.

Table 2 Example of a Bracketing Design

Strength		50 mg			75 mg			100 mg		
Batch		1	2	3	1	2	3	1	2	3
Container size	15 mL bottle	T	T	T				T	T	T
	100 mL bottle									
	500 mL bottle	T	T	T				T	T	T

T = Sample tested

Table 3 Example of a Matrixing Design on Time Points for a Product with Two Strengths
''One-Half Reduction''

Time point (months)			0	3	6	9	12	18	24	36
Strength	S1	Batch 1	T	T		T	T		T	T
		Batch 2	T	T		T	T	T		T
		Batch 3	T		T		T	T		T
	S2	Batch 1	T		T		T		T	T
		Batch 2	T	T		T	T	T		T
		Batch 3	T		T		T		T	T

T = Sample tested

4. Photostability testing

Photostability testing is a part of stress testing evaluating the photostability characteristics of drug substances and products.

4.1. Light sources

The light sources described below may be used for photostability testing.

(i) Option 1 Any light source that is designed to produce an output similar to the D_{65}/ID_{65} emission standard such as an artificial daylight fluorescent lamp combining visible and ultraviolet (UV) outputs, xenon, or metal halide lamp.

(ii) Option 2 For option 2 the same sample should be exposed to both the cool white fluorescent and near ultraviolet fluorescent lamp.

1. A cool white fluorescent lamp designed to produce an output similar to that specified in ISO10977(1993); and

2. A near ultraviolet fluorescent lamp having a spectral distribution from 320 nm to 400 nm with a maximum energy emission between 350 nm and 370 nm; a significant proportion of energy emission should be in both bands of 320 to 360 nm and 360 to 400 nm.

4.2. Light exposure level and testing condition

For drug substances, photostability testing should consist of two parts: forced degradation testing and confirmatory testing. The purpose of forced degradation testing studies is to evaluate the overall photosensitivity of the material for method development purposes and/or degradation pathway elucidation. This testing may involve the drug substance alone and/or in simple solutions/suspensions to validate the analytical procedures. In these forced degradation testing studies, a variety of exposure conditions may be used, depending on the photosensitivity of the drug substance involved and the intensity of the light sources used. For development and validation purposes it is appropriate to limit exposure and end the studies if extensive decomposition occurs. For photostable materials, studies may be terminated after an appropriate exposure level has been used. The design of these experiments is left to the applicant's discretion although the exposure levels used should be justified. Confirmatory studies of drug substance should then be undertaken to provide the information necessary for handling, packaging, and labeling. For confirmatory studies, samples should be exposed to light providing an overall illumination of not less than 1.2 million lx·h and an integrated near ultraviolet energy of not less than 200 $W \cdot h/m^2$ to allow direct comparisons to be made between the drug substance and drug product. Efforts should be made, such as cooling and/or placing the samples in sealed containers, to ensure that the effects of the changes in physical states such as sublimation, evaporation or melting are minimized. Containers used should not interfere with the exposure of a test sample as much as possible, and avoid materials that cause interference with the testing such as interaction between the sample and the materials. As a direct challenge for samples of solid drug substances, an appropriate amount of sample should be taken and placed in a suitable glass or plastic dish and protected with a suitable transparent cover if considered necessary. Powder drug substances should be spread across the container to give a thickness of typically not more than 3 mm. Drug substances that are liquids should be exposed in chemically inert and transparent containers. Where practicable when testing samples of the drug product outside of the primary pack, these should be presented in a

way similar to the conditions mentioned for the drug substance. The samples should be positioned to provide maximum area of exposure to the light source. For example, tablets, capsules, etc., should be spread in a single layer. If direct exposure is not practical (e.g., due to oxidation of a product), the sample should be placed in a suitable protective inert transparent container (e.g., quartz). If testing of the drug product in the immediate container or as marketed is needed, the samples should be placed horizontally or transversely with respect to the light source, whichever provides for the most uniform exposure of the samples. Some adjustment of testing conditions may have to be made when testing large volume containers (e.g., dispensing packs).

5. Evaluation of stability data

In the stability data evaluation, data from long term and accelerated testing, also if needed from intermediate testing and, as appropriate, supporting data (data of stability testing using drug substances and products in developing stage) should be evaluated to determine the critical quality attributes likely to influence the quality and performance of the drug substance or product. Each attribute should be assessed separately, and an overall assessment should be made of the findings for the purpose of proposing a re-test period or shelf life. An approach for analyzing data of a quantitative attribute that is expected to change with time is to determine the time at which the 95% one-sided confidence limit for the mean curve intersects the acceptance criterion. The re-test period or shelf life proposed should not exceed that predicted for any single attribute.

Basic Requirements and Terms for the Packaging of Pharmaceutical Products *<G0-5-170>*

This chapter describes the basic requirements for the packaging of pharmaceutical products as well as the terms and their definitions used for the packaging as taking into account the aspects in quality assurance of the pharmaceutical products and the point of view of international harmonization.[1)]

In this chapter, the concept of packaging for pharmaceutical products, or packaging, includes putting or holding the drugs in container. In addition, the information presented as basic requirements shall be a central focus on packaging for drug products, as well as for ensuring quality on the transportation and storage of drug substances or additives.

1. Basic requirements of packaging for pharmaceutical products

For the packaging for pharmaceutical products, it is important to settle the requirements of the packaging based on the evaluation of the packaging suitability in the development stage so as to be able to ensure the quality standards of preparations defined over the shelf life of preparations. The suitability of packaging for pharmaceutical products must be maintained through the product life cycle on the basis of the requirements of packaging settled in the development stage.

For the packaging of drug products, it is also necessary to consider for the suitability for proper use and for ensuring safe application in addition to quality assurance. The stringency of the evaluation for suitability of packaging for preparations differs depending on the degree of the risk according to the route of administration, such as intravenous administration, oral administration, or dermal administration and the risk due to interaction between the products and the primary packaging according to injections, liquids and solutions, semi-solid or solid dosage forms.

1.1. Suitability evaluation and requirements of packaging in the design stage

The packaging suitability to be evaluated in the design stage includes protection, compatibility, safety, and performance.

The basic items to be evaluated as suitability are described in the course of the packaging design.

1.1.1. Safety of materials used for packaging

Leachables or migrants, such as the monomers of the polymer resins, additives or metal impurities, from the materials used for the primary packaging such as plastic or glass containers should not deteriorate drug safety. The amount of leachable or migratable chemical substances from the primary packaging materials to the contents must be sufficiently small from a safety perspective.

The primary packaging materials of the containers that are in direct contact with the drugs should be used which quality such as on toxicity has been appropriately evaluated by the suppliers according to ''Basic Requirements for Plastic Containers for Pharmaceutical Use and Rubber Closures for Containers for Aqueous Infusions *<G7-2-162>*'' in General Information or the like. In the design stage, the information on quality evaluation of the packing materials is desirable to be obtained as far as possible from the suppliers.

1.1.2. Compatibility with the contents

The primary packaging must not reduce the quality of pharmaceutical products over the shelf life of preparations. The contents adsorbing onto the surface of primary packaging, or migrating inside of the materials, must not lead to a drug concentration change of more than a certain level. Moreover, the interaction between the contents and the materials must not lead to degradation of drugs.

The primary packaging should not be deformed, deteriorated, or degraded by the contents.

In the design stage, the compatibility of the primary packaging with the content is examined by the combination of individual candidate material and the content drugs, together with other evaluation items. And chose the applicable material based on the results of the study on the prototype primary packaging for complying with the essential requirements, i.e. the design specifications, for the issues about the protection from moisture and light, the sorption to and leaching from the primary packaging, etc., based on the data from the experiments and/or information from the scientific documentation. When selecting the primary packaging material, the suitability of the material for the secondary packaging is also to be evaluated as needed.

1.1.3. Protection by packaging

The packaging should be able to prevent loss, efflorescence, deliquescence, or evaporation of the contents and to protect the contents by the addition of moisture resistance, light shielding, or a gas barrier, depending on the characteristic of the contents. In the case of not being able to ensure the quality of the contents by the primary packaging alone, it should be ensured by the combination of multiple packaging materials, including the secondary packaging. Furthermore, the containers for injections or ophthalmic solutions are preferable to be made of a high transparency material, so that foreign matter contamination can be observed visually.

For pharmaceutical products susceptible to moisture such as by hydrolysis, the packaging used with desiccants or primary packaging materials with gas-barrier function can be moisture-proof packaging. For preparations susceptible to evaporation of water, gas-barrier materials for the primary

packaging can be used. For the pharmaceutical products that are easily oxidized, the packaging with deoxidants or the low-gas-permeability materials can be used for the primary packaging to protect the pharmaceutical products from oxygen in the air.

Protection by the packaging should be evaluated in the packaging design stage, and finally confirmed by stability tests. Resistance to physical shock during transportation is also necessary to be verified.

1.1.4. Container integrity (microbial contamination prevention)

The packaging should be able to protect the contents from microbial contamination, depending on the characteristics of the content drug or dosage form, and especially for the containers used for sterile preparations, the integrity of primary packaging, through the tests such as the fitting compatibility tests for containers and closures, must be confirmed.

In the case of pharmaceutical products that must be sterilized, the primary packaging must meet the above-mentioned suitability for safety, compatibility and protection even after the sterilization. There should not be any residue or generation of toxic substances of more than a certain safety level after the sterilization. In addition, the primary packaging should have a structure and/or material that must prevent any microbial contamination of the pharmaceutical products contained therein during storage and transportation after sterilization.

1.1.5. Packaging performance

The packaging design with consideration for discrimination, usability, and disposal should be performed.

With regard to discrimination, for example, a display should be considered for patients so that the proper administration and use of the drugs can be ensured and even for aged patients to be able to idenify easily. An easy-to-understand display or container for preventing accidental misuse or a prank, such as tamper-resistant packaging and child-resistant packaging, is preferable.

With regard to usability, items such as easy handling of the drug in dispensing, easy dosaging for children with small doses, easy removal from the container when the drug is administered or used, successful administration, and preferable storage and portability should be considered for each preparation.

On packaging-related waste, the choice or determination of the containers must be considered for disposal, since paying attention to the effect of use of resources, following the Containers and Packaging Recycling Act and the rules of each local government, and striving to reduce wastes are required. In the primary packaging, the recycled packaging materials that are not assured for material composition must not be used.

1.1.6. Requirements of packaging

Based on the test methods and/or the evaluation techniques used for study of the packaging suitability in the design stage of pharmaceutical preparations, the necessary and sufficient items of the quality control for maintaining the packaging suitability are established. Generally, the requirements of packaging are composed of the control of the material quality, specifications and test methods, in-process tests, and the like.

1.2. Examples of suitability evaluation in the design stage of packaging for pharmaceutical products

The following are the examples of the suitability evaluation in the design stage.

1.2.1. Suitability evaluation of packaging to be used for solid oral dosage forms

For the suitability evaluation of the packaging for solid oral dosage forms, the following tests should be included.

- If bottles are used, the measurement of opening torques with selected stoppers should be performed.
- If PTP packaging or strip packaging is used, the moisture permeability test should be performed.

1.2.2. Suitability evaluation of containers to be used for injections

For the suitability evaluation of containers to be used for injections, the following tests should be included.

- The injections using ampules should perform pinhole tests, and the integrity must be confirmed.
- The injections using vials, rubber closures, or prefilled glass syringes, except ampules, should perform the fitting compatibility tests, and the integrity as a container must be confirmed.
- The plastic containers for pharmaceutical products used for injections (prefilled syringes, plastic bottles, plastic bags, etc.) should be verified as ''tight containers in which microorganisms will not be contaminated'' over the shelf life of preparations.

1.2.3. Suitability evaluation on metal impurities for a container closure system

If leaching of metal impurities from the primary packaging materials used for injections, liquids, or semi-solid preparations is suspected, it is necessary to confirm that the amount of metal impurities contained in preparations is sufficiently low from the viewpoint of safety using Atomic Absorption Spectrophotometry *〈2.23〉*, Inductively Coupled Plasma Emission Spectrometry and Inductively Coupled Plasma-Mass Spectrometry *〈2.63〉*, etc.

1.2.4. Suitability evaluation of kit products

If dispensing devices, such as prefilled syringes, injection cartridges, or metered-dose preparations for inhalation, are used, the accurate dose with reproducibility in conditions as close as possible to the product usage must be verified.

1.2.5. Suitability evaluation of light resistant packaging

If active substances are susceptible to the light and the formulation design alone cannot overcome the effect of light, the light resistant packaging including containers should be considered. A selection of an appropriate light resistant packaging must be verified using a photostability test and the like as severe tests.

1.3. Selection, change control, stability monitoring, etc. of the packaging materials in the packaging process development

To maintain appropriately the quality assurance of pharmaceutical products by packaging, the properness of the requirements of the packaging must be confirmed through the appropriate change control, stability monitoring and the like in the packaging process development and production stages, together with the suitability evaluation performed in the design stage.

For the primary packaging materials used in the manufacture of preparations, whether the quality of material is changed by the supplier must be appropriately managed. Therefore, all information concerning the manufacturing process of containers including the information about substances added is desirable to be obtained.

The finally selected packaging for pharmaceutical products should be evaluated whether it meets the requirements as designed. If it does not meet the requirements, the packaging form or material must be changed through change control.

1.3.1. Selection or change of packaging materials in the packaging process development

The packaging materials should be selected in consideration of the manufacturability and the prevention capability

of adhesion of foreign matters or insects in addition to meeting the requirements determined in the design stage. For the finally selected packaging, it should be verified to withstand against temperature change during storage and transportation, physical shock during transportation, and the like.

The suitability of the selected packaging materials shall be confirmed, using the applicable tests in the General Tests, 7. Test for Containers and Packing Materials. For the tests that are not described in the General Tests, set the applicable test and judge the suitability.

1.3.2. Stability monitoring, etc.

It must be confirmed that the packaging form does not adversely affect the stability of preparations through stability monitoring or reference sample stored. If the packaging is considered to affect the quality of stability of the preparations, the applicable packaging and management should be selected to ensuring quality, where the quality of the lots using this should be monitored, and the packaging should be improved through change control, if necessary.

1.4. Examples of quality control in the packaging process of pharmaceutical products

To maintain the suitability of the packaging of pharmaceutical products, it must be confirmed by performing the test such as process control test before shipment that the packaging meets the requirements.

The following shows the examples.

1.4.1. Examples of solid oral dosage forms

The PTP packaged tablets must be confirmed whether the integrity of the seal as designed is secured in the airtight tests for PTP sheets (e.g. water pressure reduction tests).

1.4.2. Examples of injections

- Aqueous injections using ampules should be checked if it has no pinholes.
- The plastic containers for pharmaceutical products used for injections (prefilled syringes, plastic bottles, plastic bags, etc.) should be checked when they are shipped to the market if they have been produced as tight containers, as designed, where microorganisms are not contaminated.

2. Terms of packaging for pharmaceutical products

2.1. Basic terms

Primary packaging: Any packaging that is in direct contact with active substances, excipients, or preparations, and should not give a physical or chemical change to the contents. The primary packaging holds the quality of the pharmaceutical products and provides better performance including convenience.

For example, the primary packaging includes an ampule that is an ''immediate container'' for injections, and a PTP packaging which is an ''inner bag'' for tablets or capsules.

Outside container or outside wrapper: A container or wrapper that is used to contain or to wrap immediate containers or immediate wrappers for the pharmaceutical products for sale or distribution and has a legal label by ordinance[2] on it.

Tight container: A container that protects the contents from extraneous solids or liquids, from loss of the contents, and from efflorescence, deliquescence, or evaporation under ordinary or customary conditions of handling, shipment, and storage. Where a tight container is specified, it may be replaced by a hermetic container. (General Notices 44)

The tight container includes containers made of plastic resins as the examples of container and packaging often used (bottles, vials, syringes, blister (PTP) packaging, strip packaging, etc.).

Final packaging or marketed packaging: Any packaging that is for pharmaceutical products for sale or distribution and a form of the shipped products to the market by labeling as defined by ordinance[2,3].

The final packaging may be used for irradiation when a radiation sterilization method is used.

Labeling and packaging materials[4]: Product's containers, wrappers, and labeling including package insert.

Immediate wrapper: A container in which pharmaceutical products are contained directly (papers, clothes, plastics, and aluminum bags). It can be sold or distributed as it is by labeling as defined by ordinance[3]. There is an inner bag as an example of immediate wrapper that it is not sold or distributed as it is and does not require a legal label by ordinance[3].

The immediate wrapper in which the pharmaceutical products are contained directly is also referred to as the primary packaging.

Immediate container: A solid container in which the drugs are directly contained (cans, bottles, ampules, vials, tubes, containers for eye drops, boxes, etc.). It can be sold or distributed as it is by labeling as defined by ordinance[3]. In addition, the paper boxes will be the immediate container, if they use the PTP packaging as an inner bag like tablets.

The immediate container in which the drugs are contained directly is also referred to as a primary packaging.

Inner bag: For example, a plastic bag used under the wrapper for moisture-proof and light resistance and a drug bag that contains each single dose of powder. It is referred as a plastic bag, strip packaging, blister packaging (such as a PTP packaging), and a plastic container for suppositories. In addition, when the pharmaceutical products are contained directly in the inner bag, it corresponds to an immediate packaging, however, if it is not sold or distributed as it is, a legal label by ordinance[3] is not required.

The inner bag in which the drugs are contained directly is also referred to as a primary packaging.

Secondary packaging: Any packaging that is a single or multiple packaging to compensate for a primary packaging and is not in direct contact with active substances, excipients, or preparations. Any secondary packaging can keep the quality of pharmaceutical products and add performance, such as preventing errors and convenience in the use of pharmaceutical products.

Wrapper: A container or parcel made of soft materials such as paper, cloth, plastic, and aluminum bag. As examples of the wrapper for pharmaceutical products, there are medicine envelope, plastic bag, strip packaging, and blister packaging (such as a PTP packaging).

Labeling[5]: A labeling defined by ordinance[2,3], which is a product label and package insert.

Sealing: Sealing from which drugs cannot be taken out unless opened and does not allow its original state to be easily restored after being opened, according to ordinance.[6]

Packaging[7]: The appropriate materials, containers or wrappers to keep the quality of pharmaceutical products under ordinary or customary conditions of handling, shipment, storage, or usage, and techniques to hold the products in them, or a packaged state.

Hermetic container: A container that is impervious to air or any other gas under ordinary or customary conditions of handling, shipment, and storage. (General Notices 45)

For the injections, ampules, container closure systems, such as vials/rubber closures, glass prefilled syringes may be used as this container. For the other dosage forms, blister (PTP) packaging with both sides of aluminum and metal extrusion tubes may be used as this container.

Well-closed container: A container that protects the contents from extraneous solids and from loss of the drug under ordi-

nary or customary conditions of handling, shipment, and storage. Where a well-closed container is specified, it may be replaced by a tight container. (General Notices 43)

The well-closed container includes paper or plastic bags with one opening made of a flexible material, and cans with metal or plastic resins, etc., as the examples of often used.

Container: A device that holds drugs. The stopper or cap is included as a part of the container. The containers have no physical and chemical reactivity affecting the specified description and quality of the contents (General Notices 42).

As examples of the container for pharmaceutical products, there are cans, bottles, tubes, ampules, vials, and boxes.

Container closure system: A packaging form that consists of the materials used for a primary packaging that is in direct contact with active substances, excipients, or preparations and composed of other materials. A container closure system should be considered in combination with the contents, where the quality cannot be guaranteed with primary packaging alone, the materials used for a secondary packaging should be included.

2.2. Terms of individual packaging or containers

Ampule: A container that is made of a clear or colored glass or plastic that encapsulates drug solution such as injections, or freeze-dried contents. The opening is usually sealed or welded.

Collapsible tube[7]**:** A container that has a nozzle and cap at one end, and the other end is closed, having flexibility to extrude the contents of ointments. This includes metal tubes, plastic tubes, and laminated tubes, etc.

Syringe: A container that is composed of an external cylinder (barrel), a gasket, a pusher (plunger), and a top cap. This may include a needle. It is used for prefilled syringes.

Strip packaging[7]**:** A packaging in which tablets, capsules, powder, and granules are directly tucked between two materials and bonded to the surrounding. It is also referred to as the SP packaging and corresponds to an inner bag or primary packaging as the pharmaceutical products are directly contained.

Vial: A container, a type of bottle, that is made of a clear or colored glass, or plastic used for injections. This is sealed with a rubber closure and aluminum cap.

PTP packaging[7] **(Press through packaging):** Any packaging that is a kind of a blister packaging, using collapsible materials such as aluminum foils to extrude the opening of the plastic forming products. This corresponds to an inner bag or primary packaging as the capsules or tablets are directly contained.

Pillow type packaging[7]**:** Any packaging that is a kind of bag-shaped. For example, in which the vertical central portion is bonded and the top and bottom edges are sealed. Where the primary packaging alone is difficult to ensure quality, a secondary packaging, which consists of composite films laminated with aluminum foils for protection from moisture and lights, is often used.

Plastic bags: A soft container that uses polyethylene or polypropylene resins as single or composite materials, and which has one or more openings. This usually uses a rubber closure as a plug body. It is used as a large volume injection container such as parenteral infusions.

Blister packaging[7]**:** Any packaging where plastic or aluminum foils are heat formed and given one or more pockets and the preparations are put therein, the opening is covered with plastic films, sheets, or aluminum foil, and the periphery to the substrate is bonded or fixed. It refers to the form that is carried out by peeling the film or foil as the preparations are removed, and used for capsules, tablets, prefilled syringes agent, kit products containing a plurality of ampules.

In addition, where the tables, etc. are directly contained, it corresponds to an inner bag or primary packaging.

Single-dose packages: Preparations in single-dose packages. For example, a strip packaging which contains powders or granules for a single dose corresponds to this.

2.3. Terms of packaging performance

Gas barrier packaging[7]**:** Any packaging that gives the function of suppressing gas permeability aimed. This is a low-gas-permeability packaging.

Light resistant container and packaging: A container or packaging which prevents light permeability to protect if the light affects the quality of the contents under ordinary or customary conditions of handling, shipment, and storage. (General Notices 46)

In addition to colored containers to be used, the containers may be covered with shrink films.

Tamper-resistant packaging, tamper-proof packaging[7]**:** Any packaging that is designed to prevent a risk if a person unintentionally handles, or ''plays a prank''.

Child-resistant packaging, childproof packaging[7]**:** Any packaging that is intended for prevention of accidental ingestion by children and may be used for adults properly not to let children open it accidentally.

Moisture-proof packaging[7]**:** Any packaging that uses a material with moisture-proof performance to protect the pharmaceutical products from the effects of moisture, if necessary, using desiccant to keep the inside dry.

3. Reference

1) FDA Guidance for Industry ''Container Closure Systems for Packaging Human Drugs and Biologics'', May 1999.
2) November 25, 2014 enforcement, ''The Law on Securing Quality, Efficacy and Safety of Products including Pharmaceuticals and Medical Devices'' provide in Article 51
3) ''The Law on Securing Quality, Efficacy and Safety of Products including Pharmaceuticals and Medical Devices'' provided in Article 50
4) MHLW Ministerial Ordinance No. 179, ''Ministerial Ordinance on Standards for Manufacturing Control and Quality Control for Drugs and Quasi-drugs'' of December 24, 2004 provided in Article 2 Paragraph 2
5) PFSB/CND Notification No. 0830 – 1 Office Memorandum, ''Ministerial Ordinance on Standards for Manufacturing Control and Quality Control for Drugs and Quasi-drugs'' of August 30, 2013
6) ''The Law on Securing Quality, Efficacy and Safety of Products including Pharmaceuticals and Medical Devices'' provided in Article 58
7) Japanese Industrial Standards JIS Z 0108: 2012 ''Packaging-Vocabulary''

Glossary for Quality by Design (QbD), Quality Risk Management (QRM) and Pharmaceutical Quality System (PQS) *<G0-6-172>*

1. Introduction

The purpose of this glossary is to define terms, used for developing the new concept of quality assurance in ICH Q8 to 11 guidelines so-called Q quartet, and to explain the con-

cept. The terms shown here are determined as the result of discussion for long time in ICH, and are most important to understand the concept of systematic quality assurance based on science and quality risk management, as shown by the guidelines. The usage may not necessarily accord with general usage, however it is necessary to keep in mind that the following definition is used in the regulatory application of pharmaceuticals. The terms used in ICH Q8 to Q11 are shown below in their order. For terms explained in more than one guideline, the name of duplicated guideline is described in parentheses at the end of the corresponding sentence.

2. Glossary

[ICH Q8 Guideline]

Control Strategy: A planned set of controls, derived from current product and process understanding, that ensures process performance and product quality. The controls can include parameters and attributes related to drug substance and drug product materials and components, facility and equipment operating conditions, in-process controls, finished product specifications, and the associated methods and frequency of monitoring and control (ICH Q10, Q11). A control strategy is expected irrespective of development approaches. Under the development approach using Quality by Design, testing, monitoring or controlling can be shifted earlier into the process.

Quality by Design (QbD): A systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management.

Continuous Process Verification: An alternative approach to process validation in which manufacturing process performance is continuously monitored and evaluated. Process validation protocol can use Continuous Process Verification (CPV) to the process validation protocol for the initial and ongoing commercial production (ICH Q11). Generally, for initial process validation, CPV is more appropriate when QbD approach has been applied. However, it can also be used when extensive process knowledge has been gained through commercial manufacturing experience.

Process Robustness: Ability of a process to tolerate variability of materials and changes of the process and equipment without negative impact on quality (ICH Q11).

Critical Process Parameter (CPP): A process parameter whose variability has an impact on a critical quality attribute and therefore should be monitored or controlled to ensure the process produces the desired quality.

Critical Quality Attribute (CQA): A physical, chemical, biological or microbiological property or characteristic that should be within an appropriate limit, range, or distribution to ensure the desired product quality (ICH Q11). For example, CQAs of solid oral dosage forms are typically those aspects affecting product purity, strength, drug release and stability as described in ICH Q8, however it is usual to include product purity and strength itself in CQAs.

Formal Experimental Design: A structured, organized method for determining the relationship between factors affecting a process and the output of that process. Also known as ''Design of Experiments'' (DoE). The factors to be studied in a DoE could come from the risk assessment exercise or prior knowledge.

Design Space (DS): The multidimensional combination and interaction of input variables (e.g., material attributes) and process parameters that have been demonstrated to provide assurance of quality. Working within the design space is not considered as a change. Movement out of the design space is considered to be a change and would normally initiate a regulatory post approval change process. Design space is proposed by the applicant and is subject to regulatory assessment and approval (ICH Q10, Q11). Design space can be updated over the lifecycle as additional knowledge is gained. Since Proven Acceptable Range (PAR) from only univariate experimentation may lack an understanding of interactions between process parameters and/or material attributes, it should be noted that a combination of PAR does not constitute a design space.

Quality: The degree to which a set of inherent properties of a product, system or process fulfills requirements (ICH Q6A, Q8, Q10). The suitability of either a drug substance or a drug product for its intended use. This term includes such attributes as the identity, strength, and purity (ICH Q6A, Q8, Q9, Q10).

Process Analytical Technology (PAT): A system for designing, analyzing, and controlling manufacturing through timely measurements (i.e., during processing) of critical quality and performance attributes of raw and in-process materials and processes with the goal of ensuring final product quality.

Quality Target Product Profile (QTPP): A prospective summary of the quality characteristics of a drug product that ideally will be achieved to ensure the desired quality, taking into account safety and efficacy of the drug product. Quality Target Product Profile describes the design criteria for the product, and should therefore form the basis for development of the product (ICH Q8).

Lifecycle: All phases in the life of a product from the initial development through marketing until the product's discontinuation (ICH Q11).

Real Time Release Testing (RTRT): The ability to evaluate and ensure the quality of in-process and/or final product based on process data, which typically include a valid combination of measured material attributes and process controls (ICHQ11). Parametric release is one type of Real Time Release Testing. It is based on process data rather than testing of material and/or a sample for a specific attribute. For details, refer to ''Basic Concepts for Quality Assurance of Drug Substances and Drug Products <*G0-1-172*>'' in General Information.

Proven Acceptable Range (PAR): A characterized range of a process parameter for which operation within this range, while keeping other parameters constant, will result in producing a material meeting relevant quality criteria.

[ICH Q9 Guideline]

Decision Maker(s): Person(s) with the competence and authority to make appropriate and timely quality risk management decisions.

Harm: Damage to health, including the damage that can occur from loss of product quality or availability.

Trend: A statistical term referring to the direction or rate of change of a variable(s).

Detectability: The ability to discover or determine the existence, presence, or fact of a hazard.

Severity: A measure of the possible consequences of a hazard.

Product Lifecycle: All phases in the life of the product from

the initial development through marketing until the product's discontinuation.

Hazard: The potential source of harm (ISO/IEC Guide 51).

Quality System: The sum of all aspects of a system that implements quality policy and ensures that quality objectives are met.

Requirements: The explicit or implicit needs or expectations of the patients or their surrogates (e.g., health care professionals, regulators and legislators). In this document (ICH Q9), ''requirements'' refers not only to statutory, legislative, or regulatory requirements, but also to such needs and expectations.

Stakeholder: Any individual, group or organization that can affect, be affected by, or perceive itself to be affected by a risk. Decision makers might also be stakeholders. For the purposes of this guideline, the primary stakeholders are the patient, healthcare professional, regulatory authority, and industry.

Risk: The combination of the probability of occurrence of harm and the severity of that harm (ISO/IEC Guide 51).

Risk Assessment: A systematic process of organizing information to support a risk decision to be made within a risk management process. It consists of the identification of hazards and the analysis and evaluation of risks associated with exposure to those hazards.

Risk Communication: The sharing of information about risk and risk management between the decision maker and other stakeholders.

Risk Control: Actions implementing risk management decisions (ISO Guide 73).

Risk Acceptance: The decision to accept risk (ISO Guide 73).

Risk Reduction: Actions taken to lessen the probability of occurrence of harm and the severity of that harm.

Risk Identification: The systematic use of information to identify potential sources of harm (hazards) referring to the risk question or problem description.

Risk Evaluation: The comparison of the estimated risk to given risk criteria using a quantitative or qualitative scale to determine the significance of the risk.

Risk Analysis: The estimation of the risk associated with the identified hazards.

Risk Management: The systematic application of quality management policies, procedures, and practices to the tasks of assessing, controlling, communicating and reviewing risk.

Risk Review: Review or monitoring of output/results of the risk management process considering (if appropriate) new knowledge and experience about the risk.

[ICH Q10 Guideline]

Innovation: The introduction of new technologies or methodologies.

Pharmaceutical Quality System (PQS): Management system to direct and control a pharmaceutical company with regard to quality (ICH Q10 based upon ISO 9000:2005) .

Outsourced Activities: Activities conducted by a contract acceptor under a written agreement with a contract giver.

State of Control: A condition in which the set of controls consistently provides assurance of continued process performance and product quality.

Performance Indicators: Measurable values used to quantify quality objectives to reflect the performance of an organization, process or system, also known as ''performance metrics'' in some regions.

Continual Improvement: Recurring activity to increase the ability to fulfil requirements (ISO 9000:2005).

Senior Management: Person(s) who direct and control a company or site at the highest levels with the authority and responsibility to mobilize resources within the company or site (ICH Q10 based in part on ISO 9000:2005).

Capability of a Process: Ability of a process to realize a product that will fulfil the requirements of that product. The concept of process capability can also be defined in statistical terms (ISO 9000:2005).

Product Realization: Achievement of a product with the quality attributes appropriate to meet the needs of patients, health care professionals, and regulatory authorities (including compliance with marketing authorization) and internal customers requirements.

Corrective Action: Action to eliminate the cause of a detected non-conformity or other undesirable situation. NOTE: Corrective action is taken to prevent recurrence whereas preventive action is taken to prevent occurrence (ISO 9000:2005).

Enabler: A tool or process which provides the means to achieve an objective.

Knowledge Management: Systematic approach to acquiring, analyzing, storing, and disseminating information related to products, manufacturing processes and components.

Quality Planning: Part of quality management focused on setting quality objectives and specifying necessary operational processes and related resources to fulfil the quality objectives (ISO 9000:2005).

Quality Policy: Overall intentions and direction of an organization related to quality as formally expressed by senior management (ISO 9000:2005).

Quality Manual: Document specifying the quality management system of an organization (ISO 9000:2005).

Quality Objectives: A means to translate the quality policy and strategies into measurable activities.

Quality Risk Management (QRM): A systematic process for the assessment, control, communication and review of risks to the quality of the drug (medicinal) product across the product lifecycle (ICH Q9, Q10). For details, refer to ''Basic Concept of Quality Risk Management *<G0-2-170>*'' in General Information.

Feedback/Feedforward: The modification or control of a process or system by its results or effects. Feedback/feedforward can be applied technically in process control strategies and conceptually in quality management. Feedback is to reflect results to a previous process (for example: a control of the supply of materials in a previous process), and feedforward is to reflect results to a subsequent process (for example: a control of time for drying in a subsequent process).

Change Management: A systematic approach to proposing, evaluating, approving, implementing and reviewing changes.

Preventive Action: Action to eliminate the cause of a potential non-conformity or other undesirable potential situation.

Note: Preventive action is taken to prevent occurrence whereas corrective action is taken to prevent recurrence (ISO 9000:2005).

[ICH Q11 Guideline]
Chemical Transformation Step: For Chemical Entities, a step involved in the synthesis of the chemical structure of the drug substance from precursor molecular fragments. Typically it involves C—X or C—C bond formation or breaking.

Contaminants: Any adventitiously introduced materials (e.g., chemical, biochemical, or microbial species) not intended to be part of the manufacturing process of the drug substance or drug product (ICH Q6B).

3. References

1) ICH: Guideline for Q8(R2), Pharmaceutical Development.
2) ICH: Guideline for Q9, Quality Risk Management.
3) ICH: Guideline for Q10, Pharmaceutical Quality Systems.
4) ICH: Guideline for Q11, Development and Manufacture of Drug Substance (Chemical Entities and Biotechnological/Biological Entities).
5) ICH: Quality Implementation Working Group, Points to Consider (R2), ICH-Endorsed Guide for ICH Q8/Q9/Q10 Implementation
6) ICH: Quality Implementation Working Group on Q8, Q9 and Q10 Questions & Answers (R4)

G1 Physics and Chemistry

Validation of Analytical Procedures
〈G1-1-130〉

The validation of an analytical procedure is the process of confirming that the analytical procedure employed for a test of pharmaceutics is suitable for its intended use. In other word, the validation of an analytical procedure requires us to demonstrate scientifically that risks in decision by testing caused by errors from analytical steps are acceptably small. The performance of an analytical procedure is established by various kinds of validation characteristics. The validity of a proposed analytical procedure can be shown by demonstrating experimentally that the validation characteristics of the analytical procedure satisfy the standards set up according to the acceptable limits of testing.

When an analytical procedure is to be newly carried in the Japanese Pharmacopoeia, when a test carried in the Japanese Pharmacopoeia is to be revised, and when the test carried in the Japanese Pharmacopoeia is to be replaced with a new test according to regulations in general notices, analytical procedures employed for these tests should be validated according to this document.

1. Required data for analytical procedures to be carried in the Japanese Pharmacopoeia

1.1. Outline

This section should provide a brief explanation of the principle of a proposed analytical procedure, identify the necessity of the analytical procedure and its advantage compared with other procedures, and summarize the validation. When an analytical procedure is revised, the limitation of the current analytical procedure and the advantage offered by the new analytical procedure should be described.

1.2. Analytical procedure

This section should contain a complete description of the analytical procedure to enable skilled persons to evaluate correctly the analytical procedure and replicate it if necessary. Analytical procedures include all important operating procedures for performing analyses, the preparation of standard samples, reagents and test solutions, precautions, procedures to verify system suitability (e.g. the verification of the separating performance of a chromatographic system), formulas to obtain results, the number of replications and so forth. Any instruments and apparatus that are not stated in the Japanese Pharmacopoeia should be described in detail. The physical, chemical or biological characteristics of any new reference standards should be clarified and their testing methods should be established.

1.3. Data showing the validity of analytical procedures

This section should provide complete data showing the validity of the analytical procedures. This includes the experimental design to determine the validation characteristics, experimental data, calculation results and results of hypothesis tests.

2. Validation characteristics

The definition of typical validation characteristics to be assessed in validation of analytical procedures and examples of assessing procedures are given below.

The terminology and definitions of the validation characteristics may possibly vary depending upon the fields to which analytical procedures are applied. The terminology and definitions shown in this document are established for the purpose of the Japanese Pharmacopoeia. Typical methods for assessing the validation characteristics are shown in the item of assessment. Various kinds of methods to determine the validation characteristics have been proposed and any methods that are widely accepted will be accepted for the present purpose. However, since values of the validation characteristics may possibly depend upon methods of determination, it is required to present the methods of determining the validation characteristics, the data and calculation methods in sufficient detail.

Although robustness is not listed as a validation characteristic, it should be considered during the development of analytical procedures. Studying the robustness may help to improve analytical procedures and to establish appropriate analytical conditions including precautions.

2.1. Accuracy/Trueness

2.1.1. Definition

The accuracy is a measure of the bias of observed values obtained by an analytical procedure. The accuracy is expressed as the difference between the average value obtained from a large series of observed values and the true value.

2.1.2. Assessment

The estimate of accuracy of an analytical method is expressed as the difference between the total mean of observed values obtained during investigation of the reproducibility and the true value. A certified value or a consensus value may be used as the true value. When an analytical procedure for a drug product is considered, the observed value of the standard solution of the drug substance may be used as the consensus value.

It may be inferred from specificity data that an analytical procedure is unbiased.

A 95% confidence interval of the accuracy should be calculated using the obtained estimate of accuracy and the standard error based on the reproducibility (intermediate precision). It should be confirmed that the confidence interval includes zero or that the upper or lower confidence limits

are within the range of the accuracy required of the analytical procedure.

2.2. Precision

2.2.1. Definition

The precision is a measure of the closeness of agreement between observed values obtained independently from multiple samplings of a homogenous sample and is expressed as the variance, standard deviation or relative standard deviation (coefficient of variation) of observed values.

The precision should be considered at three levels with different repetition conditions; repeatability, intermediate precision and reproducibility.

(i) Repeatability/Intra-assay precision

The repeatability expresses the precision of observed values obtained from multiple samplings of a homogenous sample over a short time interval within a laboratory, by the same analyst, using the same apparatus and instruments, lots of reagents and so forth (repeatability conditions).

(ii) Intermediate precision

The intermediate precision expresses the precision of observed values obtained from multiple samplings of a homogenous sample by changing a part of or all of the operating conditions including analysts, experimental dates, apparatus and instruments and lots of reagents within a laboratory (intermediate precision condition).

(iii) Reproducibility

The reproducibility expresses the precision of observed values obtained from multiple samplings of a homogenous sample in different laboratories (reproducibility condition).

2.2.2. Assessment

A sufficient volume of a homogenous sample should be prepared before studying the precision. The solution is assumed to be homogenous. When it is difficult to obtain a homogenous sample, the following samples may be used as homogenous samples; e.g., a large amount of drug products or mixture of drug substance and vehicles that are crushed and mixed well until they can be assumed to be homogenous.

Suitable experimental designs such as one-way layout may be employed when more than one level of precision is to be investigated simultaneously. A sufficient number of repetitions, levels of operating conditions and laboratories should be employed. Sources of variations affecting analytical results should be evaluated as thoroughly as possible through the validation.

It is required to show the variance, standard deviation and relative standard deviation (coefficient of variation) of each level of precision. The 90% confidence interval of the variance and corresponding intervals of the standard deviation should also be established. The validity of the proposed analytical procedure for its intended use may be confirmed by comparing obtained values with the reference values of precision required for the analytical procedure. Whether the proposed analytical procedure is acceptable may normally be decided based on the reproducibility.

2.3. Specificity

2.3.1. Definition

The specificity is the ability of an analytical procedure to measure accurately an analyte in the presence of components that may be expected to be present in the sample matrix. The specificity is a measure of discriminating ability. Lack of specificity of an analytical procedure may be compensated by other supporting analytical procedures.

2.3.2. Assessment

It should be confirmed that the proposed analytical procedure can identify an analyte or that it can accurately measure the amount or concentration of an analyte in a sample. For example, the specificity may be assessed by comparing analytical results obtained from a sample containing the analyte only with results obtained from samples containing excipients, related substances or degradation products, and including or excluding the analyte. If reference standards of impurities are unavailable, samples that are expected to contain impurities or degradation products may be used (e.g. samples after accelerated or stress tests).

2.4. Detection limit

2.4.1. Definition

The detection limit is the lowest amount or concentration of the analyte in a sample that is detectable, but not necessarily quantifiable.

2.4.2. Assessment

The detection limit should be normally determined so that producer's and consumer's risks are less than 5%. The detection limit may be calculated using the standard deviation of responses of blank samples or samples containing an analyte close to the detection limit and the slope of the calibration curve close to the detection limit. The following equation is an example to determine the detection limit using the standard deviation of responses of blank samples and the slope of the calibration curve.

$$DL = 3.3\sigma/slope$$

DL: detection limit
σ: the standard deviation of responses of blank samples
slope: slope of the calibration curve

The noise level may be used as the standard deviation of responses of blank samples in chromatographic methods.

It should be ensured that the detection limit of the analytical procedure is lower than the specified limit for testing.

2.5. Quantitation limit

2.5.1. Definition

The quantitation limit is the lowest amount or concentration of the analyte in a sample that can be determined. The precision expressed as the relative standard deviation of samples containing an analyte at the quantitation limit is usually 10%.

2.5.2. Assessment

The quantitation limit may be calculated using the standard deviation of responses of blank samples or samples containing an analyte close to the quantitation limit and the slope of the calibration curve close to the quantitation limit. The following equation is an example to determine the quantitation limit using the standard deviation of responses of blank samples and the slope of the calibration curve.

$$QL = 10\sigma/slope$$

QL: quantitation limit
σ: the standard deviation of responses of blank samples
slope: slope of the calibration curve

The noise level may be used as the standard deviation of responses of blank samples in chromatographic methods.

It should be ensured that the quantitation limit of the analytical procedure is lower than the specified limit for testing.

2.6. Linearity

2.6.1. Definition

The linearity is the ability of an analytical procedure to elicit responses linearly related to the amount or concentration of an analyte in samples. A well-defined mathematical transformation may sometimes be necessary to obtain a linear relationship.

2.6.2. Assessment

Responses are obtained after analyzing samples repeatedly with various amounts or concentrations of an analyte according to described operating procedures. The linearity may

be evaluated in terms of the correlation coefficient and the regression equation. It may be also helpful for evaluating the linearity to plot residual errors from the regression line against the amount or concentration and to confirm that there is no particular tendency in the graph. Samples with five different amounts or concentrations of an analyte should be usually investigated.

2.7. Range

2.7.1. Definition

The range for the validation of analytical procedures is the interval between the lower and upper limits of the amount or concentration of an analyte providing sufficient accuracy and precision. The range for the validation of analytical procedures for an analytical procedure with linearity is the interval between the lower and upper limits providing sufficient accuracy, precision and linearity.

2.7.2. Assessment

When the range for the validation of analytical procedures is investigated, 80 to 120% of specified limits of testing should be usually considered. The accuracy, precision and linearity should be evaluated using samples containing the lower and upper limits and in the middle of the range.

3. Categories of tests employing analytical procedures

Tests covered with this document are roughly classified into three categories shown below according to their purposes. The table lists the normally required validation characteristics to be evaluated in the validation of analytical procedures used in these tests. This list should be considered to represent typical validation characteristics. This is a principle, and validation characteristics to be assessed vary depending upon the characteristics of analytical procedures and their intended use.

(i) Type I Identification. Tests for identifying major components in pharmaceuticals according to their characteristics.

(ii) Type II Impurity tests. Tests for determination of impurities in pharmaceuticals.

(iii) Type III Tests for assaying components in pharmaceuticals.

(Additives such as stabilizing agents and preservatives are included in components.) Tests for determining performance of pharmaceuticals, such as dissolution testing.

Table Lists of validation characteristics required to be evaluated in tests of each type

Validation characteristics \ Type of test	Type I	Type II		Type III
		Quantitation test	Limit test	
Accuracy/Trueness	−	+	−	+
Precision				
Repeatability	−	+	−	+
Intermediate precision	−	−*	−	−*
Reproducibility	−	+*	−	+*
Specificity**	+	+	+	+
Detection limit	−	−	+	−
Quantitation limit	−	+	−	−
Linearity	−	+	−	+
Range	−	+	−	+

− Usually need not to be evaluated.

\+ Usually need to be evaluated.

* Either intermediate precision or reproducibility should be evaluated depending upon circumstances in which analytical procedures or tests are performed. The latter should be normally evaluated in the validation of analytical procedures proposed to be included in the Japanese Pharmacopoeia.

** The lack of the specificity of an analytical procedure may be compensated by other relevant analytical procedures

4. Terminology used in the validation of analytical procedures

(i) Analytical procedure: This document covers analytical procedures applied to identification, and ones that provides responses depending upon the amount or concentration of analytes in samples. Analytical procedures in this document mean analytical processes of tests.

(ii) Laboratory: The laboratory means an experimental room or facility where tests are performed. In this document different laboratories are expected to perform an analytical procedure using different analysts, different experimental apparatus and instruments, different lots of reagents and so forth.

(iii) Number of replications: The number of replications is one that is described in analytical procedures. An observed value is often obtained by more than one measurement in order to achieve good precision of analytical procedures. Analytical procedures including the number of replications should be validated.

This is different from repetition in the validation of analytical procedures to obtain precision.

(iv) Observed value: The value of a characteristic obtained as the result of performing an analytical procedure.

(v) Consumer's risk: This is the probability that products out of the specification of tests are decided to be accepted after testing. It is usually expressed as β, and is called the probability of type II error or the probability of false negative in impurity tests.

(vi) Producer's risk: This is the probability that products satisfying the specification of tests are decided to be rejected after testing. It is usually expressed as α, and is called the probability of type I error or the probability of false positive in impurity tests.

(vii) Robustness: The robustness is a measure of the capacity to remain unaffected by small but deliberate variations in analytical conditions. The stability of observed values may be studied by changing various analytical conditions within suitable ranges including pH values of solutions, reaction temperature, reaction time or amount of reagents added. When observed values are unstable, the analytical procedure should be improved. Results of studying robustness may be reflected in the developed analytical procedure as precautions or significant digits describing analytical conditions.

(viii) Test: Tests mean various tests described in general tests and official monographs in the Japanese Pharmacopoeia such as impurity tests and assay. They include sampling methods, specification limits and analytical procedures.

System Suitability 〈*G1-2-152*〉

In order to ensure the reliability on the results of drug analyses, it is essential to verify that the test method to be applied to the test, including the method prescribed in the Japanese Pharmacopoeia (JP), can give the results adequate for its intended use using the analytical system in the laboratory in which the test is to be performed, then to carry out system suitability testing for confirming that the analytical system maintains the state suitable for the quality test.

1. Definition and role of system suitability

''System Suitability'' is the concept for ensuring that the

performance of the analytical system is as suitable for the analysis of the drug as was at the time when the verification of the test method was performed using the system. Usually, system suitability testing should be carried out at every series of drug analysis. The test procedures and acceptance criteria of system suitability testing must be prescribed in the test methods of drugs. The results of drug analyses are not acceptable unless the requirements of system suitability have been met.

System suitability testing is an integral part of test methods using analytical instruments, and based on the concept that the equipments, electronic data processing systems, analytical operations, samples to be analyzed and operators constitute an integral system that can be evaluated, when the test procedures and acceptance criteria of system suitability testing are prescribed in the test methods.

2. Points to consider in setting system suitability

Parameters of system suitability testing to be prescribed in the test method depend on the intended use and type of analytical method. Since system suitability testing is to be carried out in a routine manner, it is preferable to select the parameters necessary for ensuring that the analytical system maintains the state suitable for the analysis of the drug and to prescribe its test procedure able to carry out easily and rapidly.

For example, in the case of quantitative purity tests using liquid chromatography or gas chromatography, the evaluation of parameters such as ''System performance'' (to confirm the ability to analyze target substance specifically), ''System repeatability'' (to confirm that the degree of variation in the analytical results of target substance in replicate injections is within the allowable limit) and ''Test for required detectability'' (to confirm the linearity of chromatographic response around the specification limit) are usually required.

The followings are supplements to the section of system suitability prescribed in ''Liquid Chromatography''.

2.1. System repeatability of HPLC and GC

2.1.1. Allowable limit of system repeatability

It is described in the section of system suitability in ''Liquid Chromatography'' that ''In principle, total number of replicate injections should be 6'', and ''The allowable limit of ''System repeatability'' should be set at an appropriate level based on the data when suitability of the method for the evaluation of quality of the drug was verified, and the precision necessary for the quality test''. Based on the above description, an allowable limit of system repeatability for 6 replicate injections should be set in consideration with the following descriptions. However, in the case that the test method prescribed in the JP monograph is used for the test, the allowable limit of system repeatability prescribed in the monograph should be applied.

(i) Assay for drug substance (for drug substance with the content nearby 100%): An adequate allowable limit should be set at the level that the chromatographic system is able to give the precision suitable for the evaluation of variation in the content of active ingredient within and among the batches of drug substance. For example, the allowable limit of ''not more than 1.0%'' is usually recommended for the drug substances whose width of content specification are not more than 5%, as is in the case of content specification of 98.0 – 102.0% which is often observed in the assay using liquid chromatography.

(ii) Assay for drug products: An adequate allowable limit should be set considering the width of content specification of the drug product and the allowable limit prescribed in the assay of drug substance (when the drug product is analyzed by a method with the same chromatographic conditions as those used for the analysis of drug substance).

(iii) Purity test for related substances: An adequate allowable limit should be set considering the concentration of active ingredients in the solution used for the system repeatability testing such as a standard solution or solution for system suitability testing. In the case that a solution with active ingredient concentration of 0.5 – 1.0% is used for the test of system repeatability, an allowable limit of ''not more than 2.0%'' is usually recommended.

Recommendations for allowable limits described above should not be applicable to gas chromatography.

2.1.2. Method for decreasing the number of replicate injections without losing the quality of system repeatability testing

It is described in the section of system suitability in ''Liquid Chromatography'' that ''In principle, total number of replicate injections should be 6. However, in the case that a long time is necessary for one analysis, such as the analysis using the gradient method, or the analysis of samples containing late eluting components, it may be acceptable to decrease the number of replicate injections by adopting new allowable limit of ''System repeatability'' which can guarantee a level of ''System repeatability'' equivalent to that at 6 replicate injections.'' In consideration of the above description, a method for decreasing the number of replicate injections without losing the quality of system repeatability testing is described below. One can set the test for system repeatability with reduced number of replicate injections by utilizing this method, if necessary, and routine quality tests can be performed based on the same approach.

The following table shows the allowable limits to be attained in the test at 3 – 5 replicate injections (n = 3 – 5) to

Table Allowable limits to be attained in the test at 3 – 5 replicate injections (n = 3 – 5) to keep the quality of test equivalent to that of test at n = 6*

		Allowable limit (RSD)					
Allowable limit prescribed in the test of n = 6		1%	2%	3%	4%	5%	10%
Allowable limit to be attained	n = 5	0.88%	1.76%	2.64%	3.52%	4.40%	8.81%
	n = 4	0.72%	1.43%	2.15%	2.86%	3.58%	7.16%
	n = 3	0.47%	0.95%	1.42%	1.89%	2.37%	4.73%

* The probability for inadequate analytical systems to meet the requirements of system suitability testing, is supposed to be 5%.

keep the quality test equivalent to that of test at $n = 6$.

However, it should be kept in mind that since decrease in the number of replicate injections results in increase in the weight of each injection, it becomes more important to perform the test by the experienced operator, and to maintain the equipment in a suitable state.

3. Points to consider at the change of analytical system (Change control of analytical system)

When the test method and analytical system verified is continuously used for the quality test without any change, it is sufficient to confirm the compliance to the requirements of system suitability at every series of drug analysis.

However, when the test is performed for a long period, a situation in which some changes in the analytical system are inevitable, may occur. These changes don't affect the quality of the product itself, but they affect the scale in the evaluation of product quality. If the change in the analytical system may induce a significant deviation of the scale, it may lead to the acceptance of products with inadequate quality and/or the rejection of products with adequate quality. Thus, at the time of change in the analytical system, it is necessary to check whether the change is appropriate or not, to avoid the deviation of the scale in the evaluation of product quality.

In the case of the change of test method, it is required to perform an adequate validation depending on the extent of the change. On the other hand, in the case of the change of analytical system in a laboratory, such as renewal of apparatus or column of liquid chromatography, and the change of operator, it is necessary to perform at least system suitability testing using the system after change, and to confirm that the equivalency of the results before and after change.

In the case that equivalent results would not be obtained after change, for example, when a renewal of column of liquid chromatograph may induce a significant change of elution pattern, such as the reversal of elution order between target ingredient of the test and substance for checking resolution, it is required to perform a revalidation of the analytical system for the test using new column, since it is uncertain whether the specificity and/or other validation characteristics necessary for estimating target ingredient is kept or not.

Near Infrared Spectrometry
<G1-3-161>

Near infrared spectrometry (NIR) is one of spectroscopic methods used to qualitatively and quantitatively evaluate substances from analysis of data obtained by determining their absorption spectrum of light in the near-infrared range.

The near-infrared range lies between the visible light and infrared light, typically of wavelengths (wave numbers) between 750 and 2500 nm (13,333 – 4000 cm^{-1}). The absorption of near-infrared light occurs due to harmonic overtones from normal vibration or combination tones in the infrared range (4000 to 400 cm^{-1}), primarily absorption of O-H, N-H, C-H and S-H that involve hydrogen atoms, in particular. For instance the asymmetrical stretching vibration of N-H occurs in the vicinity of 3400 cm^{-1}, but the absorption due to the first harmonic overtone occurs in the vicinity of 6600 cm^{-1} (wavelength 1515 nm), which is near double 3400 cm^{-1}.

Absorption in the near-infrared range is far weaker than absorption due to normal vibration that occurs in the infrared range. Furthermore, in comparison with visible light, near-infrared light has longer wavelength, which makes it possible for the light to penetrate to a depth of several mm into solid specimens including fine particles. This method is often utilized as a nondestructive analysis, as changes occurring with absorbed light spectrum (transmitted light or reflected light) in this process provide physical and chemical information pertaining to specimens.

Conventional spectrometry, such as calibration curve method, is used as a method for analyzing near-infrared absorption spectrum whenever applicable. Ordinarily, however, chemometrics methods are used for analysis. Chemometrics ordinarily involve quantification of chemical data, as well as numerical and statistical procedures for computerization of information. Chemometrics for near-infrared spectrometry includes various types of multivariate analysis such as multiple regression analysis, to perform qualitative or quantitative evaluation of active substances.

Near-infrared spectrometry is used as a rapid and nondestructive method of analysis that replaces conventional and established analysis methods for water determinations or substance verifications. It is necessary to perform a comparison test to evaluate this method against an existing analysis method, to verify that this method is equivalent to such existing analysis method, before using this analysis method as a quality evaluation test method in routine tests.

Applications of near-infrared spectrometry in the pharmaceutical field include qualitative or quantitative evaluation of ingredients, additives or water contents of active substances or preparations. Furthermore, near-infrared spectrometry can also be used for evaluation of physical conditions of substances, such as crystal forms, crystallinity, particle diameters. It is also possible to perform spectrometry on samples that are located in a remote location away from equipment main units, without sampling, by using optical fibers. It can therefore be used as an effective means to perform pharmaceutical manufacturing process control online (or in-line).

1. Equipment

Near-infrared spectrophotometers can either be a distributed near-infrared spectrophotometer or a Fourier transform near-infrared spectrophotometer[1]. Interference filter-type near-infrared spectrophotometers that use interference filter in the spectrometry section are also available, however, this type of equipment is hardly used in the field of pharmaceutical quality control.

1.1. Distributed near-infrared spectrophotometer

This equipment is comprised of light source section, sample section, spectrometry section, photometry section, signal processing section, data processing section, display-record-output section. Halogen lamps, tungsten lamps, light emitting diodes and other such devices that can emit high intensity near-infrared light in a stable manner are used in the light source section. The sample section is comprised of a sample cell and a sample holder. Equipment that has an optical fiber section that is comprised of optical fibers and a collimator are equipped with a function for transmitting light to sample section, which is remotely located away from the spectrophotometer main unit. Quartz is ordinarily used as material for optical fibers.

The spectrometry section is intended to extract light of required wavelength, using dispersive devices and is comprised of slits, mirrors and dispersive devices. Potential dispersive devices include prisms, diffraction grating, acousto-optical tunable filters (AOTF), or liquid crystal tunable filters (LCTF). The photometry section is comprised of detectors and amplifiers. Sensors include semiconductor detectors (silicon, lead sulfide, indium-gallium-arsenic, indium-anti-

mony), as well as photomultiplier tubes. Detecting methods that use semiconductor detectors generally perform detections with single elements, but there are also occasions where arraytype detectors that use multiple elements are used. Such detectors are capable of simultaneously detecting multiple wavelengths (wave numbers). The signal processing section separates signals required for measurements from output signals fed by amplifiers and then outputs such isolated signals. The data processing section performs data conversions and spectral analysis, etc. The display-record-output section outputs data, analysis results and data processing results to a printer.

1.2. Fourier transform near-infrared spectrophotometer

The configuration of the equipment is fundamentally same as that of the distributed-type equipment described in Section 1.1., except for the spectrometry section and the signal processing section.

The spectrometry section is comprised of interferometers, sampling signal generators, detectors, amplifiers, A/D conversion devices, etc. Interferometers include Michelson interferometers, transept interferometers and polarization interferometers. The signal processing section is equipped with functions that are required for spectrometer, as well as a function for translating acquired interference waveform (interferogram) into absorption spectrum by Fourier transformation.

2. Determination

There are three types of measurement methods that are used with near-infrared spectrometry: transmittance method, diffuse reflectance method and transmittance reflectance method. The selection of measurement methods relies on the shape of samples and applications. The transmittance method or diffuse reflectance method is used for solid samples, including fine particles. The transmittance method or transmittance reflectance method is used for liquid samples.

2.1. Transmittance method

The degree of decay for incident light intensity as the light from a light source passes through a sample, is represented as transmittance rate T (%) or absorbance A with the transmittance method. A sample is placed in the light path between a light source and a detector, the arrangement of which is ordinarily same as that of the spectroscopic method.

$$T = 100t$$
$$t = I/I_0 = 10^{-\alpha cl}$$

I_0: Incident light intensity
I: Transmitted light intensity
α: Absorptivity
c: Solution concentration
l: Layer length (sample thickness)

$$A = -\log t = \log (1/t) = \log (I_0/I) = \alpha cl$$

This method is applied for taking measurements of samples that are liquids and solutions. Quartz glass cells and flow cells are used, with the layer length of 1 – 5 mm along. Furthermore, this method can also be applied for taking measurements of samples that are solids, including fine particles. It is also known as diffuse transmittance method. Selecting appropriate layer length is critical for this method, since the transmitted light intensity varies depending on grain sizes and surface condition of samples.

2.2. Diffuse reflectance method

The ratio of the reflection light intensity I, emitted from the sample in a wide reflectance range and a control reflection light intensity I_r emitted from surface of a substance, is expressed as reflectance R (%) with the diffuse reflectance method. The near-infrared light penetrates to a depth of several mm into solid samples, including fine particles. In that process, transmission, refraction, reflection and dispersion are repeated, and diffusion takes place, but a portion of the diffused light is emitted again from the surface of the sample and captured by a detector. The spectrum for the diffuse reflectance absorbance (A_r) can ordinarily be obtained by plotting logarithm of inverse numbers for reflectance ($1/r$) against wavelengths (wave numbers).

$$R = 100r$$
$$r = I/I_r$$

I: Reflection light intensity of light, diffuse reflected off the sample
I_r: Control reflection light intensity of light emitted from surface of reference substance

$$A_r = \log (1/r) = \log (I_r/I)$$

The intensity of diffuse reflectance spectrum can also be expressed with the Kubelka-Munk (K-M) function. The K-M function is derived, based on the existence of a sample with sufficient thickness, and expressed in terms of light scattering coefficient, which is determined by absorptivity, grain size, shape and fill condition (compression).

This method is applied to solid samples, including fine particles, and requires a diffuse reflector.

2.3. Transmittance reflectance method

The transmittance reflectance method is a combination of the transmittance method and reflectance method. A mirror is used to re-reflect a light that has passed through a sample in order to take a measurement of transmittance reflectance rate, T^* (%). Light path must be twice the thickness of the sample. On the other hand, the light reflected by a mirror and being introduced into a detector is used as the control light. When this method is applied to suspended samples, however, a metal plate or a ceramic reflector with rough surface that causes diffuse reflectance is used instead of a mirror.

Transmittance reflectance absorbance (A^*) is obtained by the following formula with this method:

$$T^* = 100t^*$$
$$t^* = I/I_T$$

I: Intensity of transmitted and reflected light, in cases where a sample is placed
I_T: Intensity of reflected light, in cases where there is no sample

$$A^* = \log (1/t^*)$$

This is a method that is applied to solid samples, including fine particles, as well as liquids and suspended samples. The thickness of a sample must be adjusted when applying this method to a solid sample. Ordinarily adjustment is made by setting absorbance to 0.1 – 2 (transmittance of 79 – 1%), which provides the best linearity and SN ratio of detector. A cell with appropriate layer length, according to the grain size of the fine particle, must be selected when applying the method to a fine particle sample.

3. Factors that affect spectrum

Following items must be considered as factors that can affect spectrum when applying near-infrared spectrometry, particularly when conducting quantitative analysis.

(i) Sample temperature: A significant change (wavelength shift, for example) can occur when the temperature varies by a several degree (°C). Care must be taken, particu-

larly when the sample is a solution or contains water.

(ii) Water or residual solvent: Water or residual solvent contents of a sample, as well as water (humidity) in the environment wherein measurements are taken, can significantly affect absorption band of the near-infrared range.

(iii) Sample thickness: The thickness of a sample is a factor for spectral changes and therefore needs to be controlled at a certain thickness. A sample may be considered to be of adequate thickness for the diffuse reflectance method, however, if the thickness is less than a certain amount, for example, the sample may have to be placed on a support plate with high reflectance to take measurements by the transmittance reflectance method.

(iv) Fill condition of sample: The condition of sample fill can potentially affect a spectrum, when taking measurements of samples that are solids or fine particles. Care must be taken with filling samples in a cell, to ensure that a certain amount is filled through a specific procedure.

(v) Optical characteristics of samples: When a sample is physically, chemically or optically uneven, relatively large beam size must be used, multiple samples must be used, measurements must be taken at multiple points on the same samples, or a sample must be pulverized to ensure averaging of the sample. Grain size, fill condition, as well as roughness of surface can also affect fine particle samples.

(vi) Polymorphism: Variations in crystal structures (polymorphism) can also affect spectrum. In cases where multiple crystal forms exist, it is necessary to have consideration for characteristics of samples to be considered and care must be taken to ensure that even standard samples for calibration curve method have diversified distributions similar to that of samples that are subject to analysis.

(vii) Temporal changes in characteristics of samples: Samples can potentially undergo chemical, physical or optical property changes, due to passing of time or storage after sampling, and such changes affect spectrum in a subtle manner. For instance even with identical samples, if elapsed times differ, then their characteristics of near-infrared spectrum can vary significantly. In creating calibration curves, therefore, the samples to be used must be prepared with adequate considerations for reducing the time to be measured, such as the measurement is carried out offline in a laboratory or online in manufacturing process (or inline).

4. Control of equipment performance[2,3]

4.1. Accuracy of wavelengths (wave numbers)

The accuracy of wavelengths (wave numbers) of an equipment is derived from the deviation of the absorption peaks of substances for which peak absorption wavelengths (wave numbers) have been defined, such as polystyrene, mixture of rare earth oxides (dysprosium, holmium and erbium; 1:1:1) or steam, from the figures indicated on the equipment. Tolerance figures in the vicinity of 3 peaks are ordinarily set in the following manner, though appropriate tolerance figures can be set, depending on the intended purpose:

1200 ± 1 nm (8300 ± 8 cm^{-1})
1600 ± 1 nm (6250 ± 4 cm^{-1})
2000 ± 1.5 nm (5000 ± 4 cm^{-1})

Since the location of absorption peaks vary, depending on the substance used as reference, absorption peaks of wavelengths (wave numbers) that are closest to the above 3 peaks are selected for suitability evaluations. A mixture of rare earth oxides, for instance, would indicate characteristic absorption peaks at 1261 nm, 1681 nm and 1971 nm.

Absorption peaks at 1155 nm, 1417 nm, 1649 nm, 2352 nm (layer length: 1.0 nm) can be used, when taking measurements with the transmittance method that involve the use of dichloromethane as reference. The absorption peak of steam at 7306.7 cm^{-1} can be used with a Fourier transformation-type spectrophotometer, as its wave number resolution ability is high.

Other substances can also be used as reference, so long as their adequacy for the purpose can be verified.

4.2. Spectroscopic linearity

Appropriate standard plates, such as plate-shaped polymer impregnated with varying concentrations of carbon (Carbon-doped polymer standards), can be used to evaluate spectroscopic linearity. In order to verify linearity, however, standard plates with not less than 4 levels of concentration within the reflectance of 10 – 90% must be used. When measurements are expected to be taken with absorbance of no less than 1.0, it is necessary to add standard plates with reflectance of either 2% or 5% or both.

In order to plot absorbance (A_{OBS}) of such standard plates at locations in the vicinity of wavelengths 1200 nm, 1600 nm and 2000 nm against absorbance (A_{REF}) assigned to each standard plate, verifications must be made to ensure that the gradient of linearity obtained are ordinarily within the range 1.0 ± 0.05 for each of these wavelengths and 0 ± 0.05 for ordinate intercept. Depending on the intended purpose, appropriate tolerance figures can be set.

4.3. Spectrophotometric noise

The spectrophotometric noise of the equipment can be checked using appropriate reflectance standard plates, such as white-colored reflecting ceramic tiles or reflective thermoplastic resin (such as polytetrafluoroethylene).

4.3.1. High flux noise

Spectrophotometric noise is evaluated by using standard plates with high reflectance, such as reflectance of 99%. Standard plates are used to take measurements for both samples and control samples. Generally, the average value obtained from calculation of mean square root (*RMS*) of noise for each 100 nm segments in the wavelength range of 1200 – 2200 nm ordinarily must not be more than 0.3×10^{-3} and individual values must not exceed 0.8×10^{-3}. Depending on the intended purpose, appropriate tolerance figures can be set.

$$RMS = \{1/N \cdot \Sigma (A_i - A_m)^2\}^{1/2}$$

N: Number of measurement points per segment
A_i: Absorbance at each measurement point of segment
A_m: Average absorbance for segment

4.3.2. Low flux noise

Spectrophotometric noise is evaluated by using standard plates with low reflectance, such as reflectance of 10%, when the amount of light is low. In such cases, light source, optical system, detector and electronic circuit systems all have some impact on noise. Similar to the cases of high flux noise, generally, the average value obtained from calculation of *RMS* for each 100 nm segments in the wavelength range of 1200 – 2200 nm ordinarily must not be more than 1.0×10^{-3} and individual values must not exceed 2.0×10^{-3}. Depending on the intended purpose, appropriate tolerance figures can be set.

5. Application of qualitative or quantitative analysis

Unlike in the infrared range, mainly harmonic overtones and combinations manifest as spectrum in the near-infrared range. Such absorbance spectrums are often observed as overlay of absorption bands of functional groups and atomic groups. The near-infrared spectrometry, therefore, differs from conventional analysis methods that correspond to each application, by preparing model analysis methods using

methodologies of chemometrics, such as multivariate analysis, and needs to establish analytical methods depending on the intended purpose.

Characteristics of near-infrared absorption spectrum must be emphasized and effects of complexities of spectrums, as well as overlay of absorption bands must be reduced by performing mathematical preprocesses, such as primary or secondary spectral differentiation processes or normalizations, which becomes one of vital procedures in establishing analysis methods that use methodologies of chemometrics. While there are many chemometrics methodologies and mathematical preprocessing methods for data, appropriate combinations must be selected that suit the purposes of intended analysis.

Evaluation of validity based on analysis parameters is ordinarily required for the analysis validation when establishing a near-infrared analysis method. Selection of parameters that are appropriate for applications must be made for its intended use. Furthermore, following issues must be considered, in conformity with attributes of the near-infrared spectrometry.

(i) Whether or not wavelengths (wave numbers) intended for the particular analysis method, are suitable for evaluation of characteristics of a sample in performing analysis under given conditions.

(ii) Whether or not the method is adequately robust to deal with variables such as handling of samples (for instance fill condition for fine particle samples, etc.) and configuration matrix.

(iii) Whether or not about the same level of accuracy or precision can be obtained, in comparison with the existing and established analysis methods, which are available as standards.

(iv) Sustaining and managing performance of an analysis method, once established, are critical. Continuous and systematic maintenance and inspection work must therefore be implemented. Furthermore, it must be determined whether or not appropriate evaluation procedures are available to deal with change controls or implementation of re-validation on changes made in manufacturing processes or raw materials, as well as changes arising from replacement of major components in equipment.

(v) Whether or not there are appropriate evaluation procedures in place to verify validity of transferring implementation of an analysis, which presupposed the use of a specific equipment, from such originally intended equipment to another equipment (model transfer) for the purpose of sharing the analysis method.

5.1. Qualitative analysis

Qualitative analysis, such as verification of substances, is performed after preparing a reference library that includes inter-lot variations within tolerance range and establishing analytical methods by using chemometrics methodologies, such as multivariate analysis. Minute quality characteristic variations between lots can also be established by using this method.

Furthermore, multivariate analysis includes direct analysis methods that consider wavelengths (wave numbers) and absorption as variables, such as wavelength correlation method, residual sum of squares, range sum of squares, along with factor analysis method, cluster analysis method, discriminant analysis method, as well as SIMCA (Soft independent modeling of class analogy), which are applied after preprocessing such as principal component analysis.

It is also possible to consider the overall near-infrared absorption spectrum as a single pattern and to identify parameters obtained by applying multivariate analysis methods or peak heights at characteristic wavelengths (wave numbers) peaks of the sample substance as indices for monitoring, for the purpose of manufacturing process control for active substances or preparations.

5.2. Quantitative analysis

Quantitative analysis uses spectrums of sample groups and analysis values obtained through the existing and established analysis methods, to obtain quantitative models with methodologies of chemometrics. These are used to calculate concentrations of individual ingredients and material values of samples being measured, using conversion formulas. Chemometrics methodologies for obtaining quantitative models include multiple regression analysis method, main ingredient regression analysis method and PLS (Partial least squares) regression analysis method.

In cases where the composition of a sample is simple, concentrations of ingredients in the sample that are subject to analysis can be calculated, by plotting a calibration curve using the absorbance of a specific wavelength (wave number) or the correlating relationship between the parameters and concentration, using samples for preparation of calibration curves with known concentrations (calibration curve method).

6. References

1) JIS K 0134 (2002), Japanese Industrial Standards, General Rules for Near-infrared Spectrophotometric Analysis
2) Near-Infrared Spectrophotometry, 2.2.40, European Pharmacopoeia 5.0 (2005)
3) Near-Infrared Spectrophotometry, ⟨*1119*⟩, US Pharmacopeia 30 (2007)

G2 Solid-state Properties

Solid and Particle Densities ⟨*G2-1-171*⟩

Density of a solid or a powder as a state of aggregation has different definitions depending on the way of including of the interparticulate and intraparticulate voids that exist between the particles or inside the powder. Different figures are obtained in each case, and there are different practical meanings. Generally, there are three levels of definitions of the solid or powder density.

(1) Crystal density: It is assumed that the system is homogeneous with no intraparticulate void. Crystal density is also called true density.

(2) Particle density: The sealed pores or the experimentally non-accessible open pores are also included as a part of the volumes of the solid or the powder.

(3) Bulk density: The interparticulate void formed in the powder bed is also included as a part of the volumes of the solid or the powder. Bulk density is also called apparent density. Generally, the powder densities at loose packing and at tapping are defined as the bulk density and the tapped density, respectively.

Generally, the densities of liquid or gas are affected only by temperature and pressure, but the solid or powder density is affected by the state of aggregation of the molecules or the particles. Therefore, the solid or powder densities naturally vary depending on crystal structure or crystallinity of the substance concerned, and also varies depending on the method of preparation or handling if the sample is amor-

phous form or partially amorphous. Consequently, even in a case that two solids or powders are chemically identical, it may be possible that the different figures of density are obtained if their crystal structures are different. As the solid or powder particle densities are important physical properties for the powdered pharmaceutical drugs or the powdered raw materials of drugs, the Japanese Pharmacopoeia specifies each density determination as ''Powder Particle Density Determination'' for the particle density and as ''Determination of Bulk and Tapped Densities'' for the bulk density.

The solid or powder densities are expressed in mass per unit volume (kg/m^3), and generally expressed in g/cm^3 (1 g/cm^3 = 1000 kg/m^3).

Crystal Density

The crystal density of a substance is the average mass per unit volume, exclusive of all voids that are not a fundamental part of the molecular packing arrangement. It is an intrinsic property concerning the specific crystal structure of the substance, and is not affected by the method of determination. The crystal density can be determined either by calculation or by simple measurement.

A. The calculated crystal density is obtained using:
   1) For example, the crystallographic data (volume and composition of the unit cell) obtained by the perfect crystal X-ray diffraction data from single crystal or indexing the powder X-ray diffraction data.
   2) Molecular mass of the substance.

B. The measured crystal density is obtained as the mass to volume ratio after measuring the single crystal mass and volume.

Particle Density

The particle density takes account both the crystal density and the intraparticulate porosity (sealed and/or experimentally non-accessible open pores) as a part of the particle volume. The particle density depends on the value of the volume determined, and the volume in turn depends on the method of measurement. Concerning the determination of particle density, the Japanese Pharmacopoeia specifies the pycnometry as the ''Powder Particle Density Determination''.

The pycnometric density is obtained by assuming that the volume of the gas displaced, which is measured with the gas displacement pycnometer, is equivalent to that of a known mass of the powder. In pycnometric density measurements, any volume with the open pores accessible to the gas is not included as a part of volume of the powder, but the sealed pores or pores inaccessible to the gas is included as a part of the volume of the powder. Due to the high diffusivity of helium which can penetrate to most open pores, it is recommendable as the measurement gas of particle density. Therefore, the pycnometric particle density of a finely milled powder is generally not very different from the crystal density. Hence, the particle density by this method is the best estimate of the true density of an amorphous or partially crystalline sample, and can be widely used for manufacturing control of the processed pharmaceutical powder samples.

Bulk Density and Tapped Density

The bulk density of a powder includes the contribution of interparticulate void volume as a part of the volume of the powder. Therefore, the bulk density depends on both the powder particle density and the space arrangement of particles in the power bed. Further, since the slightest disturbance of the bed may result in variation of the space arrangement, it is often very difficult to determine the bulk density with good reproducibility. Therefore, it is essential to specify how the determination was made upon reporting the bulk density.

The Japanese Pharmacopoeia specifies ''Determination of Bulk and Tapped Densities''.

A. The bulk density is determined by measuring the apparent volume of a known mass of powder sample that has been passed through a screen in a graduated cylinder (constant mass method). Separately, the Pharmacopoeia specifies the method of determining bulk density by measuring the mass of powder in a vessel having a known volume (constant volume method).

B. The tapped density is obtained by mechanically tapping a measuring cylinder containing a powder sample. After determining the initial bulk volume, carry out tapping under a fixed measurement condition (tapping rate and drop height), and the measurement is carried out repeatedly until the bulk volume variation obtained at consecutive two measurements is within an acceptable range (constant mass method). Separately, the Pharmacopoeia specifies the method of determining the tapped density by measuring the mass of a fixed volume of the tapped powder (constant volume method).

Powder Fineness <*G2-2-171*>

This classification is harmonized with the European Pharmacopoeia and the U.S. Pharmacopeia.

Information on the harmonization with the European Pharmacopoeia and the U.S. Pharmacopeia is available on the website of the Pharmaceuticals and Medical Devices Agency.

A simple descriptive classification of powder fineness is provided in this chapter. Sieving is most suitable where a majority of the particles are larger than about 75 μm, although it can be used for some powders having smaller particle sizes where the method can be validated. Light diffraction is also a widely used technique for measuring the size of a wide range of particles. Where the cumulative distribution has been determined by analytical sieving or by application of other methods, particle size may be characterized in the following manner:

x_{90}: Particle size corresponding to 90% of the cumulative undersize distribution
x_{50}: Median particle size (i.e. 50% of the particles are smaller and 50% of the particles are larger)
x_{10}: Particle size corresponding to 10% of the cumulative undersize distribution

It is recognized that the symbol d is also widely used to designate these values. Therefore, the symbols d_{90}, d_{50} and d_{10} may be used.

The following parameters may be defined based on the cumulative distribution.

$Q_r(x)$: cumulative distribution of particles with a dimension less than or equal to x where the subscript r reflects the distribution type

r	Distribution type
0	Number
1	Length
2	Area
3	Volume

Therefore, by definition:

$Q_r(x) = 0.90$ when $x = x_{90}$
$Q_r(x) = 0.50$ when $x = x_{50}$
$Q_r(x) = 0.10$ when $x = x_{10}$

An alternative but less informative method of classifying powder fineness is by use of the descriptive terms in the following table.

Classification of powders by fineness

Descriptive term	x_{50} (μm)	Cumulative distribution by volume basis, $Q_3(x)$
Coarse	> 355	$Q_3(355) < 0.50$
Moderately fine	180 – 355	$Q_3(180) < 0.50$, $Q_3(355) \geqq 0.50$
Fine	125 – 180	$Q_3(125) < 0.50$, $Q_3(180) \geqq 0.50$
Very fine	≦ 125	$Q_3(125) \geqq 0.50$

Powder Flow <*G2-3-171*>

This test is harmonized with the European Pharmacopoeia and the U.S. Pharmacopeia.

Information on the harmonization with the European Pharmacopoeia and the U.S. Pharmacopeia is available on the website of the Pharmaceuticals and Medical Devices Agency.

The widespread use of powders in the pharmaceutical industry has generated a variety of methods for characterizing powder flow. Not surprisingly, scores of references appear in the pharmaceutical literature, attempting to correlate the various measures of powder flow to manufacturing properties. The development of such a variety of test methods was inevitable; powder behavior is multifaceted and thus complicates the effort to characterize powder flow. The purpose of this chapter is to review the methods for characterizing powder flow that have appeared most frequently in the pharmaceutical literature. In addition, while it is clear that no single and simple test method can adequately characterize the flow properties of pharmaceutical powders, this chapter proposes the standardization of test methods that may be valuable during pharmaceutical development.

Four commonly reported methods for testing powder flow are ''(1) angle of repose'', ''(2) compressibility index or Hausner ratio'', ''(3) flow rate through an orifice'', and ''(4) shear cell''. In addition, numerous variations of each of these basic methods are available. Given the number of test methods and variations, standardizing the test methodology, where possible, would be advantageous.

With this goal in mind, the most frequently used methods are discussed below. Important experimental considerations are identified and recommendations are made regarding standardization of the methods. In general, any method of measuring powder flow should be practical, useful, reproducible, sensitive, and yield meaningful results. It bears repeating that no one simple powder flow method will adequately or completely characterize the wide range of flow properties experienced in the pharmaceutical industry. An appropriate strategy may well be the use of multiple standardized test methods to characterize the various aspects of powder flow as needed by the pharmaceutical scientist.

1. Angle of repose

The angle of repose has been used in several branches of science to characterize the flow properties of solids. Angle of repose is a characteristic related to interparticulate friction, or resistance to movement between particles. Angle of repose test results are reported to be very dependent upon the method used. Experimental difficulties arise due to segregation of material and consolidation or aeration of the powder as the cone is formed. Despite its difficulties, the method continues to be used in the pharmaceutical industry, and a number of examples demonstrating its value in predicting manufacturing problems appear in the literature.

The angle of repose is the constant, three-dimensional angle (relative to the horizontal base) assumed by a cone-like pile of material formed by any of several different methods (described briefly below).

1.1. Basic methods for angle of repose

A variety of angle of repose test methods are reported in the literature. The most common methods for determining the static angle of repose can be classified based on two important experimental variables:

(i) The height of the ''funnel'' through which the powder passes may be fixed relative to the base, or the height may be varied as the pile forms.

(ii) The base upon which the pile forms may be of fixed diameter or the diameter of the powder cone may be allowed to vary as the pile forms.

1.2. Variations in angle of repose methods

In addition to the above methods, variations of them have been used to some extent.

(i) Drained angle of repose: This is determined by allowing an excess quantity of material positioned above a fixed diameter base to drain from the container. Formation of a cone of powder on the fixed diameter base allows determination of the drained angle of repose.

(ii) Dynamic angle of repose: This is determined by filling a cylinder (with a clear, flat cover on one end) and rotating it at a specified speed. The dynamic angle of repose is the angle (relative to the horizontal) formed by the flowing powder. The internal angle of kinetic friction is defined by the plane separating those particles sliding down the top layer of the powder and those particles that are rotating with the drum (with roughened surface).

1.3. Angle of repose general scale of flowability

While there is some variation in the qualitative description of powder flow using the angle of repose, much of the pharmaceutical literature appears to be consistent with the classification by Carr[1)], which is shown in Table 1. There are examples of formulations with an angle of repose in the range of 40 to 50 degrees that manufactured satisfactorily. When the angle of repose exceeds 50 degrees, the flow is rarely acceptable for manufacturing purposes.

Table 1 Flow properties and corresponding angles of repose[1)]

Flow property	Counter measure to prevent crosslink	Angle of repose (degrees)
Excellent		25 – 30
Good		31 – 35
Fair	aid not needed	36 – 40
Passable	may hang up	41 – 45
Poor	most agitate, vibrate	46 – 55
Very poor		56 – 65
Very, very poor		>66

1.4. Experimental considerations for angle of repose

Angle of repose is not an intrinsic property of the powder, that is to say, it is very much dependent upon the method used to form the cone of powder. On this subject, the existing literature raises these important considerations:

(i) The peak of the cone of powder can be distorted by the impact of powder from above. By carefully building the powder cone, the distortion caused by impact can be minimized.

(ii) The nature of the base upon which the powder cone is formed influences the angle of repose. It is recommended that the powder cone be formed on a ''common base'', which can be achieved by forming the cone of powder on a layer of powder. This can be done by using a base of fixed diameter with a protruding outer edge to retain a layer of powder upon which the cone is formed.

1.5. Recommended procedure for angle of repose

Form the angle of repose on a fixed base with a retaining lip to retain a layer of powder on the base. The base should be free of vibration. Vary the height of the funnel to carefully build up a symmetrical cone of powder. Care should be taken to prevent vibration as the funnel is moved. The funnel height should be maintained approximately 2 – 4 cm from the top of the powder pile as it is being formed in order to minimize the impact of falling powder on the tip of the cone. If a symmetrical cone of powder cannot be successfully or reproducibly prepared, this method is not appropriate. Determine the angle of repose by measuring the height of the cone of powder and calculating the angle of repose, α, from the following equation:

$$\tan \alpha = \text{height}/(\text{diameter of base} \times 0.5)$$

2. Compressibility index and Hausner ratio

In recent years the compressibility index and the closely related Hausner ratio have become the simple, fast and popular methods of predicting powder flow characteristics. The compressibility index has been proposed as an indirect measure of bulk density, size and shape, surface area, moisture content, and cohesiveness of materials because all of these can influence the observed compressibility index. The compressibility index and the Hausner ratio are determined by measuring both the bulk volume and tapped volume of a powder.

2.1. Basic methods for compressibility index and Hausner ratio

While there are some variations in the method of determining the compressibility index and Hausner ratio, the basic procedure is to measure (1) the unsettled apparent volume, V_o, and (2) the final tapped volume, V_f, of the powder after tapping the material until no further volume changes occur. The compressibility index and the Hausner ratio are calculated as follows:

$$\text{Compressibility Index} = (V_o - V_f)/V_o \times 100$$
$$\text{Hausner Ratio} = V_o/V_f$$

Alternatively, the compressibility index and Hausner ratio may be calculated using measured values for bulk density (ρ_{bulk}) and tapped density (ρ_{tapped}) as follows:

$$\text{Compressibility Index} = (\rho_{tapped} - \rho_{bulk})/\rho_{tapped} \times 100$$
$$\text{Hausner Ratio} = \rho_{tapped}/\rho_{bulk}$$

In a variation of these methods, the rate of consolidation is sometimes measured rather than, or in addition to, the change in volume that occurs on tapping. For the compressibility index and the Hausner ratio, the generally accepted scale of flowability is given in Table 2.

Table 2 Scale of flowability[1)]

Compressibility index (%)	Flow character	Hausner ratio
≦10	Excellent	1.00 – 1.11
11 – 15	Good	1.12 – 1.18
16 – 20	Fair	1.19 – 1.25
21 – 25	Passable	1.26 – 1.34
26 – 31	Poor	1.35 – 1.45
32 – 37	Very poor	1.46 – 1.59
>38	Very, very poor	>1.60

2.2. Experimental considerations for the compressibility index and Hausner ratio

Compressibility index and Hausner ratio are not intrinsic properties of the powder, that is to say, they are dependent upon the methodology used. The existing literature points out several important considerations affecting the determination of the (1) unsettled apparent volume, V_o, (2) the final tapped volume, V_f, (3) the bulk density, ρ_{bulk}, and (4) the tapped density, ρ_{tapped}:

(i) The diameter of the cylinder used

(ii) The number of times the powder is tapped to achieve the tapped density

(iii) The mass of material used in the test

(iv) Rotation of the sample during tapping

2.3. Recommended procedure for compressibility index and Hausner ratio

Use a 250-mL volumetric cylinder with a test sample weight of 100 g. Smaller weights and volumes may be used, but variations in the method should be described with the results. An average of three determinations is recommended.

3. Flow through an orifice

The flow rate of a material depends upon many factors, some of which are particle-related and some related to the process. Monitoring the rate of flow of material through an orifice has been proposed as a better measure of powder flowability. Of particular significance is the utility of monitoring flow continuously since pulsating flow patterns have been observed even for free flowing materials. Changes in flow rate as the container empties can also be observed. Empirical equations relating flow rate to the diameter of the opening, particle size, and particle density have been determined. However, determining the flow rate through an orifice is useful only with free-flowing materials.

The flow rate through an orifice is generally measured as the mass per time flowing from any of a number of types of containers (cylinders, funnels, hoppers). Measurement of the flow rate can be in discrete increments or continuous.

3.1. Basic methods for flow through an orifice

There are a variety of methods described in the literature. The most common for determining the flow rate through an orifice can be classified based on three important experimental variables:

(1) The type of container used to contain the powder. Common containers are cylinders, funnels and hoppers from production equipment.

(2) The size and shape of the orifice used. The orifice diameter and shape are critical factors in determining powder flow rate.

(3) The method of measuring powder flow rate. Flow rate can be measured continuously using an electronic balance and with some sort of recording device (strip chart recorder, computer). It can also be measured in discrete samples (for example, the time it takes for 100 g of powder to

pass through the orifice to the nearest tenth of a second or the amount of powder passing through the orifice in 10 seconds to the nearest tenth of a gram).

3.2. Variations in methods for flow through an orifice

Either mass flow rate or volume flow rate can be determined. Mass flow rate is the easier of the methods, but it biases the results in favor of high-density materials. Since die fill is volumetric, determining volume flow rate may be preferable. A vibrator is occasionally attached to facilitate flow from the container, however, this appears to complicate interpretation of results. A moving orifice device has been proposed to more closely simulate rotary press conditions. The minimum diameter orifice through which powder flows can also be identified.

3.3. General scale of flowability for flow through an orifice

No general scale is available because flow rate is critically dependent on the method used to measure it. Comparison between published results is difficult.

3.4. Experimental considerations for flow through an orifice

Flow rate through an orifice is not an intrinsic property of the powder. It is very much dependent upon the methodology used. The existing literature points out several important considerations affecting these methods:

(i) The diameter and shape of the orifice
(ii) The type of container material (metal, glass, plastic)
(iii) The diameter and height of the powder bed.

3.5. Recommended procedure for flow through an orifice

Flow rate through an orifice can be used only for materials that have some capacity to flow. It is not useful for cohesive materials. Provided that the height of the powder bed (the 'head' of powder) is much greater than the diameter of the orifice, the flow rate is virtually independent of the powder head. Use a cylinder as the container because the cylinder material should have little effect on flow. This configuration results in flow rate being determined by the movement of powder over powder rather than powder along the wall of the container. Powder flow rate often increases when the height of the powder column is less than two times the diameter of the column. The orifice should be circular and the cylinder should be free of vibration. General guidelines for dimensions of the cylinder are as follows:

(i) Diameter of opening >6 times the diameter of the particles
(ii) Diameter of the cylinder >2 times the diameter of the opening

Use of a hopper as the container may be appropriate and representative of flow in a production situation. It is not advisable to use a funnel, particularly one with a stem, because flow rate will be determined by the size and length of the stem as well as the friction between the stem and the powder. A truncated cone may be appropriate, but flow will be influenced by the powder—wall friction coefficient, thus, selection of an appropriate construction material is important.

For the opening in the cylinder, use a flat-faced bottom plate with the option to vary orifice diameter to provide maximum flexibility and better ensure a powder-over-powder flow pattern. Rate measurement can be either discrete or continuous. Continuous measurement using an electronic balance can more effectively detect momentary flow rate variations.

4. Shear cell methods

In an effort to put powder flow studies and hopper design on a more fundamental basis, a variety of powder shear testers and methods that permit more thorough and precisely defined assessment of powder flow properties have been developed. Shear cell methodology has been used extensively in the study of pharmaceutical materials. From these methods, a wide variety of parameters can be obtained, including the yield loci representing the shear stress-shear strain relationship, the angle of internal friction, the unconfined yield strength, the tensile strength, and a variety of derived parameters such as the flow factor and other flowability indices. Because of the ability to more precisely control experimental parameters, flow properties can also be determined as a function of consolidation load, time, and other environmental conditions. The methods have been successfully used to determine critical hopper and bin parameters.

4.1. Basic methods for shear cell

One type of shear cell is the cylindrical shear cell which is split horizontally, forming a shear plane between the lower stationary base and the upper movable portion of the shear cell ring. After powder bed consolidation in the shear cell (using a well-defined procedure), the force necessary to shear the powder bed by moving the upper ring is determined. Annular shear cell designs offer some advantages over the cylindrical shear cell design, including the need for less material. A disadvantage, however, is that because of its design, the powder bed is not sheared as uniformly because material on the outside of the annulus is sheared more than material in the inner region. A third type of shear cell (parallel-plate type) consists of a thin sandwich of powder between a lower stationary rough surface and an upper rough surface that is moveable.

All of the shear cell methods have their advantages and disadvantages, but a detailed review is beyond the scope of this chapter. As with the other methods for characterizing powder flow, many variations are described in the literature. A significant advantage of shear cell methodology in general is a greater degree of experimental control. The methodology generally is rather time-consuming and requires significant amounts of material and a well-trained operator.

4.2. Recommendations for shear cell

The many existing shear cell configurations and test methods provide a wealth of data and can be used very effectively to characterize powder flow. They are also helpful in the design of equipment such as hoppers and bins. Because of the diversity of available equipment and experimental procedures, no specific recommendations regarding methodology are presented in this chapter. It is recommended that the results of powder flow characterization using shear cell methodology include a complete description of equipment and methodology used.

5. References

1) Carr R.L.: Evaluating flow properties of solids. *Chem. Eng*. 1965; 72: 163–168.

Measurement of the Diameter of Particles Dispersed in Liquid by Dynamic Light Scattering

〈G2-4-161〉

This method is used for measuring average particle diameter and particle diameter distribution of submicron-sized particles dispersed in a liquid by means of dynamic light scattering.

The average particle diameter and the particle diameter

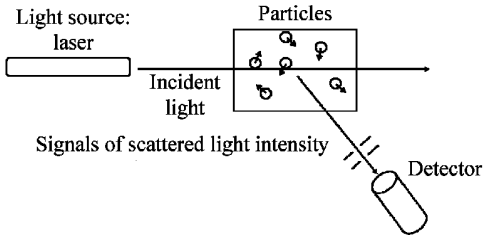


Fig. 1 Schematic illustration of the measurement principle

distribution obtained by this method are important characteristics mainly of colloidal dispersion formulations, such as emulsion injections, suspension injections, and liposome formulations.

There are two ways of analyzing the detected signals in dynamic light scattering: photon correlation spectroscopy and frequency analysis. Dynamic light scattering is applied to the analysis of particles whose diameters range from nm scale to approximately 1 μm or particles free from the influence of sedimentation.

1. Principle

When particles in Brownian motion in solution or in suspension are irradiated with laser light, scattered light from the particles fluctuates depending on their diffusion coefficients. The intensity of the scattered light from larger particles fluctuates more slowly, because the larger particles move more slowly. On the other hand, the intensity of the scattered light from smaller particles fluctuates more rapidly, because they move faster.

In dynamic light scattering measurements, the particle diameter is determined by applying the Stokes-Einstein equation to analysis of the detected fluctuations of scattered light intensity, which reflect the diffusion coefficient of the particles.

$$d = \frac{kT}{3\pi\eta D} \times 10^{12}$$

d: Particle diameter (nm)
k: Boltzmann constant (1.38×10^{-23} J·K^{-1})
T: Absolute temperature (K)
η: Viscosity (mPa·s)
D: Diffusion coefficient (m^2·s^{-1})

In photon correlation spectroscopy, the time-dependent changes (fluctuation) in the scattered light intensity, namely the observed signals of the scattered light intensity, are transmitted to the correlator. The average particle diameter and the polydispersity index are obtained from the autocorrelation function of the scattered light intensity, which is calculated based on the data processed by the correlator.

In frequency analysis, the average particle diameter and the polydispersity index are obtained from the frequency power spectrum, which is the Fourier transform of the frequency components included in the signals of the scattered light intensity.

Major terms used in this method are as follows.

(i) Average particle diameter: dynamic light scattering harmonic intensity-weighted arithmetic averaged particle diameter, whose unit is nanometer (nm).

(ii) Polydispersity index: dimensionless indicator of the broadness of the particle diameter distribution.

(iii) Scattering volume: observation volume defined by the light-receiving optics and the incident laser light. This value may be given in the specifications of the instrument. Its order of magnitude is typically 10^{-12} m^3.

(iv) Count rate: number of the photon pulses per second detected in the light-receiving optics in photon correlation spectroscopy. This value is proportional to the detected scattered light intensity. The unit is cps (count per second).

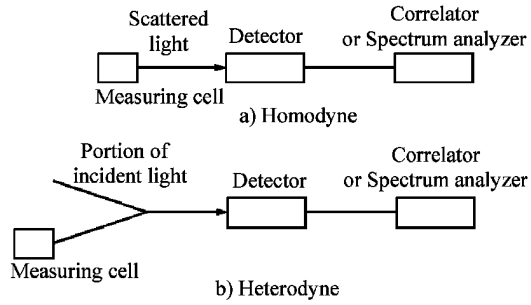


Fig. 2 Different optical arrangements of the apparatus

(v) Signal of scattered light fluctuation: signal detected by the light-receiving optics in the frequency analysis. The signal is proportional to the scattered light intensity, and includes frequency components depending on the distribution of the particle diameter.

2. Apparatus

2.1. Constitution of the apparatus

The measuring apparatus generally consists mainly of a laser, sample holder, light-receiving optics and detector, and correlator or spectrum analyzer. There are two types of optical detection according to the optical arrangements: (a) homodyne detection in which only the scattered light is measured, and (b) heterodyne detection in which the scattered light and a portion of the incident light are measured simultaneously.

(i) Laser: a monochromatic laser polarized with its electric field component perpendicular to the plane formed by the incident light and light-receiving optical axes (vertical polarization).

(ii) Sample holder: a holder whose temperature can be measured and controlled within an accuracy of ± 0.3°C.

(iii) Measuring cell: a rectangular or cylindrical cell made of optical glass or optical plastic, which can be placed in the sample holder. The cell is integrated with the sample holder in some apparatus.

(iv) Light-receiving optics and detector: light optics and detector which capture the scattered light from the sample at a single scattering angle between 90° to 180° and convert the captured light to a photon pulse (digitized signal). In the case that a polarization analyzer is included, it shall be positioned so that the transmittance of the vertically polarized light is maximized.

(v) Correlator: a device which calculates the autocorrelation function from the number of photon pulses in a certain time.

(vi) Spectrum analyzer: a device which calculates the frequency power spectrum by performing Fourier transformation of the frequency components present in the scattered light fluctuation signals.

(vii) Computation unit: data processor for determining the particle diameter distribution from the autocorrelation function obtained by the correlator or from the frequency power spectrum. Some computation units also function as a correlator or spectrum analyzer.

2.2. Validation and reproducibility of the instrument

Because the particle diameter obtained by dynamic light scattering is not a relative value calculated using standard particles but an absolute value based on a fundamental principle, calibration of the value is unnecessary.

However, it is necessary to confirm the performance of

the instrument by using particles with certified diameter, when the instrument is first installed or if abnormal performance is suspected. In addition, it is desirable to confirm the proper performance of the instrument at least every year thereafter.

As standard particles of known diameter, polystyrene latex particles with a narrow distribution of diameter shall be used, whose average particle size is certified to be approximately 100 nm as determined by dynamic light scattering. The measured average diameter of these particles must be within 2% of the stated diameter range, and the relative standard deviation must be less than 2%. In addition, the measured polydispersity index must be less than 0.1.

3. Measurement

3.1. Choice of the dispersion liquid

The dispersion liquid shall fulfill all of the following requirements.

(i) It shall be non-absorbing at the wavelength of the laser.

(ii) It shall not cause damage such as corrosion to the materials of the instrument.

(iii) It shall not dissolve, swell or coagulate the particles.

(iv) It shall have a refractive index different from that of the particulate material.

(v) Its refractive index and viscosity shall be known within an accuracy of 0.5%.

(vi) It shall be clean enough not to interfere with the measurements.

3.2. Cleaning the measuring cell

The degree of cell washing required depends on the conditions of the measurement.

When an individually packaged clean disposable cell is used, cleaning by blowing off dust with compressed clean air is sufficient. When a cell is intended to be washed rigorously, the cell is fully rinsed beforehand with water to remove water-rinsable adhesion substances and is washed with a nonabrasive detergent.

3.3. Sample preparation

It is necessary to prepare a sample whose concentration is within an appropriate range to eliminate the influence of the multiple scattering of light. In addition, it is important to remove dust, which may affect the measurement, and to prevent their re-introduction during the preparation.

When the sample is shaken, dust-laden air is entrapped in the sample and air is dissolved in the solvent. The invisible small air bubbles scatter light more strongly than do the sample particles to be measured. It is necessary not to shake the sample violently after preparation, but to swirl it gently. A homogeneous sample solution can be prepared quickly by adding diluent to the concentrated sample droplet rather than dropping the sample droplet into the diluent.

3.4. Measurement procedure

1) Switch the instrument on and allow it to warm up. A period of approximately 30 minutes is typically required for stabilizing the laser intensity and bringing the sample holder to the desired temperature.
2) Choose the appropriate dispersion liquid, and record the count rate or the amplitude of the signals of scattered light fluctuation from the dispersion liquid.
3) Place the sample containing the dispersed particles in the instrument, and wait until temperature equilibrium is established between the sample and the sample holder. It is desirable to control and measure the temperature within an accuracy of $\pm 0.3°C$.
4) Perform a preliminary measurement of the sample, and set the particle concentration within the appropriate range based on 5.2.
5) Perform the measurement with the appropriate measuring time and number of integrations.
6) Record the average particle diameter and the polydispersity index for each measurement.
7) If the measured values are dependent on the particle concentration, adopt the extrapolated infinite dilution values of the average particle diameter and the polydispersity index (or the measured values at the lowest particle concentration).
8) Confirm that no significant sedimentation has occurred in the sample at the end of the measurement. The presence of sediment indicates that the sample may have aggregated or precipitated, or that the sample may be unsuitable for measurement by dynamic light scattering.
9) Perform the measurement for each sample at least three times.

3.5. Repeatability

The repeatability of the determination of the average particle diameter, evaluated in terms of relative standard deviation, must be less than 5%.

4. Data analysis

The dispersion that is the target for the measurement is irradiated with the laser light. Phases of the light scattered by each particle fluctuate because the dispersed particles are in Brownian motion. The observed scattered intensity, which is the sum of the scattered light (result of interference), fluctuates along the time axis. Analyzing the fluctuation of the scattered light intensity as a function of time provides information on the motion of the dispersed particles.

Analysis by photon correlation spectroscopy is performed using the autocorrelation function of the scattered light intensity. This autocorrelation function depends only on the time difference (correlation time) and is independent of the time at which the measurement is started. For a large number of monodisperse particles in Brownian motion in a scattering volume, the autocorrelation function of the scattered light intensity is basically an exponential decay function of the correlation time. Polydispersity index is a parameter indicating the distribution of the decay constant, and is also a scale indicating the broadness of the distribution of particle diameter.

Frequency analysis is performed using the frequency power spectrum calculated from the scattered light intensity. The amplitude of the frequency power spectrum is proportional to the scattered light intensity and the concentration of the sample, and the characteristic frequency is inversely proportional to the particle diameter. The decay constant and the characteristic frequency are related to the translational diffusion coefficient of homogeneous spherical particles in Brownian motion. The diffusion constant of the spherical particles dispersed in the dispersion liquid is related to the particle diameter according to the Stokes-Einstein equation in the absence of inter-particle interaction. The polydispersity index determined by frequency analysis is a measure of the broadness of the particle diameter distribution calculated from the particle diameter distribution based on the scattered light intensity, and might differ from the polydispersity index determined by photon correlation spectroscopy.

Records of data shall include the average particle diameter and polydispersity index, and in addition, shall also state the principle of measurement (photon correlation spectroscopy or frequency analysis), optical configuration (homodyne or heterodyne), observation angle, temperature of the sample, refractive index and viscosity of the dispersion liquid, meas-

uring time or number of integrations, and sample concentration.

5. Points to note regarding the measurement

5.1. Shape of particles

The particles are assumed to be homogeneous and spherical in the data analysis of dynamic light scattering.

5.2. Particle concentration

For measurement, it is necessary to prepare a sample whose concentration falls in the range satisfying the following conditions.

(i) The sample consists of dispersion liquid and particles well-dispersed in the liquid.

(ii) The range of the particle concentration is determined so that consistent results can be obtained in particle diameter measurements. The range is determined beforehand based on measurements of systematically diluted samples.

5.3. Purification of the dispersion liquid

Scattered light signals from the dispersion liquid used for sample dilution must normally be undetected or very weak. If the situations described in cases (i) or (ii) below are found, particulate substances are likely to have become mixed in the sample, and in such cases the dispersion liquid shall be further purified (by filtration, distillation, and so on) before use. The lower limit of the particle concentration is determined mainly so that scattered light from the dispersion liquid and contaminating substances will not affect the measurement. When water is chosen as the dispersion liquid, use of fresh distilled water (prepared by quartz-glass distillation) or desalted and filtered (pore size 0.2 μm) water is recommended.

(i) Large fluctuations of the count rate or of the amplitude of the scattered light fluctuation signals, accompanied by abnormally strong signals, are recorded.

(ii) Light spots appear in the path of the laser light in the sample.

5.4. Others

(i) When particles are highly charged with electricity, long-range interactions between the particles may affect the measurement result, and in such cases, a small amount of salt (for example, sodium chloride: around 10^{-2} mol/L) may be added to the dispersion liquid to reduce the effect.

(ii) Traceable polystyrene latex particles for use in the validation of the instrument are commercially available.

6. Reference

1) JIS Z8826: 2005 Particle size analysis—Photon correlation spectroscopy
2) ISO 13321: 1996 Particle size analysis—Photon correlation spectroscopy
3) ISO 22412: 2008 Particle size analysis—Dynamic light scattering (DLS)

G3 Biotechnological/Biological Products

Basic Concept of the Quality Control on Biotechnological Products (Biopharmaceuticals) *<G3-1-180>*

Introduction

This document provides general principle to ensure the quality of biotechnological products (hereinafter referred to as ''biopharmaceuticals'') focusing on the elements peculiar to biopharmaceuticals on the basis of the recommendations in a series of so-called Q-quartet guidelines from ICH Q8 to Q11 and those in Q5A to Q5E and Q6B guidelines[1-6] on the quality of biopharmaceuticals. The general concepts for assurance of drug substances and drug products are described in the General Information, ''Basic Concepts for Quality Assurance of Drug Substances and Drug Products *<G0-1-172>*''.

The principles of this General Information apply to biopharmaceuticals: proteins and peptides, their derivatives, and products of which they are components. These proteins and polypeptides are produced from recombinant or non-recombinant cell-culture expression systems. The principles outlined in this document may also apply to other types of biotechnological/biological products.

In the case of biopharmaceuticals, an inherent degree of structural heterogeneity occurs in molecular structure due to the biosynthetic processes used by living organisms to produce them. In addition to post-translational modification such as glycosylation, they may receive various modifications such as oxidation and deamidation during the production process and storage periods. Impurities that may remain in the drug substance of biopharmaceuticals include those with molecular diversity, such as proteins derived from cells used for production, and there is a risk of contamination such as viruses. Such quality profiles can vary due to various factors on the manufacturing process.

In order to ensure the quality of biopharmaceuticals, it is necessary to establish an appropriate quality control strategy in consideration of the above characteristics. ''Basic Concepts for Quality Assurance of Drug Substances and Drug Products *<G0-2-170>*'' described in General Information G0 is useful for this. First, the quality attributes are clarified by thorough characteristic analysis. Then, identify the critical quality attributes (CQAs) in consideration of the quality target product profile (QTPP), and construct a quality control strategy to keep the CQAs within the appropriate ranges, limits and distributions. If the manufacturing process is to be changed during the development period or the post-marketing period, conduct comparability exercise of before and after the change made in the manufacturing process, and check the validity of the change by verifying that the change will not have adverse impact on the quality, safety and efficacy of the drug product. New manufacturing and analytical technology of biopharmaceuticals are continuously being developed day by day, and desired to be utilized for continuous improvement of product quality throughout the product life cycle.

1. Quality evaluation and control of biopharmaceuticals

1.1. Quality evaluation

1.1.1. Characterization

Characterization of products is an essential step in identifying CQAs and establishing quality control strategies. In the characterization of biopharmaceuticals, detailed analysis is performed as much as possible for the structure and physicochemical properties, biological activity, molecular variants of a desired product, process-related impurities, and so on. The desired product is the protein which has an expected structure and is expected from the DNA sequence, the protein which is expected from anticipated post-translational modification, and/or expected from the intended downstream processing/modification. Among the molecular variants of the desired product, those with properties comparable to the desired product with respect to biological activity, efficacy and safety can be classified as product-related substances. Otherwise, those without the same properties can be

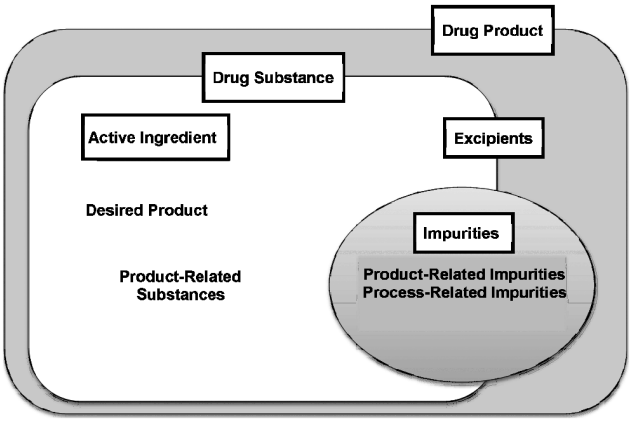


Fig. 1 Components of biopharmaceuticals.

*In biopharmaceuticals, the drug substance contains excipients such as buffer solution components for stabilization of the active ingredient.

classified as product-related impurities. The active ingredient consists of the desired product and the product-related substances, and generally, the active ingredient of biopharmaceuticals has heterogeneity. (Fig. 1).

a. Structure and physicochemical properties

Analyzes amino acid sequence and amino acid composition, terminal amino acid sequence, sulfhydryl group and disulfide bond, carbohydrate composition and structure, glycation, oxidation, deamidation, and so on. Oligosaccharides in glycoproteins may be associated with stabilization of structure, biological activity, antigenicity and pharmacokinetics, and so on. Their profiles are susceptible to variations of manufacturing process. It is necessary to analyze in detail by monosaccharide analysis, oligosaccharide analysis/oligosaccharide profiling, glycopeptide analysis, glycoform analysis etc. Molecular variants generated by oxidization and deamidation, etc. may be analyzed by peptide mapping. The charge profiles may be evaluated by ion exchange chromatography or isoelectric focusing. The molecular heterogeneity occurs not only during the culture process but also during subsequent manufacture and storage of the drug substance and drug product. Therefore, scientific understanding of the manufacturing process obtained by characterizing the degree of heterogeneity and profile, and evaluating the impact of the variations in process parameters on the degree of heterogeneity, will be useful to construct the quality control strategy.

Physicochemical properties are analyzed in terms of molecular weight, molecular size, molar absorbance coefficient, etc. Information on the secondary structure and higher-order structure of the desired product can be obtained by spectroscopic methods such as circular dichroism, Fourier transform infrared absorption spectrum and NMR, or by thermodynamic methods such as differential scanning calorimetry.

b. Biological activities

Biological activity is an indicator of the specific ability or capacity of a product to achieve a defined biological effect. It is difficult to determine higher-order structure by physicochemical analysis, because that an active ingredient of biopharmaceutical is a large molecule having complex structure, and is a mixture of various molecular entities as mentioned above. Therefore, the confirmation that the biopharmaceutical has an expected structure is usually obtained by biological activity. Biological assays for measuring biological activity include biochemical assays (measurement of enzyme activity, measurement of binding activity, etc.), cell culture-based biological assays, animal-based biological assays, and so on. The tests are selected by considering characteristics of active ingredient, mechanism of action and its pharmacological activity to the disease to be treated. For example, enzyme activity if the active ingredient is enzymes, cell proliferation activity if the active ingredient is growth factors, antigen binding activity, antigen neutralizing activity, antibody dependent cytotoxicity, and complement dependent cytotoxicity etc. if the active ingredient is antibodies, are evaluated.

In biological assays, the potency is expressed as a unit or relative activity (%) to the reference material by comparing the response obtained from the sample to that from the standard. The potency is the quantitative measure of biological activity based on the attribute of the product which is linked to the relevant biological properties. The biological activity used for potency measurement should, in principle, be the same as or similar to that expected in the clinical situation. The correlation between the expected clinical response and the activity in the biological assay should be established in pharmacodynamic or clinical studies.

c. Molecular variants of desired product (product-related substances and product-related impurities)

In the characterization of drug substances and drug products, we analyze as much as possible such as the structure, biological activity and binding activity of the contained molecular variants. In products with large molecular masses and complex structures, it is often difficult to clearly separate the product-related substances and product-related impurities, and it is difficult to control the proportion of individual molecular variants in the drug substance and the drug product. In such cases, profiles (oligosaccharide profiles and charge profiles) obtained by appropriate analytical methods should be clarified. Typical molecular variants classified as product-related impurities are aggregates (multimers) and fragments. In addition, deamidated, isomerized, oxidized, mismatched S-S linked disulfide bond mismatched, glycated forms, etc. may be considered as impurities derived from the desired product. Aggregates and fragments are evaluated for their content by size exclusion chromatography, SDS polyacrylamide gel electrophoresis, SDS capillary gel electrophoresis, and so on.

d. Process-related impurities

The process-related impurities are classified into those derived from cell substrates (e.g., host cell proteins and host cell DNA), impurities derived from cell cultures (e.g., antibiotics and insulin), impurities derived from downstream processing such as extraction, separation, processing, purification and formulation steps (ligands of carries for chromatography such as protein A, enzymes, chemical modification reagents, solvents, etc.). Regarding process-related impurities, if it is possible to guarantee that impurities are constantly removed by the control of process parameters, or if the in-process tests are set, in some cases, it is not necessary to set specifications for drug substances and drug products. Particular attention should be given to process-related impurities that exhibit pharmacological activity and may have immunogenicity.

1.1.2. Identification of CQA

For each quality attribute revealed by the characterization, risk level is estimated with respect to the impact and uncertainty that their variation has on efficacy and safety, and then the CQAs to be controlled are identified. For example, in many cases, from the viewpoint of 1. biological activity or efficacy, 2. pharmacokinetics, 3. immunogenicity, 4. safety, if the score obtained by multiplication of scores of impact and uncertainty of each attribute is above a certain value,

that attribute is identified as CQA. Risk level can also be estimated from the severity and probability of their impact on efficacy and safety.

1.2. Establishment of quality control strategy

The quality control strategy defines a set of controls to bring the CQAs within appropriate limits, ranges and distributions. Quality control strategies include such as raw material control, manufacturing process control, specifications and stability testing. Acceptable ranges and target management criteria of CQAs are set based on characterization results, lot analysis results based on specifications, stability tests results, and clinical tests results, and so on. In stability testing, the analytical results of the quality attributes (i.e. the proportion of molecular variants such as fragments, oxidized forms and deamidated forms) and biological activity of the forced degradation samples in the stress stability testing and accelerated stability testing, are useful for setting acceptable ranges and target management criteria of each CQA. In the course of developing the manufacturing process of biopharmaceuticals, identify the raw material characteristics and process parameters affecting the CQAs, and construct the control method of the manufacturing process so that the CQAs are within the target range. Based on these results, construct an appropriate quality control strategy consisting of raw material specifications, process parameter control, in-process testing, drug substance and/or drug product specifications.

1.2.1. Raw materials control

Raw materials for biopharmaceuticals include cell banks, media used in culture processes, media additives, resins and carriers for chromatography used in purification processes, buffers, washing solutions, and filters, and they also include PEGylation reagents used in the modification process, additives used in the formulation process, and the like.

a. Evaluation and control of cell bank (including evaluation of gene expression construct)

Cell substrates are generally controlled in a two-tiered cell bank, where Working Cell Banks are prepared from a Master Cell Bank, and their characteristics are clarified by conducting characterization and purity tests. Also, confirm that the cell substrate is suitable for pharmaceutical production. In addition, the same evaluation is performed at the upper limit of in vitro cell age that can be used for production, and the stability of the cell substrate during the culture period is confirmed. In the purity test, it is evaluated that the cell bank is not contaminated with adventitious microbial contaminants (see 1.2.3 for virus). In the characterization tests, cell morphology, viable cell number, expression of target protein, etc. are evaluated. In the case of a cell line to which a gene expression construct is introduced, the gene expression construct should be evaluated for copy number and insertions or deletions, and coding sequence of desired protein, etc.

b. Control of other raw materials

Raw materials used in the manufacturing process are used after confirming that they fulfill the criteria for their intended use. When using raw materials derived from humans or animals, such as serum and enzymes, make sure that they meet the ''Biological Raw Material Standards''.

1.2.2. Manufacturing process control

The manufacturing process of biopharmaceuticals consists mainly of drug substance process containing culture process and purification process, and formulation process. Because process parameters of culture process and purification process may affect the heterogeneity profile and impurity profile, etc., sufficient understanding of these processes and the establishment of appropriate control methods (setting and evaluation of process parameters, in-process test etc.) are essential for quality consistency. The constructed manufacturing process is qualified by process validation/evaluation. Process validation is usually performed on a commercial-scale, however it can be performed on a small-scale model that has been qualified for investigating the ability to remove and inactivate virus and the number of reuses of purification columns and so on.

a. Process parameter control

The process parameters to be controlled and their control ranges in each manufacturing process are set based on the previous manufacturing results and univariate experiments, or on the relationships between the process parameters and the CQAs clarified by a systematic approach. When developing a manufacturing process with the latter method, estimate the risk level related to the impact of each process parameter on each CQA, and set the operating range of each process parameter so that each CQA does not exceed its acceptable range. When estimating the risk level during the establishment of the control strategy, consider the severity, probability, and detectability of the impact that the parameter variations may have on each CQA. As an example of process parameters to be controlled, in the culture process, temperature, medium additives concentration, dissolved oxygen concentration, dissolved carbon dioxide concentration, pH, stirring speed, culture time, etc., and in the purification process, column size, loading amount, buffer solution composition, flow velocity, etc. can be mentioned. The acceptable range of purification process parameters is set in consideration of the impact on the heterogeneity profile and the impurity removal efficiency. In addition, it is considered necessary to ensure that the characteristics of each process, such as cell density and viability in the culture process and recovery rate in the purification process, fall within a certain range. Processes that have a particularly large impact on quality are regarded as critical processes. Major examples of critical processes include production culture processes, virus inactivation and removal processes, and affinity chromatography processes.

b. In-process tests

In the quality control of biopharmaceuticals, in-process tests are considered possible or appropriate to control contamination with such as process-related impurities, viruses and adventitious infectious factors. Examples of in-process testing include adventitious virus test after production culture, filter integrity tests of virus removal filters and sterile filters, testing for process-related impurities such as host cell proteins and host cell DNA, bioburden test, and so on. In-process tests are also evaluated for their validity by appropriate approaches such as analytical procedure validation.

1.2.3. Evaluation and control of contaminants

Contaminants are substances that should not be present in manufacturing processes, such as adventitious chemicals, biochemical materials, or microorganisms. From the viewpoint of ensuring safety, contaminants should be strictly avoided, and after constructing an appropriate manufacturing process. As mentioned above, it should be appropriately controlled by raw material control, in-process testing or specifications.

Viruses may contaminate as an adventitious factor from production processes and may be present as an endogenous factor in cell substrates used. The following three major complementary approaches are taken as rational measures to prevent the virus contamination specific to products using biological origin and to ensure the safety. 1) Selecting and testing cell lines and other raw materials, including media components, for the absence of undesirable viruses which may be infectious and/or pathogenic for humans. 2) Assess-

ing the capacity of production processes to clear infectious viruses. 3) Testing the product at appropriate steps of production for absence of contaminating infectious viruses. The details are described in General Information: ''Basic Requirements for Viral Safety of Biotechnological/Biological Products listed in Japanese Pharmacopoeia <*G3-13-141*>''.

1.2.4. Specifications

a. Justification of specifications

The items and test methods adopted for the specification differ depending on the quality control strategy established. It is necessary to clarify the justification of the acceptance criteria. The acceptance criteria are set on the basis of the data obtained from lots used in clinical trials, the data obtained from lots used to indicate the consistency of production, stability test data, and appropriate data in the product development stages.

b. Description

It qualitatively defines the physical state (e.g., solid, liquid) and color of a drug substance and drug product. Prescribe the transparency of a drug product if it is solution.

c. Identification test

Set up specific tests based on the structural features and specific properties of an active ingredient(s). In order to confirm the identity, not less than two types of tests (physical and chemical test, biological test, immunochemical test, etc.) are usually to be set up for a drug substance. For a drug product, one type of test may be sufficient, but some products may require more than one type of test.

d. Specific physical and/or chemical values

The quality attributes to be set as the specific physical and/or chemical values include oligosaccharide, charge, molecular mass/size, and so on. In the case where the product-related substances and the product-related impurities are difficult to separate and can not be set as the purity tests, the heterogeneity profiles are specified as the physical and/or chemical values. Typical examples include oligosaccharide profiles and charge profiles. In addition, set the specification for attributes that are important in ensuring the quality of a drug substance and drug product. Examples of the items include pH and osmotic pressure, etc.

e. Purity tests

Purity is usually assessed by a combination of analytical methods. In the choice and optimization of test methods for impurities, emphasis should be placed on separating or identifying the desired product and product-related substances from impurities (product-related impurities and process-related impurities).

f. Biological activities

Specifications for biopharmaceuticals should usually contain the tests for biological activity. Considering the action mechanism of the active ingredient, a suitable one of the methods used for the characterization is set as a biological activity test. The acceptance criterion is expressed in units/mL when the potency in the solution is used as an index, and when using the potency per protein amount as an index, it is expressed in units/mg. The potency per amount of protein is called the specific activity. Besides these, the specific activity may be compared with a standard material, and this may be expressed as a percentage (%) to obtain an acceptance criterion. In recent years, there has been an increasing number of cases where the ratio (%) of specific activity to a reference material is set as an acceptance criterion without setting a unit.

g. Quantity

The quantity of active ingredient contained in a drug substance and drug product is expressed as protein content (mass) or potency (unit). As it is a critical factor of product quality, measure it using an appropriate quantitative method. For tests to determine the protein content, the methods described in the General Information ''Total Protein Assay <*G3-12-172*>'', the method comparing peak areas with the reference material using HPLC, etc. are used. A biological test is used to determine the potency.

If physicochemical testing has provided sufficient physicochemical information on the product, including information on higher order structure, and proper correlation with biological activity has been well demonstrated, in addition, if the manufacturing experience is well established, biological tests for determining potency can be replaced by physicochemical testing. For insulins, etc., the quantity (unit) of the active ingredient in a sample is determined by comparing the peak area with the reference material indicated by the unit by the quantitative method using HPLC.

h. Tests for preparations

Conduct tests for preparations according to the dosage form. Since most biopharmaceuticals are injections, sterility test, bacterial endotoxin test, test for extractable volume of parenteral preparations, insoluble particulate matter test for injections and foreign insoluble matter test for injections, etc. are conducted.

1.2.5. Stability testing

a. Conditions of stability testing

The shelf life of biopharmaceuticals is usually set based on the results of long term testing at actual storage temperatures for the actual storage period of the product to be applied. The accelerated testing and the stress testing can provide supplementary information for setting the shelf life and useful information for elucidating the mechanism of quality change, as well as evaluating the validity of the analytical methods and the influence of storage conditions on the quality such as during transportation.

b. Attributes to be evaluated

During the storage of biopharmaceuticals, the bioactivity and the physicochemical properties may change, so it is necessary to comprehensively evaluate the quality characteristics by various analytical methods. In the stability testing, usually, adopting the appropriate attributes and tests used in characterization, evaluate the changes according to the characteristics of the product, such as biological activity, molecular heterogeneity, and product-related impurities.

2. Comparability of biopharmaceuticals subject to changes in their manufacturing process

When changing the manufacturing process of biopharmaceuticals, evaluation work on comparability will be carried out in order to ensure the quality, efficacy and safety of the drug product produced by the changed manufacturing process. The demonstration of comparability does not necessarily mean that the quality attributes of the pre-change and post-change product are identical, but that they are highly similar and that the existing knowledge is sufficiently predictive to ensure that any differences in quality attributes have no adverse impact upon safety and efficacy of the drug product. However, where the relationship between specific quality attributes and safety or efficacy has not been established, it might be appropriate to include a combination of quality, nonclinical, and/or clinical studies in the comparability exercise.

2.1. Considerations for the comparability exercise

The extent to which the test to prove the comparability before and after the change should be conducted is considered on the production step where the changes were introduced, on the risk level of potential impact of the changes on the

quality characteristics, on the suitability of the analytical techniques used, and on the relationship between quality attributes and safety or efficacy based on overall nonclinical and clinical experience. The judgment of product comparability should be done by considering characterization data, stability data providing insight into potential product differences in the changes and the degradation of the protein, and data of lots used for demonstration of manufacturing consistency. The historical data that provide insight into changes of quality attributes with respect to safety and efficacy following manufacturing process change, and nonclinical or clinical characteristics of the drug product and its therapeutic indications should also be considered.

2.2. Quality considerations

By re-executing all or part of the characterization that has already been carried out (if it is a part, it is necessary to explain its appropriateness), compare the quality characteristics before and after the change directly, to obtain the data needed to determine the comparability. However, it is necessary to evaluate the meaning of the difference by additional characterization, such as when difference in heterogeneity and/or impurity profile are found before and after the change. Even when evaluating the same quality attributes, it is necessary to apply multiple analysis methods and analysis methods having different measurement principles, and devise to be able to detect the change of the quality characteristics that may occur due to the change of manufacturing process. In addition, changes in the manufacturing process, even minor one, may affect the stability of the product, so when changing the manufacturing process that possibly affect the quality characteristics, also evaluate the influence on the stability of the product.

2.3. Manufacturing process considerations

Confirm that the process controls in the modified process provide at least similar or more effective control of the product quality, compared to those of the original process. A careful consideration of potential impacts of the planned change on steps downstream and quality attributes related to these steps is extremely important. The modified process steps should be re-evaluated and/or re-validated, as appropriate.

3. References

1) ICH: Guideline for Q5A (R1), Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin.
2) ICH: Guideline for Q5B, Quality of Biotechnological Products: Analysis of the Expression Construct in Cells Used for Production of R-DNA Derived Protein Products.
3) ICH: Guideline for Q5C, Quality of Biotechnological Products: Stability Testing of Biotechnological/Biological Products.
4) ICH: Guideline for Q5D, Derivation and Characterization of Cell Substrates Used for Production of Biotechnological/Biological Products.
5) ICH: Guideline for Q5E, Comparability of Biotechnological/Biological Products Subject to Changes in Their Manufacturing Process.
6) ICH: Guideline for Q6B, Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products.

Amino Acid Analysis ⟨*G3-2-171*⟩

This test is harmonized with the European Pharmacopoeia and the U.S. Pharmacopeia.

Information on the harmonization with the European Pharmacopoeia and the U.S. Pharmacopeia is available on the website of the Pharmaceuticals and Medical Devices Agency.

Amino acid analysis refers to the methodology used to determine the amino acid composition or content of proteins, peptides, and other pharmaceutical preparations. Proteins and peptides are macromolecules consisting of covalently bonded amino acid residues organized as a linear polymer. The sequence of the amino acids in a protein or peptide determines the properties of the molecule. Proteins are considered large molecules that commonly exist as folded structures with a specific conformation, while peptides are smaller and may consist of only a few amino acids. Amino acid analysis can be used to quantify protein and peptides, to determine the identity of proteins or peptides based on their amino acid composition, to support protein and peptide structure analysis, to evaluate fragmentation strategies for peptide mapping, and to detect atypical amino acids that might be present in a protein or peptide. It is necessary to hydrolyze a protein/peptide to its individual amino acid constituents before amino acid analysis. Following protein/peptide hydrolysis, the amino acid analysis procedure can be the same as that practiced for free amino acids in other pharmaceutical preparations. The amino acid constituents of the test sample are typically derivatized for analysis.

Apparatus

Methods used for amino acid analysis are usually based on a chromatographic separation of the amino acids present in the test sample. Current techniques take advantage of the automated chromatographic instrumentation designed for analytical methodologies. An amino acid analysis instrument will typically be a low-pressure or high-pressure liquid chromatograph capable of generating mobile phase gradients that separate the amino acid analytes on a chromatographic column. The instrument must have postcolumn derivatization capability, unless the sample is analyzed using precolumn derivatization. The detector is usually an ultraviolet-visible or fluorescence detector depending on the derivatization method used. A recording device (e.g., integrator) is used for transforming the analog signal from the detector and for quantitation. It is preferred that instrumentation be dedicated particularly for amino acid analysis.

General Precautions

Background contamination is always a concern for the analyst in performing amino acid analysis. High purity reagents are necessary (e.g., low purity hydrochloric acid can contribute to glycine contamination). Analytical reagents are changed routinely every few weeks using only high-pressure liquid chromatography (HPLC) grade solvents. Potential microbial contamination and foreign material that might be present in the solvents are reduced by filtering solvents before use, keeping solvent reservoirs covered, and not placing amino acid analysis instrumentation in direct sunlight.

Laboratory practices can determine the quality of the amino acid analysis. Place the instrumentation in a low traffic area of the laboratory. Keep the laboratory clean. Clean and calibrate pipets according to a maintenance schedule. Keep pipet tips in a covered box; the analysts may not handle pipet tips with their hands. The analysts may wear

powder-free latex or equivalent gloves. Limit the number of times a test sample vial is opened and closed because dust can contribute to elevated levels of glycine, serine, and alanine.

A well-maintained instrument is necessary for acceptable amino acid analysis results. If the instrument is used on a routine basis, it is to be checked daily for leaks, detector and lamp stability, and the ability of the column to maintain resolution of the individual amino acids. Clean or replace all instrument filters and other maintenance items on a routine schedule.

Reference Standard Material

Acceptable amino acid standards are commercially available for amino acid analysis and typically consist of an aqueous mixture of amino acids. When determining amino acid composition, protein or peptide standards are analyzed with the test material as a control to demonstrate the integrity of the entire procedure. Highly purified bovine serum albumin has been used as a protein standard for this purpose.

Calibration of Instrumentation

Calibration of amino acid analysis instrumentation typically involves analyzing the amino acid standard, which consists of a mixture of amino acids at a number of concentrations, to determine the response factor and range of analysis for each amino acid. The concentration of each amino acid in the standard is known. In the calibration procedure, the analyst dilutes the amino acid standard to several different analyte levels within the expected linear range of the amino acid analysis technique. Then, replicates at each of the different analyte levels can be analyzed. Peak areas obtained for each amino acid are plotted versus the known concentration for each of the amino acids in the standard dilution. These results will allow the analyst to determine the range of amino acid concentrations where the peak area of a given amino acid is an approximately linear function of the amino acid concentration. It is important that the analyst prepare the samples for amino acid analysis so that they are within the analytical limits (e.g., linear working range) of the technique employed in order to obtain accurate and repeatable results.

Four to six amino acid standard levels are analyzed to determine a response factor for each amino acid. The response factor is calculated as the average peak area or peak height per nmol of amino acid present in the standard. A calibration file consisting of the response factor for each amino acid is prepared and used to calculate the concentration of each amino acid present in the test sample. This calculation involves dividing the peak area corresponding to a given amino acid by the response factor for that amino acid to give the nmol of the amino acid. For routine analysis, a single-point calibration may be sufficient; however, the calibration file is updated frequently and tested by the analysis of analytical controls to ensure its integrity.

Repeatability

Consistent high quality amino acid analysis results from an analytical laboratory require attention to the repeatability of the assay. During analysis of the chromatographic separation of the amino acids or their derivatives, numerous peaks can be observed on the chromatogram that correspond to the amino acids. The large number of peaks makes it necessary to have an amino acid analysis system that can repeatedly identify the peaks based on retention time and integrate the peak areas for quantitation. A typical repeatability evaluation involves preparing a standard amino acid solution and analyzing many replicates (i.e., six analyses or more) of the same standard solution. The relative standard deviation (RSD) is determined for the retention time and integrated peak area of each amino acid. An evaluation of the repeatability is expanded to include multiple assays conducted over several days by different analysts. Multiple assays include the preparation of standard dilutions from starting materials to determine the variation due to sample handling. Often the amino acid composition of a standard protein (e.g., bovine serum albumin) is analyzed as part of the repeatability evaluation. By evaluating the replicate variation (i.e., RSD), the laboratory can establish analytical limits to ensure that the analyses from the laboratory are under control. It is desirable to establish the lowest practical variation limits to ensure the best results. Areas to focus on to lower the variability of the amino acid analysis include sample preparation, high background spectral interference due to quality of reagents and/or laboratory practices, instrument performance and maintenance, data analysis and interpretation, and analyst performance and habits. All parameters involved are fully investigated in the scope of the validation work.

Sample Preparation

Accurate results from amino acid analysis require purified protein and peptide samples. Buffer components (e.g., salts, urea, detergents) can interfere with the amino acid analysis and are removed from the sample before analysis. Methods that utilize postcolumn derivatization of the amino acids are generally not affected by buffer components to the extent seen with precolumn derivatization methods. It is desirable to limit the number of sample manipulations to reduce potential background contamination, to improve analyte recovery, and to reduce labor. Common techniques used to remove buffer components from protein samples include the following methods: (1) injecting the protein sample onto a reversed-phase HPLC system, eluting the protein with a volatile solvent containing a sufficient organic component, and drying the sample in a vacuum centrifuge; (2) dialysis against a volatile buffer or water; (3) centrifugal ultrafiltration for buffer replacement with a volatile buffer or water; (4) precipitating the protein from the buffer using an organic solvent (e.g., acetone); and (5) gel filtration.

Internal Standards

It is recommended that an internal standard be used to monitor physical and chemical losses and variations during amino acid analysis. An accurately known amount of internal standard can be added to a protein solution prior to hydrolysis. The recovery of the internal standard gives the general recovery of the amino acids of the protein solution. Free amino acids, however, do not behave in the same way as protein-bound amino acids during hydrolysis because their rates of release or destruction are variable. Therefore, the use of an internal standard to correct for losses during hydrolysis may give unreliable results. It will be necessary to take this point under consideration when interpreting the results. Internal standards can also be added to the mixture of amino acids after hydrolysis to correct for differences in sample application and changes in reagent stability and flow rates. Ideally, an internal standard is an unnaturally occurring primary amino acid that is commercially available and inexpensive. It should also be stable during hydrolysis, its response factor should be linear with concentration, and it needs to elute with a unique retention time without overlapping other amino acids. Commonly used amino acid standards include norleucine, nitrotyrosine, and α-aminobutyric acid.

Protein Hydrolysis

Hydrolysis of protein and peptide samples is necessary for amino acid analysis of these molecules. The glassware used for hydrolysis must be very clean to avoid erroneous results. Glove powders and fingerprints on hydrolysis tubes may cause contamination. To clean glass hydrolysis tubes, boil tubes for 1 hour in 1 mol/L hydrochloric acid or soak tubes in concentrated nitric acid or in a mixture of concentrated hydrochloric acid and concentrated nitric acid (1:1). Clean hydrolysis tubes are rinsed with high-purity water followed by a rinse with HPLC grade methanol, dried overnight in an oven, and stored covered until use. Alternatively, pyrolysis of clean glassware at 500°C for 4 hours may also be used to eliminate contamination from hydrolysis tubes. Adequate disposable laboratory material can also be used.

Acid hydrolysis is the most common method for hydrolyzing a protein sample before amino acid analysis. The acid hydrolysis technique can contribute to the variation of the analysis due to complete or partial destruction of several amino acids. Tryptophan is destroyed; serine and threonine are partially destroyed; methionine might undergo oxidation; and cysteine is typically recovered as cystine (but cystine recovery is usually poor because of partial destruction or reduction to cysteine). Application of adequate vacuum (≦less than 200 μm of mercury or 26.7 Pa) or introduction of an inert gas (argon) in the headspace of the reaction vessel can reduce the level of oxidative destruction. In peptide bonds involving isoleucine and valine the amido bonds of Ile-Ile, Val-Val, Ile-Val, and Val-Ile are partially cleaved; and asparagine and glutamine are deamidated, resulting in aspartic acid and glutamic acid, respectively. The loss of tryptophan, asparagine, and glutamine during an acid hydrolysis limits quantitation to 17 amino acids. Some of the hydrolysis techniques described are used to address these concerns. Some of the hydrolysis techniques described (i.e., *Methods 4-11*) may cause modifications to other amino acids. Therefore, the benefits of using a given hydrolysis technique are weighed against the concerns with the technique and are tested adequately before employing a method other than acid hydrolysis.

A time-course study (i.e., amino acid analysis at acid hydrolysis times of 24, 48, and 72 hours) is often employed to analyze the starting concentration of amino acids that are partially destroyed or slow to cleave. By plotting the observed concentration of labile amino acids (i.e., serine and threonine) versus hydrolysis time, the line can be extrapolated to the origin to determine the starting concentration of these amino acids. Time-course hydrolysis studies are also used with amino acids that are slow to cleave (e.g., isoleucine and valine). During the hydrolysis time course, the analyst will observe a plateau in these residues. The level of this plateau is taken as the residue concentration. If the hydrolysis time is too long, the residue concentration of the sample will begin to decrease, indicating destruction by the hydrolysis conditions.

An acceptable alternative to the time-course study is to subject an amino acid calibration standard to the same hydrolysis conditions as the test sample. The amino acid in free form may not completely represent the rate of destruction of labile amino acids within a peptide or protein during the hydrolysis. This is especially true for peptide bonds that are slow to cleave (e.g., Ile-Val bonds). However, this technique will allow the analyst to account for some residue destruction. Microwave acid hydrolysis has been used and is rapid but requires special equipment as well as special precautions. The optimal conditions for microwave hydrolysis must be investigated for each individual protein/peptide sample. The microwave hydrolysis technique typically requires only a few minutes, but even a deviation of one minute may give inadequate results (e.g., incomplete hydrolysis or destruction of labile amino acids). Complete proteolysis, using a mixture of proteases, has been used but can be complicated, requires the proper controls, and is typically more applicable to peptides than proteins.

Note: During initial analyses of an unknown protein, experiments with various hydrolysis time and temperature conditions are conducted to determine the optimal conditions.

Method 1

Acid hydrolysis using hydrochloric acid containing phenol is the most common procedure used for protein/peptide hydrolysis preceding amino acid analysis. The addition of phenol to the reaction prevents the halogenation of tyrosine.

Hydrolysis Solution 6 mol/L hydrochloric acid containing 0.1% to 1.0% of phenol.

Procedure—

Liquid Phase Hydrolysis Place the protein or peptide sample in a hydrolysis tube, and dry. [Note: The sample is dried so that water in the sample will not dilute the acid used for the hydrolysis.] Add 200 μL of *Hydrolysis Solution* per 500 μg of lyophilized protein. Freeze the sample tube in a dry ice-acetone bath, and flame seal in vacuum. Samples are typically hydrolyzed at 110°C for 24 hours in vacuum or inert atmosphere to prevent oxidation. Longer hydrolysis times (e.g., 48 and 72 hours) are investigated if there is a concern that the protein is not completely hydrolyzed.

Vapor Phase Hydrolysis This is one of the most common acid hydrolysis procedures, and it is preferred for microanalysis when only small amounts of the sample are available. Contamination of the sample from the acid reagent is also minimized by using vapor phase hydrolysis. Place vials containing the dried samples in a vessel that contains an appropriate amount of *Hydrolysis Solution*. The *Hydrolysis Solution* does not come in contact with the test sample. Apply an inert atmosphere or vacuum (≦less than 200 μm of mercury or 26.7 Pa) to the headspace of the vessel, and heat to about 110°C for a 24-hour hydrolysis time. Acid vapor hydrolyzes the dried sample. Any condensation of the acid in the sample vials is minimized. After hydrolysis, dry the test sample in vacuum to remove any residual acid.

Method 2

Tryptophan oxidation during hydrolysis is decreased by using mercaptoethanesulfonic acid (MESA) as the reducing acid.

Hydrolysis Solution 2.5 mol/L MESA solution.

Vapor Phase Hydrolysis About 1 to 100 μg of the protein/peptide under test is dried in a hydrolysis tube. The hydrolysis tube is placed in a larger tube with about 200 μL of the *Hydrolysis Solution*. The larger tube is sealed in vacuum (about 50 μm of mercury or 6.7 Pa) to vaporize the *Hydrolysis Solution*. The hydrolysis tube is heated to 170°C to 185°C for about 12.5 minutes. After hydrolysis, the hydrolysis tube is dried in vacuum for 15 minutes to remove the residual acid.

Method 3

Tryptophan oxidation during hydrolysis is prevented by using thioglycolic acid (TGA) as the reducing acid.

Hydrolysis Solution A solution containing 7 mol/L hydrochloric acid, 10% of trifluoroacetic acid, 20% of thioglycolic acid, and 1% of phenol.

Vapor Phase Hydrolysis About 10 to 50 μg of the protein/peptide under test is dried in a sample tube. The sample tube is placed in a larger tube with about 200 μL of the

Hydrolysis Solution. The larger tube is sealed in vacuum (about 50 μm of mercury or 6.7 Pa) to vaporize the TGA. The sample tube is heated to 166°C for about 15 to 30 minutes. After hydrolysis, the sample tube is dried in vacuum for 5 minutes to remove the residual acid. Recovery of tryptophan by this method may be dependent on the amount of sample present.

Method 4

Cysteine-cystine and methionine oxidation is performed with performic acid before the protein hydrolysis.

Oxidation Solution The performic acid is prepared fresh by mixing formic acid and 30 percent hydrogen peroxide (9:1), and incubated at room temperature for 1 hour.

Procedure The protein/peptide sample is dissolved in 20 μL of formic acid, and heated at 50°C for 5 minutes; then 100 μL of the *Oxidation Solution* is added. The oxidation is allowed to proceed for 10 to 30 minutes. In this reaction, cysteine is converted to cysteic acid and methionine is converted to methionine sulfone. The excess reagent is removed from the sample in a vacuum centrifuge. This technique may cause modifications to tyrosine residues in the presence of halides. The oxidized protein can then be acid hydrolyzed using *Method 1* or *Method 2*.

Method 5

Cysteine-cystine oxidation is accomplished during the liquid phase hydrolysis with sodium azide.

Hydrolysis Solution 6 mol/L hydrochloric acid containing 0.2% of phenol, to which sodium azide is added to obtain a final concentration of 0.2% (w/v). The added phenol prevents halogenation of tyrosine.

Liquid Phase Hydrolysis The protein/peptide hydrolysis is conducted at about 110°C for 24 hours. During the hydrolysis, the cysteine-cystine present in the sample is converted to cysteic acid by the sodium azide present in the *Hydrolysis Solution*. This technique allows better tyrosine recovery than *Method 4*, but it is not quantitative for methionine. Methionine is converted to a mixture of the parent methionine and its two oxidative products, methionine sulfoxide and methionine sulfone.

Method 6

Cysteine-cystine oxidation is accomplished with dimethyl sulfoxide (DMSO).

Hydrolysis Solution 6 mol/L hydrochloric acid containing 0.1% to 1.0% of phenol, to which DMSO is added to obtain a final concentration of 2% (v/v).

Vapor Phase Hydrolysis The protein/peptide hydrolysis is conducted at about 110°C for 24 hours. During the hydrolysis, the cysteine-cystine present in the sample is converted to cysteic acid by the DMSO present in the *Hydrolysis Solution*. As an approach to limit variability and compensate for partial destruction, it is recommended to evaluate the cysteic acid recovery from oxidative hydrolyses of standard proteins containing 1 to 8 mol of cysteine per mol of protein. The response factors from protein/peptide hydrolysates are typically about 30% lower than those for nonhydrolyzed cysteic acid standards. Because histidine, methionine, tyrosine, and tryptophan are also modified, a complete compositional analysis is not obtained with this technique.

Method 7

Cysteine-cystine reduction and alkylation is accomplished by a vapor phase pyridylethylation reaction.

Reducing Solution Transfer 83.3 μL of pyridine, 16.7 μL of 4-vinylpyridine, 16.7 μL of tributylphosphine, and 83.3 μL of water to a suitable container, and mix.

Procedure Add the protein/peptide (between 1 μg and 100 μg) to a hydrolysis tube, and place in a larger tube. Transfer the *Reducing Solution* to the large tube, seal in vacuum (about 50 μm of mercury or 6.7 Pa), and incubate at about 100°C for 5 minutes. Then remove the inner hydrolysis tube, and dry it in a vacuum desiccator for 15 minutes to remove residual reagents. The pyridylethylated protein/peptide can then be acid hydrolyzed using previously described procedures. The pyridylethylation reaction is performed simultaneously with a protein standard sample containing 1 to 8 mol of cysteine per mol of protein to improve accuracy in the pyridylethylcysteine recovery. Longer incubation times for the pyridylethylation reaction can cause modifications to the α-amino terminal group and the ε-amino group of lysine in the protein.

Method 8

Cysteine-cystine reduction and alkylation is accomplished by a liquid phase pyridylethylation reaction.

Stock Solutions Prepare and filter three solutions: 1 mol/L Tris hydrochloride (pH 8.5) containing 4 mmol/L disodium dihydrogen ethylendiamine tetraacetate (*Stock Solution A*), 8 mol/L guanidine hydrochloride (*Stock Solution B*), and 10% of 2-mercaptoethanol in water (*Stock Solution C*).

Reducing Solution Prepare a mixture of *Stock Solution B* and *Stock Solution A* (3:1) to obtain a buffered solution of 6 mol/L guanidine hydrochloride in 0.25 mol/L Tris hydrochloride.

Procedure Dissolve about 10 μg of the test sample in 50 μL of the *Reducing Solution*, and add about 2.5 μL of *Stock Solution C*. Store under nitrogen or argon for 2 hours at room temperature in the dark. To achieve the pyridylethylation reaction, add about 2 μL of 4-vinylpyridine to the protein solution, and incubate for an additional 2 hours at room temperature in the dark. The protein/peptide is desalted by collecting the protein/peptide fraction from a reversed-phase HPLC separation. The collected sample can be dried in a vacuum centrifuge before acid hydrolysis.

Method 9

Cysteine-cystine reduction and alkylation is accomplished by a liquid phase carboxymethylation reaction.

Stock Solutions Prepare as directed for *Method 8*.

Carboxymethylation Solution Prepare a solution containing 100 mg of iodoacetamide per mL of ethanol (95).

Buffer Solution Use the *Reducing Solution*, prepared as directed for *Method 8*.

Procedure Dissolve the test sample in 50 μL of the *Buffer Solution*, and add about 2.5 μL of *Stock Solution C*. Store under nitrogen or argon for 2 hours at room temperature in the dark. Add the *Carboxymethylation Solution* in a ratio 1.5 fold per total theoretical content of thiols, and incubate for an additional 30 minutes at room temperature in the dark. [Note: If the thiol content of the protein is unknown, then add 5 μL of 100 mmol/L iodoacetamide for every 20 nmol of protein present.] The reaction is stopped by adding excess of 2-mercaptoethanol. The protein/peptide is desalted by collecting the protein/peptide fraction from a reversed-phase HPLC separation. The collected sample can be dried in a vacuum centrifuge before acid hydrolysis. The *S*-carboxyamidomethyl-cysteine formed will be converted to *S*-carboxymethylcysteine during acid hydrolysis.

Method 10

Cysteine-cystine is reacted with dithiodiglycolic acid or dithiodipropionic acid to produce a mixed disulfide. [Note: The choice of dithiodiglycolic acid or dithiodipropionic acid

depends on the required resolution of the amino acid analysis method.]

Reducing Solution A solution containing 10 mg of dithiodiglycolic acid (or dithiodipropionic acid) per mL of 0.2 mol/L sodium hydroxide.

Procedure Transfer about 20 μg of the test sample to a hydrolysis tube, and add 5 μL of the *Reducing Solution*. Add 10 μL of isopropyl alcohol, and then remove all of the sample liquid by vacuum centrifugation. The sample is then hydrolyzed using *Method 1*. This method has the advantage that other amino acid residues are not derivatized by side reactions, and the sample does not need to be desalted prior to hydrolysis.

Method 11

Asparagine and glutamine are converted to aspartic acid and glutamic acid, respectively, during acid hydrolysis. Asparagine and aspartic acid residues are added and represented by *Asx*, while glutamine and glutamic acid residues are added and represented by *Glx*. Proteins/peptides can be reacted with bis(1,1-trifluoroacetoxy)iodobenzene (BTI) to convert the asparagine and glutamine residues to diaminopropionic acid and diaminobutyric acid residues, respectively, upon acid hydrolysis. These conversions allow the analyst to determine the asparagine and glutamine content of a protein/peptide in the presence of aspartic acid and glutamic acid residues.

Reducing Solutions Prepare and filter three solutions: a solution of 10 mmol/L trifluoroacetic acid (*Solution A*), a solution of 5 mol/L guanidine hydrochloride and 10 mmol/L trifluoroacetic acid (*Solution B*), and a freshly prepared solution of *N*,*N*-dimethylformamide containing 36 mg of BTI per mL (*Solution C*).

Procedure In a clean hydrolysis tube, transfer about 200 μg of the test sample, and add 2 mL of *Solution A* or *Solution B* and 2 mL of *Solution C*. Seal the hydrolysis tube in vacuum. Heat the sample at 60°C for 4 hours in the dark. The sample is then dialyzed with water to remove the excess reagents. Extract the dialyzed sample three times with equal volumes of *n*-butyl acetate, and then lyophilize. The protein can then be acid hydrolyzed using previously described procedures. The α,β-diaminopropionic and α,γ-diaminobutyric acid residues do not typically resolve from the lysine residues upon ion-exchange chromatography based on amino acid analysis. Therefore, when using ion-exchange as the mode of amino acid separation, the asparagine and glutamine contents are the quantitative difference in the aspartic acid and glutamic acid content assayed with underivatized and BTI-derivatized acid hydrolysis. [Note: The threonine, methionine, cysteine, tyrosine, and histidine assayed content can be altered by BTI derivatization; a hydrolysis without BTI will have to be performed if the analyst is interested in the composition of these other amino acid residues of the protein/peptide.]

Methodologies of Amino Acid Analysis General Principles

Many amino acid analysis techniques exist, and the choice of any one technique often depends on the sensitivity required from the assay. In general, about one-half of the amino acid analysis techniques employed rely on the separation of the free amino acids by ion-exchange chromatography followed by postcolumn derivatization (e.g., with ninhydrin or *o*-phthalaldehyde). Postcolumn detection techniques can be used with samples that contain small amounts of buffer components, such as salts and urea, and generally require between 5 and 10 μg of protein sample per analysis. The remaining amino acid techniques typically involve precolumn derivatization of the free amino acids (e.g., phenyl isothiocyanate; 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate or *o*-phthalaldehyde; (dimethylamino)azobenzenesulfonyl chloride; 9-fluorenylmethylchloroformate; and, 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole) followed by reversed-phase HPLC. Precolumn derivatization techniques are very sensitive and usually require between 0.5 and 1.0 μg of protein sample per analysis but may be influenced by buffer salts in the samples. Precolumn derivatization techniques may also result in multiple derivatives of a given amino acid, which complicates the result interpretation. Postcolumn derivatization techniques are generally influenced less by performance variation of the assay than precolumn derivatization techniques.

The following *Methods* may be used for quantitative amino acid analysis. Instruments and reagents for these procedures are available commercially. Furthermore, many modifications of these methodologies exist with different reagent preparations, reaction procedures, chromatographic systems, etc. Specific parameters may vary according to the exact equipment and procedure used. Many laboratories will utilize more than one amino acid analysis technique to exploit the advantages offered by each. In each of these *Methods*, the analog signal is visualized by means of a data acquisition system, and the peak areas are integrated for quantification purposes.

Method 1—Postcolumn Ninhydrin Detection General Principle

Ion-exchange chromatography with postcolumn ninhydrin detection is one of the most common methods employed for quantitative amino acid analysis. As a rule, a Li-based cation-exchange system is employed for the analysis of the more complex physiological samples, and the faster Na-based cation-exchange system is used for the more simplistic amino acid mixtures obtained with protein hydrolysates (typically containing 17 amino acid components). Separation of the amino acids on an ion-exchange column is accomplished through a combination of changes in pH and cation strength. A temperature gradient is often employed to enhance separation.

When the amino acid reacts with ninhydrin, the reactant has characteristic purple or yellow color. Amino acids, except imino acid, give a purple color, and show the maximum absorption at 570 nm. The imino acids such as proline give a yellow color, and show the maximum absorption at 440 nm. The postcolumn reaction between ninhydrin and amino acid eluted from column is monitored at 440 and 570 nm, and the chromatogram obtained is used for the determination of amino acid composition.

Detection limit is considered to be 10 pmol for most of the amino acid derivatives, but 50 pmol for proline. Response linearity is obtained in the range of 20 to 500 pmol with correlation coefficients exceeding 0.999. To obtain good composition data, samples larger than 1 μg before hydrolysis are best suited for this amino acid analysis of protein/peptide.

Method 2—Postcolumn OPA Fluorometric Detection General Principle

o-Phthalaldehyde (OPA) reacts with primary amines in the presence of thiol compound, to form highly fluorescent isoindole products. This reaction is utilized for the postcolumn derivatization in analysis of amino acids by ion-exchange chromatography. The rule of the separation is the same as *Method 1*. Instruments and reagents for this form of amino acid analysis are available commercially. Many modifications of this methodology exist.

Although OPA does not react with secondary amines (imino acids such as proline) to form fluorescent substances,

the oxidation with sodium hypochlorite allows secondary amines to react with OPA. The procedure employs a strongly acidic cation-exchange column for separation of free amino acids followed by postcolumn oxidation with sodium hypochlorite and postcolumn derivatization using OPA and thiol compound such as *N*-acetyl-L-cysteine and 2-mercaptoethanol. The derivatization of primary amino acids is not noticeably affected by the continuous supply of sodium hypochlorite.

Separation of the amino acids on an ion-exchange column is accomplished through a combination of changes in pH and cation strength. After postcolumn derivatization of eluted amino acids with OPA, the reactant passes through the fluorometric detector. Fluorescence intensity of OPA-derivatized amino acids is monitored with an excitation wavelength of 348 nm and an emission wavelength of 450 nm.

Detection limit is considered to be a few tens of picomole level for most of the amino acid derivatives. Response linearity is obtained in the range of a few picomole level to a few tens of nanomole level. To obtain good compositional data, the starting with greater than 500 ng of sample before hydrolysis is best suited for the amino acid analysis of protein/peptide.

Method 3—Precolumn PITC Derivatization General Principle

Phenylisothiocyanate (PITC) reacts with amino acids to form phenylthiocarbamyl (PTC) derivatives which can be detected with high sensitivity at 245 nm. Therefore, precolumn derivatization of amino acids with PITC followed by reversed-phase HPLC separation with UV detection is used to analyze the amino acid composition.

After the reagent is removed under vacuum, the derivatized amino acids can be stored dry and frozen for several weeks with no significant degradation. If the solution for injection is kept cold, no noticeable loss in chromatographic response occurs after three days.

Separation of the PTC-amino acids on a reversed-phase HPLC with ODS column is accomplished through a combination of changes in concentrations of acetonitrile and buffer ionic strength. PTC-amino acids eluted from the column are monitored at 254 nm.

Detection limit is considered to be 1 pmol for most of the amino acid derivatives. Response linearity is obtained in the range of 20 to 500 pmol with correlation coefficients exceeding 0.999. To obtain good compositional data, samples larger than 500 ng of protein/peptide before hydrolysis is best suited for this amino acid analysis of proteins/peptides.

Method 4—Precolumn AQC Derivatization General Principle

Precolumn derivatization of amino acids with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) followed by reversed-phase HPLC separation with fluorometric detection is used.

AQC reacts with amino acids to form stable, fluorescent unsymmetric urea derivatives (AQC-amino acids) which are readily amenable to analysis by reversed-phase HPLC. Therefore, precolumn derivatization of amino acids with AQC followed by reversed-phase HPLC separation is used to analyze the amino acid composition.

Separation of the AQC-amino acids on an ODS column is accomplished through a combination of changes in concentrations of acetonitrile and salt. Selective fluorescence detection of the derivatives with excitation wavelength at 250 nm and emission wavelength at 395 nm allows for the direct injection of the reaction mixture with no significant interference from the only major fluorescent reagent by-product, 6-aminoquinoline. Excess reagent is rapidly hydrolyzed ($t_{1/2} < 15$ seconds) to yield 6-aminoquinoline, *N*-hydroxysuccinimide and carbon dioxide, and after 1 minute no further derivatization can take place.

Peak areas for AQC-amino acids are essentially unchanged for at least 1 week at room temperature, and the derivatives have more than sufficient stability to allow for overnight automated chromatographic analysis.

Detection limit is considered to be ranging from ca. 40 to 320 fmol for each amino acid, except for Cys. Detection limit for Cys is approximately 800 fmol. Response linearity is obtained in the range of 2.5 to 200 μmol/L with correlation coefficients exceeding 0.999. Good compositional data could be obtained from the analysis of derivatized protein hydrolysates containing as little as 30 ng of protein/peptide.

Method 5—Precolumn OPA Derivatization General Principle

Precolumn derivatization of amino acids with *o*-phthalaldehyde (OPA) followed by reversed-phase HPLC separation with fluorometric detection is used. This technique does not detect amino acids that exist as secondary amines (e.g., proline).

OPA in conjunction with a thiol reagent reacts with primary amine groups to form highly fluorescent isoindole products. 2-Mercaptoethanol or 3-mercaptopropionic acid can be used as thiol. OPA itself does not fluoresce and consequently produces no interfering peaks. In addition, its solubility and stability in aqueous solution, along with the rapid kinetics for the reaction, make it amenable to automated derivatization and analysis using an autosampler to mix the sample with the reagent. However, lack of reactivity with secondary amino acids has been a predominant drawback. This method does not detect amino acids that exist as secondary amines (e.g., proline). To compensate for this drawback, this technique may be combined with another technique described in *Method 7* or *Method 8*.

Precolumn derivatization of amino acids with OPA is followed by reversed-phase HPLC separation. Because of the instability of the OPA-amino acid derivative, HPLC separation and analysis are performed immediately following derivatization. The liquid chromatograph is equipped with a fluorometric detector for the detection of derivatized amino acids. Fluorescence intensity of OPA-derivatized amino acids is monitored with an excitation wavelength of 348 nm and an emission wavelength of 450 nm.

Detection limits as low as 50 fmol via fluorescence have been reported, although the practical limit of analysis remains at 1 pmol.

Method 6—Precolumn DABS-Cl Derivatization General Principle

Precolumn derivatization of amino acids with (dimethylamino)azobenzenesulfonyl chloride (DABS-Cl) followed by reversed-phase HPLC separation with visible light detection is used.

DABS-Cl is a chromophoric reagent employed for the labeling of amino acids. Amino acids labeled with DABS-Cl (DABS-amino acids) are highly stable and show the maximum absorption at 436 nm.

DABS-amino acids, all 19 naturally occurring amino acids derivatives, can be separated on an ODS column of reversed-phase HPLC by employing gradient systems consisting of acetonitrile and aqueous buffer mixture. Separated DABS-amino acids eluted from the column are detected at 436 nm in the visible region.

This *Method* can analyze the imino acids such as proline together with the amino acids at the same degree of sensitivity, DABS-Cl derivatization method permits the simultaneous quantification of tryptophan residues by previous hydrolysis of the protein/peptide with sulfonic acids such as mercaptoethanesulfonic acid, *p*-toluenesulfonic acid or methanesulfonic acid described under *Method 2* in ''Protein Hydrolysis''. The other acid-labile residues, asparagine and glutamine, can also be analysed by previous conversion into diaminopropionic acid and diaminobutyric acid, respectively, by treatment of protein/peptide with BTI described under *Method 11* in ''Protein Hydrolysis''.

The non-proteinogenic amino acid, norleucine cannot be used as internal standard in this method, as this compound is eluted in a chromatographic region crowded with peaks of primary amino acids. Nitrotyrosine can be used as an internal standard, because it is eluted in a clean region.

Detection limit of DABS-amino acid is about 1 pmol. As little as 2 to 5 pmol of an individual DABS-amino acid can be quantitatively analyzed with reliability, and only 10 to 30 ng of the dabsylated protein hydrolysate is required for each analysis.

Method 7—Precolumn FMOC-Cl Derivatization General Principle

Precolumn derivatization of amino acids with 9-fluorenylmethyl chloroformate (FMOC-Cl) followed by reversed-phase HPLC separation with fluorometric detection is used.

FMOC-Cl reacts with both primary and secondary amino acids to form highly fluorescent products. The reaction of FMOC-Cl with amino acid proceeds under mild conditions in aqueous solution and is completed in 30 seconds. The derivatives are stable, only the histidine derivative showing any breakdown. Although FMOC-Cl is fluorescent itself, the reagent excess and fluorescent side-products can be eliminated without loss of FMOC-amino acids.

FMOC-amino acids are separated by a reversed-phase HPLC using an ODS column. The separation is carried out by gradient elution varied linearly from a mixture of acetonitrile methanol and acetic acid buffer (10:40:50) to a mixture of acetonitrile and acetic acid buffer (50:50), and 20 amino acid derivatives are separated in 20 minutes. Each derivative eluted from the column is monitored by a fluorometric detector set at an excitation wavelength of 260 nm and an emission wavelength of 313 nm.

The detection limit is in the low fmol range. A linearity range of 0.1 to 50 μmol/L is obtained for most of the amino acids.

Method 8—Precolumn NBD-F Derivatization General Principle

Precolumn derivatization of amino acids with 7-fluoro-4-nitrobenzo-2-oxa-1.3-diazole (NBD-F) followed by reversed-phase HPLC separation with fluorometric detection is used.

NBD-F reacts with both primary and secondary amino acids to form highly fluorescent products. Amino acids are derivatized with NBD-F by heating to 60°C for 5 minutes.

NBD-amino acid derivatives are separated on an ODS column of reversed-phase HPLC by employing gradient elution system consisting of acetonitrile and aqueous buffer mixture, and 17 amino acid derivatives are separated in 35 minutes. ε-Aminocaproic acid can be used as an internal standard, because it is eluted in a clean chromatographic region. Each derivative eluted from the column is monitored by a fluorometric detector set at an excitation wavelength of 480 nm and an emission wavelength of 530 nm.

The sensitivity of this method is almost the same as for precolumn OPA derivatization method (*Method 5*), excluding proline to which OPA is not reactive, and might be advantageous for NBD-F against OPA. The detection limit for each amino acid is about 10 fmol. Profile analysis was achieved for about 1.5 μg of protein hydrolysates in the final precolumn labeling reaction mixture for HPLC.

Data Calculation and Analysis

When determining the amino acid content of a protein/peptide hydrolysate, it should be noted that the acid hydrolysis step destroys tryptophan and cysteine. Serine and threonine are partially destroyed by acid hydrolysis, while isoleucine and valine residues may be only partially cleaved. Methionine can undergo oxidation during acid hydrolysis, and some amino acids (e.g., glycine and serine) are common contaminants. Application of adequate vacuum (≦0.0267 kPa) or introduction of inert gas (argon) in the headspace of the reaction vessel during vapor phase hydrolysis can reduce the level of oxidative destruction. Therefore, the quantitative results obtained for cysteine, tryptophan, threonine, isoleucine, valine, methionine, glycine, and serine from a protein/peptide hydrolysate may be variable and may warrant further investigation and consideration.

Calculations

Amino Acid Mole Percent This is the number of specific amino acid residues per 100 residues in a protein. This result may be useful for evaluating amino acid analysis data when the molecular weight of the protein under investigation is unknown. This information can be used to corroborate the identity of a protein/peptide and has other applications. Carefully identify and integrate the peaks obtained as directed for each *Procedure*. Calculate the mole percent for each amino acid present in the test sample by the formula:

$$100r_U/r,$$

in which r_U is the peak response, in nmol, of the amino acid under test; and r is the sum of peak responses, in nmol, for all amino acids present in the test sample. Comparison of the mole percent of the amino acids under test to data from known proteins can help establish or corroborate the identity of the sample protein.

Unknown Protein Samples This data analysis technique can be used to estimate the protein concentration of an unknown protein sample using the amino acid analysis data. Calculate the mass, in μg, of each recovered amino acid by the formula:

$$mM_W/1000,$$

in which m is the recovered quantity, in nmol, of the amino acid under test; and M_W is the average molecular weight for that amino acid, corrected for the weight of the water molecule that was eliminated during peptide bond formation. The sum of the masses of the recovered amino acids will give an estimate of the total mass of the protein analyzed after appropriate correction for partially and completely destroyed amino acids. If the molecular weight of the unknown protein is available (i.e., by SDS-PAGE analysis or mass spectroscopy), the amino acid composition of the unknown protein can be predicted. Calculate the number of residues of each amino acid by the formula:

$$m/(1000M/M_{WT}),$$

in which m is the recovered quantity, in nmol, of the amino acid under test; M is the total mass, in μg, of the protein; and M_{WT} is the molecular weight of the unknown protein.

Known Protein Samples This data analysis technique can be used to investigate the amino acid composition and pro-

tein concentration of a protein sample of known molecular weight and amino acid composition using the amino acid analysis data. When the composition of the protein being analyzed is known, one can exploit the fact that some amino acids are recovered well, while other amino acid recoveries may be compromised because of complete or partial destruction (e.g., tryptophan, cysteine, threonine, serine, methionine), incomplete bond cleavage (i.e., for isoleucine and valine) and free amino acid contamination (i.e., by glycine and serine).

Because those amino acids that are recovered best represent the protein, these amino acids are chosen to quantify the amount of protein. Well-recovered amino acids are, typically, aspartate-asparagine, glutamate-glutamine, alanine, leucine, phenylalanine, lysine, and arginine. This list can be modified based on experience with one's own analysis system. Divide the quantity, in nmol, of each of the well-recovered amino acids by the expected number of residues for that amino acid to obtain the protein content based on each well-recovered amino acid. Average the protein content results calculated. The protein content determined for each of the well-recovered amino acids should be evenly distributed about the mean. Discard protein content values for those amino acids that have an unacceptable deviation from the mean. Typically ≧greater than 5% variation from the mean is considered unacceptable. Recalculate the mean protein content from the remaining values to obtain the protein content of the sample. Divide the content of each amino acid by the calculated mean protein content to determine the amino acid composition of the sample by analysis.

Calculate the relative compositional error, in percentage, by the formula:

$$100m/m_S,$$

in which m is the experimentally determined quantity, in nmol per amino acid residue, of the amino acid under test; and m_S is the known residue value for that amino acid. The average relative compositional error is the average of the absolute values of the relative compositional errors of the individual amino acids, typically excluding tryptophan and cysteine from this calculation. The average relative compositional error can provide important information on the stability of analysis run over time. The agreement in the amino acid composition between the protein sample and the known composition can be used to corroborate the identity and purity of the protein in the sample.

Peptide Mapping <*G3-3-142*>

This test is harmonized with the European Pharmacopoeia and the U.S. Pharmacopeia.

Information on the harmonization with the European Pharmacopoeia and the U.S. Pharmacopeia is available on the website of the Pharmaceuticals and Medical Devices Agency.

Peptide mapping is an identity test for proteins, especially those obtained by r-DNA technology. It involves the chemical or enzymatic treatment of a protein, resulting in the formation of peptide fragments, followed by separation and identification of the fragments in a reproducible manner. It is a powerful test that is capable of identifying single amino acid changes resulting from events such as errors in the reading of complementary DNA (cDNA) sequences or point mutations. Peptide mapping is a comparative procedure because the information obtained, compared to a reference standard or reference material similarly treated, confirms the primary structure of the protein, is capable of detecting whether alterations in structure have occurred, and demonstrates process consistency and genetic stability. Each protein presents unique characteristics which must be well understood so that the scientific and analytical approaches permit validated development of a peptide map that provides sufficient specificity.

This chapter provides detailed assistance in the application of peptide mapping and its validation to characterize the desired protein product, to evaluate the stability of the expression construct of cells used for recombinant DNA products, to evaluate the consistency of the overall process and to assess product stability as well as to ensure the identity of the protein product, or to detect the presence of protein variant.

1. Peptide Map

Peptide mapping is not a general method, but involves developing specific maps for each unique protein. Although the technology is evolving rapidly, there are certain methods that are generally accepted. Variations of these methods will be indicated, when appropriate, in specific monographs.

A peptide map may be viewed as a fingerprint of a protein and is the end product of several chemical processes that provide a comprehensive understanding of the protein being analyzed. Four major steps are necessary for the development of the procedure: isolation and purification of the protein, if the protein is part of a formulation; selective cleavage of the peptide bonds; chromatographic separation of the peptides; and analysis and identification of the peptides. A test sample is digested and assayed in parallel with a reference standard or a reference material. Complete cleavage of peptide bonds is more likely to occur when enzymes such as endoproteases (e.g., trypsin) are used, instead of chemical cleavage reagents. A map should contain enough peptides to be meaningful. On the other hand, if there are too many fragments, the map might lose its specificity because many proteins will then have the same profiles.

2. Isolation and Purification

Isolation and purification are necessary for analysis of bulk drugs or dosage forms containing interfering excipients and carrier proteins and, when required, will be specified in the monograph. Quantitative recovery of protein from the dosage form should be validated.

3. Selective Cleavage of Peptide Bonds

The selection of the approach used for the cleavage of peptide bonds will depend on the protein under test. This selection process involves determination of the type of cleavage to be employed —enzymatic or chemical— and the type of cleavage agent within the chosen category. Several cleavage agents and their specificity are shown in Table 1. This list is not all-inclusive and will be expanded as other cleavage agents are identified.

3.1. Pretreatment of Sample

Depending on the size or the configuration of the protein, different approaches in the pretreatment of samples can be used. For monoclonal antibodies, the heavy and light chains will need to be separated before mapping. If trypsin is used as a cleavage agent for proteins with a molecular mass greater than 100,000 Da, lysine residues must be protected by citraconylation or maleylation; otherwise, too many peptides will be generated.

3.2. Pretreatment of the Cleavage Agent

Pretreatment of cleavage agents —especially enzymatic

Table 1 Examples of cleavage agents

Type	Agent	Specificity
Enzymatic	Trypsin (EC 3.4.21.4)	C-terminal side of Arg and Lys
	Chymotrypsin (EC 3.4.21.1)	C-terminal side of hydrophobic residues (e.g., Leu, Met, Ala, aromatics)
	Pepsin (EC 3.4.23.1 & 2)	Nonspecific digest
	Lysyl endopeptidase (Lys-C endopeptidase) (EC 3.4.21.50)	C-terminal side of Lys
	Glutamyl endopeptidase (from *S. aureus* strain V8) (EC 3.4.21.19)	C-terminal side of Glu and Asp
	Peptidyl-Asp metallo endopeptidase (Endoproteinase Asp-N) (EC 3.24.33)	N-terminal side of Asp
	Clostripain (EC 3.4.22.8)	C-terminal side of Arg
Chemical	Cyanogen bromide	C-terminal side of Met
	2-Nitro-5-thio-cyanobenzoic acid	N-terminal side of Cys
	o-Iodosobenzoic acid	C-terminal side of Trp and Tyr
	Dilute acid	Asp and Pro
	BNPS-skatole	Trp

agents— might be necessary for purification purposes to ensure reproducibility of the map. For example, trypsin used as a cleavage agent will have to be treated with tosyl-L-phenylalanine chloromethyl ketone to inactivate chymotrypsin. Other methods, such as purification of trypsin by HPLC or immobilization of enzyme on a gel support, have been successfully used when only a small amount of protein is available.

3.3. Pretreatment of the Protein

Under certain conditions, it might be necessary to concentrate the sample or to separate the protein from added substances and stabilizers used in formulation of the product, if these interfere with the mapping procedure. Physical procedures used for pretreatment can include ultrafiltration, column chromatography, and lyophilization. Other pretreatments, such as the addition of chaotropic agents (e.g., urea) can be used to unfold the protein prior to mapping. To allow the enzyme to have full access to cleavage sites and permit some unfolding of the protein, it is often necessary to reduce and alkylate the disulfide bonds prior to digestion.

Digestion with trypsin can introduce ambiguities in the tryptic map due to side reactions occurring during the digestion reaction, such as nonspecific cleavage, deamidation, disulfide isomerization, oxidation of methionine residues, or formation of pyroglutamic groups created from the deamidation of glutamine at the N-terminal side of a peptide. Furthermore, peaks may be produced by autohydrolysis of trypsin. Their intensities depend on the ratio of trypsin to protein. To avoid autohydrolysis, solutions of proteases may be prepared at a pH that is not optimal (e.g., at pH 5 for trypsin), which would mean that the enzyme would not become active until diluted with the digest buffer.

3.4. Establishment of Optimal Digestion Conditions

Factors that affect the completeness and effectiveness of digestion of proteins are those that could affect any chemical or enzymatic reactions.

(i) pH: The pH of the digestion mixture is empirically determined to ensure the optimal performance of the given cleavage agent. For example, when using cyanogen bromide as a cleavage agent, a highly acidic environment (e.g., pH 2, formic acid) is necessary; however, when using trypsin as a cleavage agent, a slightly alkaline environment (pH 8) is optimal. As a general rule, the pH of the reaction milieu should not alter the chemical integrity of the protein during the digestion and should not change during the course of the fragmentation reaction.

(ii) Temperature: A temperature between 25°C and 37°C is adequate for most digestions. The temperature used is intended to minimize chemical side reactions. The type of protein under test will dictate the temperature of the reaction milieu, because some proteins are more susceptible to denaturation as the temperature of the reaction increases. For example, digestion of recombinant bovine somatropin is conducted at 4°C, because at higher temperatures it will precipitate during digestion.

(iii) Time: If sufficient sample is available, a time course study is considered in order to determine the optimum time to obtain a reproducible map and avoid incomplete digestion. Time of digestion varies from 2 to 30 hours. The reaction is stopped by the addition of an acid which does not interfere in the tryptic map or by freezing.

(iv) Amount of Cleavage Agent: Although excessive amounts of cleavage agent are used to accomplish a reasonably rapid digestion time (i.e., 6 to 20 hours), the amount of cleavage agent is minimized to avoid its contribution to the chromatographic map pattern. A protein to protease ratio between 20:1 and 200:1 is generally used. It is recommended that the cleavage agent can be added in two or more stages to optimize cleavage. Nonetheless, the final reaction volume remains small enough to facilitate the next step in peptide mapping —the separation step. To sort out digestion artifacts that might be interfering with the subsequent analysis, a blank determination is performed, using a digestion control with all the reagents, except the test protein.

4. Chromatographic Separation

Many techniques are used to separate peptides for mapping. The selection of a technique depends on the protein being mapped. Techniques that have been successfully used for separation of peptides are shown in Table 2. In this section, a most widely used reverse-phase high performance liquid chromatography (RP-HPLC) is described as one of the procedures of chromatographic separation.

The purity of solvents and mobile phases is a critical factor in HPLC separation. HPLC-grade solvents and water that are commercially available are recommended for RP-HPLC. Dissolved gases present a problem in gradient systems where the solubility of the gas in a solvent may be less in a mixture than in a single solvent. Vacuum degassing and agitation by sonication are often used as useful degassing procedures. When solid particles in the solvents are drawn into the HPLC system, they can damage the sealing of pump valves or clog the top of the chromatographic column. Both pre- and post-pump filtration is also recommended.

4.1. Chromatographic Column

The selection of a chromatographic column is empirically determined for each protein. Columns with 100 Å or 300 Å pore size with silica support can give optimal separation. For smaller peptides, octylsilane chemically bonded to totally

Table 2 Techniques used for the separation of peptides

Reverse-Phase High Performance Liquid Chromatography (RP-HPLC)
Ion-Exchange Chromatography (IEC)
Hydrophobic Interaction Chromatography (HIC)
Polyacrylamide Gel Electrophoresis (PAGE), nondenaturating
SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)
Capillary Electrophoresis (CE)
Paper Chromatography-High Voltage (PCHV)
High-Voltage Paper Electrophoresis (HVPE)

porous silica articles, 3 to 10 μm in diameter (L7) and octadecylsilane chemically bonded to porous silica or ceramic micro-particles, 3 to 10 μm in diameter (L1) column packings are more efficient than the butyl silane chemically bonded to totally porous silica particles, 5 to 10 μm in diameter (L26) packing.

4.2. Solvent

The most commonly used solvent is water with acetonitrile as the organic modifier to which less than 0.1% trifluoroacetic acid is added. If necessary, add 2-propanol or 1-propanol to solubilize the digest components, provided that the addition does not unduly increase the viscosity of the components.

4.3. Mobile Phase

Buffered mobile phases containing phosphate are used to provide some flexibility in the selection of pH conditions, since shifts of pH in the 3.0 to 5.0 range enhance the separation of peptides containing acidic residues (e.g., glutamic and aspartic acids). Sodium or potassium phosphates, ammonium acetate, phosphoric acid, and a pH between 2 and 7 (or higher for polymer-based supports) have also been used with acetonitrile gradients. Acetonitrile containing trifluoroacetic acid is used quite often.

4.4. Gradient Selection

Gradients can be linear, nonlinear, or include step functions. A shallow gradient is recommended in order to separate complex mixtures. Gradients are optimized to provide clear resolution of one or two peaks that will become ''marker'' peaks for the test.

4.5. Isocratic Selection

Isocratic HPLC systems using a single mobile phase are used on the basis of their convenience of use and improved detector responses. Optimal composition of a mobile phase to obtain clear resolution of each peak is sometimes difficult to establish. Mobile phases for which slight changes in component ratios or in pH significantly affect retention times of peaks in peptide maps should not be used in isocratic HPLC systems.

4.6. Other Parameters

Temperature control of the column is usually necessary to achieve good reproducibility. The flow rates for the mobile phases range from 0.1 to 2.0 mL per minute, and the detection of peptides is performed with a UV detector at 200 to 230 nm. Other methods of detection have been used (e.g., postcolumn derivatization), but they are not as robust or versatile as UV detection.

4.7 Validation

This section provides an experimental means for measuring the overall performance of the test method. The acceptance criteria for system suitability depend on the identification of critical test parameters that affect data interpretation and acceptance. These critical parameters are also criteria that monitor peptide digestion and peptide analysis. An indicator that the desired digestion endpoint was achieved is by the comparison with a reference standard or reference material, which is treated exactly as the article under test. The use of a reference standard or reference material in parallel with the protein under test is critical in the development and establishment of system suitability limits. In addition a specimen chromatogram should be included with the reference standard or reference material for additional comparison purposes. Other indicators may include visual inspection of protein or peptide solubility, the absence of intact protein, or measurement of responses of a digestion-dependent peptide. The critical system suitability parameters for peptide analysis will depend on the particular mode of peptide separation and detection and on the data analysis requirements.

When peptide mapping is used as an identification test, the system suitability requirements for the identified peptides covers selectivity and precision. In this case, as well as when identification of variant protein is done, the identification of the primary structure of the peptide fragments in the peptide map provides both a verification of the known primary structure and the identification of protein variants by comparison with the peptide map of the reference standard/reference material for the specified protein. The use of a digested reference standard or reference material for a given protein in the determination of peptide resolution is the method of choice. For an analysis of a variant protein, a characterized mixture of a variant and a reference standard or reference material can be used, especially if the variant peptide is located in a less-resolved region of the map. The index of pattern consistency can be simply the number of major peptides detected. Peptide pattern consistency can be best defined by the resolution of peptide peaks. Chromatographic parameters —such as peak-to-peak resolution, maximum peak width, peak area, peak tailing factors, and column efficiency— may be used to define peptide resolution. Depending on the protein under test and the method of separation used, single peptide or multiple peptide resolution requirements may be necessary.

The replicate analysis of the digest of the reference standard or reference material for the protein under test yields measures of precision and quantitative recovery. Recovery of the identified peptides is generally ascertained by the use of internal or external peptide standards. The precision is expressed as the relative standard deviation (RSD). Differences in the recovery and precision of the identified peptides are expected; therefore, the system suitability limits will have to be established for both the recovery and the precision of the identified peptides. These limits are unique for a given protein and will be specified in the individual monograph.

Visual comparison of the relative retention times, the peak responses (the peak area or the peak height), the number of peaks, and the overall elution pattern is completed initially. It is then complemented and supported by mathematical analysis of the peak response ratios and by the chromatographic profile of a 1:1 (v/v) mixture of sample and reference standard or reference material digest. If all peaks in the sample digest and in the reference standard or reference material digest have the same relative retention times and peaks response ratios, then the identity of the sample under test is confirmed.

If peaks that initially eluted with significantly different relative retention times are then observed as single peaks in the 1:1 mixture, the initial difference would be an indication of system variability. However, if separate peaks are observed in the 1:1 mixture, this would be evidence of the nonequivalence of the peptides in each peak. If a peak in the 1:1 mixture is significantly broader than the corresponding

peak in the sample and reference standard or reference material digest, it may indicate the presence of different peptides. The use of computer-aided pattern recognition software for the analysis of peptide mapping data has been proposed and applied, but issues related to the validation of the computer software preclude its use in a compendial test in the near future. Other automated approaches have been used that employ mathematical formulas, models, and pattern recognition. Such approaches are, for example, the automated identification of compounds by IR spectroscopy and the application of diode-array UV spectral analysis for identification of peptides. These methods have limitations due to inadequate resolutions, co-elution of fragments, or absolute peak response differences between reference standard or reference material and sample fragments.

The numerical comparison of the retention times and peak areas or peak heights can be done for a selected group of relevant peaks that have been correctly identified in the peptide maps. Peak areas can be calculated using one peak showing relatively small variation as an internal reference, keeping in mind that peak area integration is sensitive to baseline variation and likely to introduce error in the analysis. Alternatively, the percentage of each peptide peak height relative to the sum of all peak heights can be calculated for the sample under test. The percentage is then compared to that of the corresponding peak of the reference standard/reference material. The possibility of auto-hydrolysis of trypsin is monitored by producing a blank peptide map, that is, the peptide map obtained when a blank solution is treated with trypsin.

The minimum requirement for the qualification of peptide mapping is an approved test procedure that includes system suitability as a test control. In general, early in the regulatory process, qualification of peptide mapping for a protein is sufficient. As the regulatory approval process for the protein progresses, additional qualifications of the test can include a partial validation of the analytical procedure to provide assurance that the method will perform as intended in the development of a peptide map for the specified protein.

5. Analysis and Identification of Peptides

This section gives guidance on the use of peptide mapping during development in support of regulatory applications.

The use of a peptide map as a qualitative tool does not require the complete characterization of the individual peptide peaks. However, validation of peptide mapping in support of regulatory applications requires rigorous characterization of each of the individual peaks in the peptide map. Methods to characterize peaks range from N-terminal sequencing of each peak followed by amino acid analysis to the use of mass spectroscopy (MS).

For characterization purposes, when N-terminal sequencing and amino acids analysis are used, the analytical separation is scaled up. Since scale-up might affect the resolution of peptide peaks, it is necessary, using empirical data, to assure that there is no loss of resolution due to scale-up. Eluates corresponding to specific peptide peaks are collected, vacuum-concentrated, and chromatographed again, if necessary. Amino acid analysis of fragments may be limited by the peptide size. If the N-terminus is blocked, it may need to be cleared before sequencing. C-terminal sequencing of proteins in combination with carboxypeptidase digestion and MALDI-TOF MS can also be used for characterization purposes.

The use of MS for characterization of peptide fragments is by direct infusion of isolated peptides or by the use of on-line LC-MS for structure analysis. In general, it includes electrospray and MALDI-TOF MS analyzer as well as fast atom bombardment (FAB). Tandem MS has also been used to sequence a modified protein and to determine the type of amino acid modification that has occurred. The comparison of mass spectra of the digests before and after reduction provides a method to assign the disulfide bonds to the various sulfhydryl-containing peptides.

If regions of the primary structure are not clearly demonstrated by the peptide map, it might be necessary to develop a secondary peptide map. The goal of a validated method of characterization of a protein through peptide mapping is to reconcile and account for at least 95% of the theoretical composition of the protein structure.

Mass Spectrometry of Peptides and Proteins <G3-4-161>

Mass spectrometry (MS) is based on the ionization of molecules and separation of the electrically charged ions according to the dimensionless quantity, m/z value, which is obtained by dividing the relative mass (m) of the ion to unified atomic mass unit by the charge number (z) of the ion. The unified atomic mass unit is defined as one twelfth of the mass of ground state ^{12}C and used to express the mass of atom, molecule and ion. The results are expressed as a mass spectrum with m/z values of the ions on the x-axis and signal intensity of the ions on the y-axis. The mass of the molecule is calculated from the m/z values and z. Tandem mass spectrometry (MS/MS) is based on the fragmentation of the precursor ion selected in the first stage mass analysis and measurement of the product ions in the second stage mass analysis. This technique provides useful information for structural analysis of the molecule. Information obtained in MS is qualitative and is sometimes used for qualification. MS and MS/MS are useful for measuring masses of peptides and proteins and for confirming amino acid sequences and post-translational modifications. Both methods are therefore used for identification of pharmaceutical peptides and proteins.

1. Instrument

A mass spectrometer is composed of an ion source, an analyzer, an ion detector, and a data system (Fig. 1). A peptide and protein sample introduced into the ion source is ionized by soft-ionization methods, such as matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI). The charged and gas phased ions are sorted according to the m/z ratio under a vacuum in the analyzer, which may be a quadrupole, time-of-flight, ion trap or

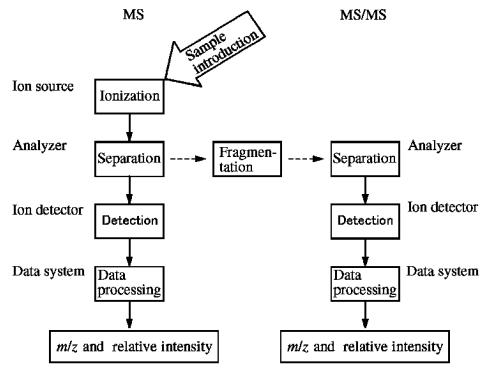


Fig. 1 Schematic diagram of mass spectrometry (MS) and tandem mass spectrometry (MS/MS)

Fourier transform ion cyclotron resonance analyzer. The ion flux collected in the detector is converted to an electric signal. Then the signal is recorded as a mass spectrum. MS/MS is carried out by using two mass spectrometers connected in series, an ion-trap mass spectrometer and Fourier transform ion cyclotron resonance mass spectrometer. The precursor ions are generally fragmented by collision-induced dissociation (CID), post-source decay (PSD), electron capture dissociation (ECO), etc.

2. Analytical mode

2.1. MS

There are two useful modes for MS:

(1) Total ion monitoring

The signals of the entire ion are acquired over the chosen range of *m/z* value. This mode provides information on the masses of the molecule of interest and different species.

(2) Selected ion monitoring

The signals of the ion at chosen *m/z* value are acquired. This mode is useful for the sensitive measurement of the chosen molecule.

2.2. MS/MS

There are four essential modes for MS/MS:

(1) Product ion analysis

The signals of all the product ions produced from the precursor ion at chosen *m/z* value are acquired. This mode provides structural information on the substrates and various co-existing species.

(2) Precursor ion scan mode

The signals of the precursor ion that yields the product ion at chosen *m/z* value are monitored. This mode is used for sorting the molecules containing a component of interest.

(3) Constant neutral loss scan mode

The signals of the precursor ion that loses the fragment at chosen *m/z* value are monitored. This mode is useful to sort the molecules containing a component of interest.

(4) Selected reaction monitoring

The signals of product ions at chosen *m/z* value that are produced from the precursor ion at chosen *m/z* value are monitored. This mode allows for sensitive and selective measurement and is used for quantification of a molecule in a complex mixture.

3. Analytical procedure

3.1. MS

In advance, it should be confirmed if the detectability and the difference between the calculated mass and observed mass meet the criteria stated in the monograph by mass measuring using a test solution specified in the system suitability in the monograph. If they do not meet the criteria, the system should be optimized by adjustment of the voltage of the ion source, analyzer and detector, as well as by calibration using appropriate mass calibrator. After confirming that the criteria are met, MS is performed according to the sample preparation and operating conditions indicated in the monograph. The general procedure is described as follows.

(1) Matrix-assisted laser desorption/ionization (MALDI)

A desalted peptide and protein sample is dissolved in an appropriate solvent, e.g., an aqueous solution of trifluoroacetic acid. A suitable matrix, such as α-ciano-4-hydroxycinnamic acid, 2,5-dihydroxybenzoic acid, or sinapic acid, is dissolved in an aqueous solution containing acetonitrile and trifluoroacetic acid. A mixture of sample solution and matrix solution is deposited on a sample plate and dried. The sample on the plate is set in the ion source, and ionized by a laser beam at suitable intensity.

(2) Electrospray ionization (ESI)

A desalted peptide and protein sample is dissolved in a suitable solvent, such as an aqueous solution containing acetic acid and methanol or acetonitrile. The sample solution is introduced by using a syringe or HPLC. The sample is ionized by applying voltage to obtain the spectrum.

3.2. MS/MS

System suitability is tested by MS/MS of the test sample specified in the monograph. The detectability and system performance should be confirmed based on the detection of the product ions specified in the monograph. The sample is ionized in the same way as for MS, and the chosen precursor is fragmented by the suitable conditions specified in the monograph. The signals are recorded as a mass spectrum. A peptide containing disulfide bonds is generally reduced by dithiothreitol, 2-mercaptethanol and tris(2-carboxyethyl) phosphine. The reduced peptides are alkylated with monoiodoacetic acid, iodoacetamide, and 4-vinylpyridine.

4. Identification test

4.1. Mass of the molecule

The monoisotopic mass of the peptide and protein molecules is measured by MS. If the monoisotopic peak is detectable, the monoisotopic mass is determined from the peak. If the monoisotopic peak is not detectable, the average mass is calculated from the top of the ion peak. Deconvolution is effective for calculating the average mass of multiply-charged ions from proteins. The mass should meet the criteria specified in the monograph.

4.2. Amino acid sequence

After measuring the mass of the sample peptide, the presence of the specified product ions that arise from the selected precursor ion is confirmed according to the conditions indicated in the monograph. Digestion of sample proteins with a suitable enzyme followed by MS/MS is sometimes effective for sequencing of the high-molecular weight proteins which provide insufficient product ions. Details of the digestion procedure are provided in ''Peptide Mapping *<G3-3-142>*'' in General Information.

5. Glossary

Ion-trap (IT)

Ion-trap refers to the quadrupole ion trap mass analyzer in a restricted sense. Ions stored in the analyzer by applying radio frequency voltage to ring electrodes are separated by subsequent ejection of the ions from the analyzer by varying the voltage on the ring electrodes. This allows multiple stage MS (MS^n) in which a selected ion is repeatedly trapped, fragmented and ejected.

Electrospray ionization (ESI)

The sample in solution is sprayed through a needle tip and held at high-voltage at atmospheric pressure. The sample is ionized by a formation of charged liquid droplets. High-molecular mass proteins are detected as multiply-charged ions. The analyzer can be connected with HPLC.

Quadrupole (Q)

The analyzer is composed of four parallel electrodes which have a hyperboloidal or corresponding cross-section. The ions transmitted to the analyzer are separated by varying the potential of direct and radio frequency components applied to the rods so that the filter for sorting the *m/z* values of ions is changed.

Collision-induced dissociation (CID)

When an ion collides with a neutral collision gas (He, Ar, N_2 and so on), some of the translational energy of the collision is converted into internal energy, thereby causing ion

excitation and dissociation. The terms low-energy CID and high-energy CID refer to those CIDs for which the translational energy of the precursor ions is lower than 1000 eV and higher than 1000 eV, respectively.

Electron capture dissociation (ECD)

Multiply-charged positive ions interact with low energy electrons producing charge-reduced radical ions, which readily dissociate. This method is primarily used for MS/MS in FT-ICR MS or IT MS.

Time-of-flight (TOF)

The ionized sample is accelerated at high-voltage and separated based on the time required for an ion to travel to the detector. There are two types of analyzer, a linear type in which ions travel linearly from the ion source to the detector, and a reflectron type where ions are inverted by a reflectron. The latter type allows high-resolution measurement by correction of the variation in the initial energy of ions.

Fourier transform ion cyclotron resonance (FT-ICR)

The analyzer is based on the principle that the cyclotron frequency of the rotating (cyclotron motion) ions in a magnetic field is inversely proportional to its m/z value. Ions are excited using radio frequency energy and their image current is detected on a detection electrode. The resulting data are devolved by applying a Fourier transform to give a mass spectrum.

Post-source decay (PSD)

Metastable ion decay occurs by excess internal energy and collision with residual gas during ion acceleration out of the MALDI ion source and prior to reaching the detector. This method is used for MS/MS by using MALDI-TOF MS with a reflectron mode.

Matrix-assisted laser desorption/ionization (MALDI)

The sample, which is mixed with a suitable matrix, is ionized by irradiation with nanosecond laser pulses. Proteins, carbohydrates, oligonucleotides, and lipids can be ionized without any dissociation. Singly-charged ions are mainly detected.

Monosaccharide Analysis and Oligosaccharide Analysis/ Oligosaccharide Profiling *〈G3-5-170〉*

Glycosylation analysis is a method to confirm the consistency of the oligosaccharides attached to glycoprotein drug substance, product or material. When oligosaccharides attached to the glycoprotein affect efficacy and safety or the possibility cannot be denied, oligosaccharides are considered as critical quality attribute, and strategy should be designed in order to ensure the consistency of glycosylation. One of the strategies is glycosylation analysis, which involves 1) analysis of released monosaccharides (monosaccharide analysis), 2) analysis of released oligosaccharides (oligosaccharide analysis/oligosaccharide profiling), 3) analysis of glycopeptides (glycopeptide analysis), and 4) analysis of intact glycoprotein (glycoform analysis). These methods provide monosaccharide compositions, oligosaccharide identities and distribution in whole glycoprotein, site-specific glycosylation identities and distribution, and overall glycosylation characteristics and distribution of glycoprotein, respectively. In the setting specification of glycosylation analysis, proper methods should be selected and used alone or in combination, in consideration of the relationship between the oligosaccharide structures and functions, such as biological activity, pharmacodynamics, pharmacokinetics, immunogenicity, stability, and solubility. Glycosylation consistency may be ensured not only by oligosaccharide analysis but also at manufacturing process. Glycosylation analysis can be also used as in-process test, and as method to confirm glycosylation consistency during process development. Methods and general consideration of monosaccharide analysis and oligosaccharide analysis/oligosaccharide profiling are described below. For glycopeptide analysis, General Test *〈2.62〉* Mass Spectrometry, and General Information Peptide Mapping and Mass Spectrometry of Peptides and Proteins would be helpful, and for glycoform analysis, General Information Isoelectric Focusing and Capillary Electrophoresis, and General Test *〈2.62〉* Mass Spectrometry would be helpful.

1. Monosaccharide analysis

Monosaccharides are released by cleavage of glycosidic bond using acid hydrolysis, exoglycosidase or methanolysis. Released monosaccharides are dried and purified if needed, and then analyzed using liquid chromatography, gas chromatography, or capillary electrophoresis. Internal standard method or absolute calibration method are used for quantitative measurement. The analytical results are typically expressed as molar ratio of individual monosaccharides to glycoprotein.

1.1. Isolation and purification of glycoprotein

Monosaccharide analysis is generally performed after glycoprotein is isolated and purified in an appropriate manner, because excipients and salts may affect hydrolysis, derivatization of monosaccharides, and chromatographic separation. When purification of the glycoprotein is required, the procedure is specified in the specific monograph.

1.2. Release of monosaccharide

1.2.1. Acid hydrolysis

Acid hydrolysis is the most common procedure to release neutral and amino sugars. In general, monosaccharides may be released by acid hydrolysis of glycosidic bond under conditions such as 2 to 7 mol/L trifluoroacetic acid at about 100°C. Since amino sugar residue directly attached to protein is difficult to release, for accurate quantification of amino sugars, acid hydrolysis should be performed separately under conditions such as 2 to 6 mol/L hydrochloride at 100°C. Because hydrolysis rate is dependent on the identity of the monosaccharide, the anomeric configuration, and position of the glycosidic linkage, it is recommended that release and degradation of individual monosaccharides are confirmed by time-course study. Because N-acetyl groups of amino sugars are removed under acid hydrolysis conditions, re-N-acetylation may be performed if needed. Since sialic acid is labile, sialic acids are released separately under conditions such as 0.1 mol/L hydrochloride, 0.1 mol/L sulfuric acid, or 2 mol/L acetic acid at 80°C.

1.2.2. Enzymatic treatment

Exoglycosidase digestion is also used for release of sialic acids from glycoprotein. Typically, sialidases with broad specificity, such as those derived from *Arthrobacter ureafaciens* or *Clostridium perfringens* are used. Digestion conditions should be optimized in consideration of the identity of sialic acids, linkage, O-acetylation and others. Other enzymes with high specificity may be used to distinguish sialic acid having different types of linkage.

1.2.3. Methanolysis

Dried sample is heated under methanolic hydrogen chlo-

Table 1 Examples of enzymatic cleavage agents

Enzyme	Specificity
N-linked oligosaccharide release	
Peptide-N^4-(*N*-acetyl-β-glucosaminyl) asparagine amidase (EC 3.5.1.52)	Hydrolysis of peptide-N^4-(*N*-acetyl-β-D-glucosaminyl) asparagine residue in which the glucosamine residue may be further glycosylated, to yield a (substituted) *N*-acetyl-β-D-glucosaminylamine and a peptide containing an aspartate residue
—Peptide N-glycosidase F (PNGase F)	Release of N-linked oligosaccharides but no release of N-linked oligosaccharides containing (α1,3)-linked core fucose
—Peptide N-glycosidase A	Release of N-linked oligosaccharides including those containing (α1,3)-linked core fucose
Mannosyl-glycoprotein endo-β-*N*-acetyl-glucosaminidase (EC 3.2.1.96)	Endohydrolysis of the *N*,*N*′-diacetylchitobiosyl unit in high-mannose glycopeptides/glycoproteins containing the—[Man(GlcNAc)$_2$]Asn structure [Man(GlcNAc)$_2$]Asn
—Endo-β-*N*-acetylglucosaminidase F (endo F)	Release of high-mannose, hybrid, and complex oligosaccharides
—Endo-β-*N*-acetylglucosaminidase H (endo H)	Release of high-mannose and hybrid oligosaccharides
O-linked oligosaccharide release	
Glycopeptide α-*N*-acetylgalactosaminidase (EC 3.2.1.97)*	Release of D-galactose-(α1,3)-*N*-acetylgalactosamine α-linked to serine/threonine residue

* This enzyme has limited usage because of its high substrate specificity.

ride. Monosaccharides are released as methyl glycosides. Degradation of released monosaccharides is low compared to acid hydrolysis.

1.3. Quantification of the released monosaccharides

1.3.1. High-pH anion-exchange chromatography with pulsed amperometric detection

Acid is removed from the hydrolysate if needed. Monosaccharides can be separated and quantified without derivatization using high-pH anion-exchange chromatography with pulsed amperometric detection. Monosaccharides have about 12 to 14 of acid dissociation constant (pKa). They are ionized under high pH conditions (pH 12 to 13), and can be separated by strong anion-exchange chromatography using polymer-based stationary phase containing quaternary ammonium groups. Amperometric detection is a method to detect electrochemically active ions by measuring the current when analyte is oxidized or reduced at electrodes. Sugar is ionized at high pH and can be selectively detected by amperometry. Because oxidized products of sugars foul the electrodes and reduce the signals, pulsed amperometry, where electrode surface is cleaned by changing positive and negative potentials after data acquisition, is used. Since amino acids are also detectable by amperometry, it is noted that analysis may be interfered in the case of the glycoprotein with low oligosaccharide contents. This analytical method can be used for oligosaccharide analysis as well as for analysis of neutral and amino sugars, and sialic acids.

1.3.2. Derivatization and liquid chromatography

(1) Neutral and amino sugars

Monosaccharides obtained by acid hydrolysis are treated to remove the acid, re-N-acetylated if needed, then reductively aminated with 2-aminobenzoic acid, 2-aminopyridine, or ethyl-4-aminobenzoate, or derivatized with 3-methyl-1-phenyl-5-pyrazolone. Impurities derived from reagents may interfere the analysis, attention should be paid to the purity of the reagents used. To prevent excessive reagents to affect test results, derivatized monosaccharides are purified if needed. The derivatized monosaccharides may be separated using reversed-phase chromatography, or anion-exchange chromatography with formation of borate complex. Separated monosaccharides are detected by fluorometric or ultraviolet detector. Underivatized monosaccharides may be separated by ion-exchange chromatography, post-column derivatization using such as arginine, and then detected.

(2) Sialic acid

Released sialic acids by mild acid hydrolysis or sialidase digestion are derivatized with 1,2-diamino-4.5-methylenedioxybenzen or 1,2-phenylenediamine, which react with α-keto acid specifically. This reaction proceeds in acidic conditions, thus acid hydrolysate can be used for derivatization without removal of the acid. Derivatized sialic acids are separated by reversed-phase chromatography and detected by fluorometric detector.

1.3.3. Gas chromatography

There are several methods for gas chromatography; monosaccharides obtained by methanolysis are re-N-acetylated and trimethylsilylated (trimethylsilyl derivatives), and the monosaccharides obtained by acid hydrolysis are reduced and then acetylated (alditol acetate derivatives). The former can quantitate sialic acids simultaneously without degradation, but each sugar gives several peaks due to α- and β-anomers and isomers, and chromatogram becomes complex.

Methylation analysis provides the structural information or the glycosidic linkage of individual monosaccharides. After all hydroxy groups in the oligosaccharide are methylated, permethylated oligosaccharide is subject to acid hydrolysis and resultant partially O-methylated monosaccharides are reduced and acetylated. Partially O-methylated alditol acetates are separated and quantified using gas chromatography.

1.4. Acceptance criteria

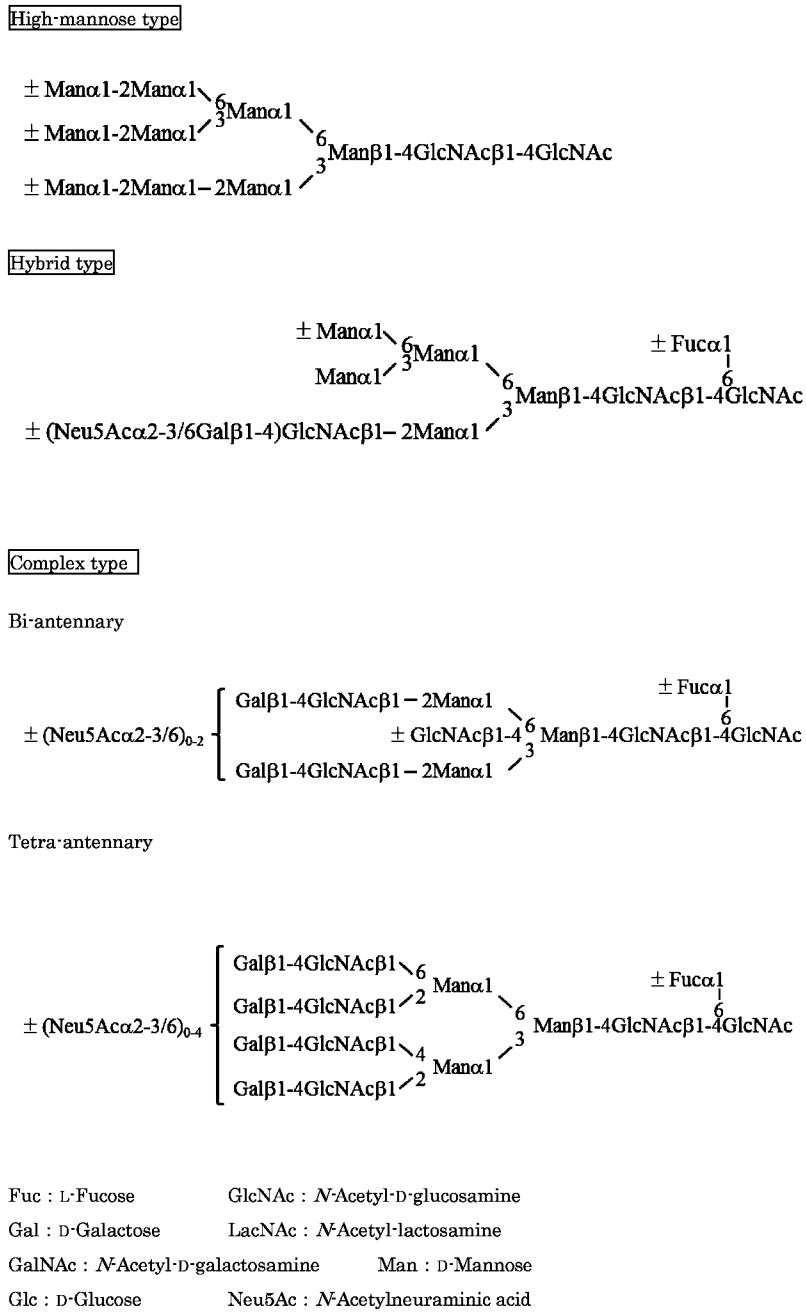
Confirmation of compliance of the test material is typically achieved by demonstrating that contents of individual monosaccharides per protein are within a specified range. In order to set acceptance criteria properly, it is needed to consider the relationship between characteristics of glycosylation, and efficacy and safety.

1.5. Monosaccharide reference materials

Monosaccharides to be analyzed are often used as reference materials for monosaccharide analysis. In this case, monosaccharide reference material mixtrure is prepared as mixing each monosaccharide equally, or at similar ratio expected in test substance.

1.6. System suitability

The solution for system suitability test should be prepared



Fuc : L-Fucose GlcNAc : *N*-Acetyl-D-glucosamine
Gal : D-Galactose LacNAc : *N*-Acetyl-lactosamine
GalNAc : *N*-Acetyl-D-galactosamine Man : D-Mannose
Glc : D-Glucose Neu5Ac : *N*-Acetylneuraminic acid

Fig. Common types of N-linked oligosaccharides

properly using monosaccharide reference materials. It may be difficult to separate each monosaccharide completely due to its similar physical property. Acceptance criteria should be set properly.

2. Oligosaccharide analysis/oligosaccharide profiling

Oligosaccharides are released from glycoprotein by enzymatic or chemical treatment, and then released oligosaccharides are analyzed or profiled by liquid chromatography, capillary electrophoresis, mass spectrometry, or combination of them. Analysis result is obtained as oligosaccharide profile, which provides the information on the identity and the distribution of oligosaccharide.

2.1. Separation and purification of glycoprotein

Interfering substance, such as excipients, salts and surfactant, are removed if needed. When purification of the glycoprotein is required, the procedure is specified in the specific monograph.

2.2. Release and isolation of oligosaccharides

Release of N-linked oligosaccharides from glycoprotein is performed by enzymatic treatment or hydrazinolysis. Release of O-linked oligosaccharides is performed by alkali β-elimination, hydrazinolysis, and O-glycanase digestion. The releasing conditions must be optimized in order to release and recover all oligosaccharides attached to the glycoprotein reproducibly, independent of their structure and their individual position in the protein. Table 1 give a non-exhaustive list of enzymatic cleavage agents and their specificity. Released oligosaccharides may be purified properly if needed.

2.2.1. Enzymatic release

For the release of N-linked oligosaccharides, peptide N-glycosidase F (PNGase F) derived from *Flavobacterium meningosepticum* or peptide N-glycosidase A (PNGase A) derived from almonds are available. These enzymes hydrolyze the amide bond between asparagine residue and *N*-

Table 2 Examples of derivatizing agents and suitable analytical techniques.

Agent	Structure	Acronym	Analytical techniques	Fluorescent or UV detection
2-Aminobenzoic acid		2-AA	LC, CE, MS	Ex: 360 nm, Em: 425 nm Ex: 325 nm, Em: 405 nm
2-Aminobenzamide		2-AB	LC, MS	Ex: 330 nm, Em: 420 nm
2-Aminopyridine		2-AP	LC, MS	Ex: 310 nm, Em: 380 nm Ex: 320 nm, Em: 400 nm
Trisodium 8-aminopyrene-1,3,6-trisulfonic salt		APTS	CE	Ex: 488 nm, Em: 520 nm
3-methyl-1-phenyl-5-pyrazolone		PMP	LC, MS	UV 245 nm

acetylglucosamine residue at reducing end of oligosaccharides to produce glycosylamine derivative and aspartic acid residue. Glycosylamine derivative is subsequently hydrolyzed non-enzymatically under weak acidic conditions to ammonia and free oligosaccharide. O-glycanase from *Diplococcus pneumoniae* is available to release O-linked oligosaccharides, but specificity of this enzyme is too narrow.

2.2.1.1. PNGase F digestion

PNGase F have an optimum pH 7 to 9. Glycoprotein is treated directly or under presence of a reducing agent, surfactant, and/or denaturing agent. Glycoprotein may be treated with PNGase F after reduced and alkylated, or after digested into glycopeptides. Glycoproteins from some insect cells and plants may have a (α1,3)-linked fucose attached to the proximal GlcNAc of the core chitobiose, and N-linked oligosaccharides containing this structure are not released by this enzyme.

2.2.1.2. PNGase A digestion

PNGase A have an optimum pH 4 to 6. Since this enzyme is difficult to release oligosaccharides directly from whole glycoprotein, a glycoprotein sample is digested with a proteolytic agent, such as endoprotease, and then glycopeptides are treated with this enzyme to release oligosaccharides.

2.2.2. Chemical cleavage

2.2.2.1. Hydrazinolysis

To well-dried glycoprotein anhydrous hydrazine is added and heated. Hydrazine cleaves glycosylamine linkage between oligosaccharide and peptide as well as peptide bond. With careful control of reaction conditions, selective release of N-linked oligosaccharide and/or O-linked oligosaccharides can be achieved. Because de-N-acetylation of amino sugar and sialic acid in oligosaccharides also occurs, amino groups are re-N-acetylated after removing hydrazine. Attention should be paid to the possibility of loss of sialic acid, and successive degradation from reducing end of released O-linked oligosaccharides (peeling reaction).

2.2.2.2. Alkali β-elimination

Heating glycoprotein under alkaline conditions results in release of O-linked oligosaccharides by β-elimination. To prevent peeling reactions, reaction is conducted in the presence of a reducing agent, such as sodium tetrahydroborate. It is noted that reducing end of obtained O-linked oligosaccharides is already reduced, thus, cannot be derivatized. There is a method to release the oligosaccharides and simultaneously to derivatize with 3-methyl-1-phenyl-5-pyrazolone.

2.3. Analysis of released oligosaccharides

Oligosaccharides are analyzed directly or after derivatized. Table 2 gives a non-exhaustive list of commonly used fluorescent labels and suitable analytical techniques. The analysis method is needed to separate and detect individual oligosaccharides or family of oligosaccharides with the structure which affects efficacy and safety.

2.3.1. Liquid chromatography *<2.01>*

2.3.1.1. Derivatization and liquid chromatography/fluorometric or UV detection

Profiling of derivatized oligosaccharides by liquid chromatography is the most common methods. Oligosaccharides derivatized with 2-aminobenzamide, 2-aminobenzoic acid, 2-aminopyridine or others are separated by hydrophilic interaction, reversed-phase, ion-exchange, or mixed-mode chromatography, and then detected by fluorometry. Oligosaccharides derivatized with 3-methyl-1-phenyl-5-pyrazolone are separated by reversed-phase chromatography, and then detected by UV spectrometry. Hydrophilic interaction liquid chromatography separates oligosaccharides on the basis of hydrophilicity (i.e. size, the number of sialic acid, ...). Reversed-phase chromatography separates oligosaccharides on the basis of hydrophobicity (i.e. type of oligosaccharide, branching, number of sialic acid, ...). Ion-exchange chromatography separates oligosaccharides on the basis of number of charges. A mix mode of ion-exchange and hydrophilic interaction separates oligosaccharides on the basis of structure as well as number of charges.

2.3.1.2. High-pH anion-exchange chromatography/pulsed amperometric detection

Released oligosaccharides are separated by strong anion-exchange chromatography using polymer-based stationary phase containing quaternary ammonium groups, and detected by pulsed amperometry. This method can separate and detect sialo-oligosaccharides according to the number of sialic acids and linkage differences. This method has often been used for profiling of sialo-oligosaccharides because of no need of derivatization (no loss of sialic acid and no loss of

oligosaccharides during procedure) and high resolution. Because response factors of individual oligosaccharides are not equal, relative peak response does not directly reflect the molar ratio of individual oligosaccharides.

2.3.2. Capillary electrophoresis

Derivatized oligosaccharides are separated by capillary zone electrophoresis using an appropriate electrolyte buffer, and then detected by a laser-induced fluorometric detector. Oligosaccharide is separated based on the sample properties such as charge, size, or shape. In general, capillary is used with the inner wall surface modified using neutral polymers covalently or dynamically in order to prevent electroosmotic flow. Derivatizing agent, and pH and additives of the electrolyte buffer are selected to achieve good separation. Capillary electrophoresis has high resolution separations and requires small amounts of sample.

2.3.3. Mass spectrometry *〈2.62〉*

Mass spectrometry is used for underivatized oligosaccharides as well as derivatized oligosaccharides. Monosaccharide compositions of oligosaccharides can be deduced from observed molecular mass of oligosaccharides. For ionization methods for oligosaccharides, soft ionization techniques, such as electrospray ionization and matrix-assisted laser desorption/ionization are used. It is noted that oligosaccharides containing sialic acid are susceptible to loss of sialic acid during mass spectrometry.

2.4. Assignment or identification of the peak

Identification of the oligosaccharides attached to the glycoprotein is important for test method development and evaluation of oligosaccharide profile. In general, structure of oligosaccharides may be deduced based on the molecular mass determined by mass spectrometry, the pattern of fragment ions obtained by tandem mass spectrometry, sensitivity to exoglycosidases or endoglycosidases with high specificity, comparison of chromatogram or electropherogram with well-characterized oligosaccharide standards, methylation analysis, and knowledge on the oligosaccharide patterns biosynthesized in the used cell line. Table 3 give a non-exhaustive list of exoglycosidases and endoglycosidases for structural assignment. During routine application, the identity of oligosaccharide peaks may be confirmed by comparison with the oligosaccharide profile obtained from the reference material.

Table 3 Examples of exoglycosidases and endoglycosidase useful for structure assignment.

Enzyme	Origin	Specificity
Exo-α-sialidase (EC 3.2.1.18)	*Arthrobactor ureafaciens*	α2-3,6,8,9
	Vibrio cholerae	α2-3,6,8
	Clostridium perfringens	α2-3,6,8
	Newcastle disease virus	α2-3
	Streptococcus pneumoniae	α2-3
β-Galactosidase (EC 3.2.1.23)	Bovine testes	β1-3,4
	Streptomyces pneumoniae	β1-4
α-L-Fucosidase (EC 3.2.1.51)	Almond meal	α1-3
	Xanthomonas sp.	α1-3,4
	Bovine kidney	α1-2,3,4,6
α-Mannosidase (EC 3.5.1.24)	Jack Bean	α1-2,3,6
α-Galactosidase (EC 3.2.1.22)	Green coffee beans	α1-3,4,6
Keratan-sulfate endo-1,4-β-galactosidase (EC 3.2.1.103)	*Bacteroides fragilis*	β1-3,4/poly LacNAc

2.5. Acceptance criteria

The oligosaccharide profile obtained from the test material is compared with that obtained in parallel using reference material, and then peak position and response ratio of individual oligosaccharides are comparable. Or peak ratio of individual oligosaccharide to total response (peak area percentage method) or relative peak response meets the acceptance criteria. In order to set specification properly, it is important to identify the oligosaccharide structure to be controlled in consideration of the relationship between oligosaccharide structure, and efficacy and safety.

2.6. Reference materials

It is important that reference material has been validated for glycosylation analysis.

2.7. System suitability

System suitability is developed depending on the purpose of oligosaccharide test. Acceptance criteria, e.g. presence of specific peaks, resolution between two adjacent peaks, the number of detectable peaks, and/or conformance to the reference oligosaccharide profile may be set for an oligosaccharide profile, obtained from the standard material, or well-characterized glycoprotein with similar properties by treating similarly. Otherwise, oligosaccharide reference material, e.g. an oligosaccharide standard prepared from the substance being tested and demonstrated to be suitable, or a system suitability oligosaccharide marker, is similarly treated. Similar acceptance criteria described above may be set for the obtained oligosaccharide profile.

Isoelectric Focusing *〈G3-6-142〉*

This test is harmonized with the European Pharmacopoeia and the U.S. Pharmacopeia.

The parts of the text that are not harmonized are marked with symbols (◆ ◆).

Information on the harmonization with the European Pharmacopoeia and the U.S. Pharmacopeia is available on the website of the Pharmaceuticals and Medical Devices Agency.

General Principles

Isoelectric focusing (IEF) is a method of electrophoresis that separates proteins according to their isoelectric point. Separation is carried out in a slab of polyacrylamide or agarose gel that contains a mixture of amphoteric electrolytes (ampholytes). When subjected to an electric field, the ampholytes migrate in the gel to create a pH gradient. In some cases gels containing an immobilized pH gradient, prepared by incorporating weak acids and bases to specific regions of the gel network during the preparation of the gel, are used. When the applied proteins reach the gel fraction that has a pH that is the same as their isoelectric point (pI), their charge is neutralized and migration ceases. Gradients can be made over various ranges of pH, according to the mixture of ampholytes chosen.

Theoretical Aspects

When a protein is at the position of its isoelectric point, it has no net charge and cannot be moved in a gel matrix by the electric field. It may, however, move from that position by diffusion. The pH gradient forces a protein to remain in its isoelectric point position, thus concentrating it; this concentrating effect is called ''focusing''. Increasing the applied voltage or reducing the sample load result in improved separation of bands. The applied voltage is limited by the heat generated, which must be dissipated. The use of thin gels and

an efficient cooling plate controlled by a thermostatic circulator prevents the burning of the gel whilst allowing sharp focusing. The separation R is estimated by determining the minimum pI difference (ΔpI), which is necessary to separate 2 neighboring bands:

$$R: \Delta pI = 3\sqrt{\frac{D(\mathrm{d}pH/\mathrm{d}x)}{E(-\mathrm{d}\mu/\mathrm{d}pH)}}$$

D: Diffusion coefficient of the protein
$\mathrm{d}pH/\mathrm{d}x$: pH gradient
E: Intensity of the electric field, in volts per centimeter
$-\mathrm{d}\mu/\mathrm{d}pH$: Variation of the solute mobility with the pH in the region close to the pI

Since D and $-\mathrm{d}\mu/\mathrm{d}pH$ for a given protein cannot be altered, the separation can be improved by using a narrower pH range and by increasing the intensity of the electric field. Resolution between protein bands on an IEF gel prepared with carrier ampholytes can be quite good. Improvements in resolution may be achieved by using immobilized pH gradients where the buffering species, which are analogous to carrier ampholytes, are copolymerized within the gel matrix. Proteins exhibiting pIs differing by as little as 0.02 pH units may be resolved using a gel prepared with carrier ampholytes while immobilized pH gradients can resolve proteins differing by approximately 0.001 pH units.

Practical Aspects

Special attention must be paid to sample characteristics and/or preparation. Having salt in the sample can be problematic and it is best to prepare the sample, if possible, in deionized water or 2 per cent ampholytes, using dialysis or gel filtration if necessary.

The time required for completion of focusing in thin-layer polyacrylamide gels is determined by placing a colored protein (e.g. hemoglobin) at different positions on the gel surface and by applying the electric field: the steady state is reached when all applications give an identical band pattern. In some protocols the completion of the focusing is indicated by the time elapsed after the sample application.

The IEF gel can be used as an identity test when the migration pattern on the gel is compared to a suitable standard preparation and IEF calibration proteins, the IEF gel can be used as a limit test when the density of a band on IEF is compared subjectively with the density of bands appearing in a standard preparation, or it can be used as a quantitative test when the density is measured using a densitometer or similar instrumentation to determine the relative concentration of protein in the bands subject to validation.

Apparatus

An apparatus for IEF consists of:

—a controllable generator for constant potential, current and power. Potentials of 2500 V have been used and are considered optimal under a given set of operating conditions. Supply of up to 30 W of constant power is recommended,

—a rigid plastic IEF chamber that contains a cooled plate, of suitable material, to support the gel,

—a plastic cover with platinum electrodes that are connected to the gel by means of paper wicks of suitable width, length and thickness, impregnated with solutions of anodic and cathodic electrolytes.

Isoelectric Focusing in Polyacrylamide Gels: Detailed Procedure

The following method is a detailed description of an IEF procedure in thick polyacrylamide slab gels, which is used unless otherwise stated in the monograph.

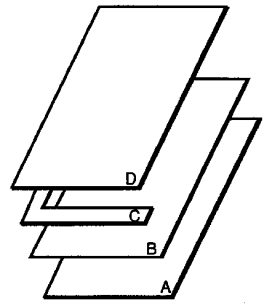


Figure. Mould

Preparation of the Gels

Mould The mould (see Figure) is composed of a glass plate (A) on which a polyester film (B) is placed to facilitate handling of the gel, one or more spacers (C), a second glass plate (D) and clamps to hold the structure together.

7.5% Polyacrylamide gel Dissolve 29.1 g of acrylamide and 0.9 g of *N,N′*-methylenebisacrylamide in 100 mL of water. To 2.5 volumes of this solution, add the mixture of ampholytes specified in the monograph and dilute to 10 volumes with water. Mix carefully and degas the solution.

Preparation of the mould Place the polyester film on the lower glass plate, apply the spacer, place the second glass plate and fit the clamps. Place 7.5% polyacrylamide gel prepared before use on a magnetic stirrer, and add 0.25 volumes of a solution of ammonium persulfate (1 in 10) and 0.25 volumes of *N,N,N′,N′*-tetramethylethylenediamine. Immediately fill the space between the glass plates of the mould with the solution.

Method

Dismantle the mould and, making use of the polyester film, transfer the gel onto the cooled support, wetted with a few millilitres of a suitable liquid, taking care to avoid forming air bubbles. Prepare the test solutions and reference solutions as specified in the monograph. Place strips of paper for sample application, about 10 mm × 5 mm in size, on the gel and impregnate each with the prescribed amount of the test and reference solutions. Also apply the prescribed quantity of a solution of proteins with known isoelectric points as pH markers to calibrate the gel. In some protocols the gel has pre-cast slots where a solution of the sample is applied instead of using impregnated paper strips. Cut 2 strips of paper to the length of the gel and impregnate them with the electrolyte solutions: acid for the anode and alkaline for the cathode. The compositions of the anode and cathode solutions are given in the monograph. Apply these paper wicks to each side of the gel several millimetres from the edge. Fit the cover so that the electrodes are in contact with the wicks (respecting the anodic and cathodic poles). Proceed with the isoelectric focusing by applying the electrical parameters described in the monograph. Switch off the current when the migration of the mixture of standard proteins has stabilized. Using forceps, remove the sample application strips and the 2 electrode wicks. Immerse the gel in ''fixing solution for isoelectric focusing in polyacrylamide gel''. Incubate with gentle shaking at room temperature for 30 minutes. Drain off the solution and add 200 mL of ''destaining solution''. Incubate with shaking for 1 hour. Drain the gel, add ''coomassie staining TS''. Incubate for 30 minutes. Destain the gel by passive diffusion with ''destaining solution'' until the bands are well visualized against a clear background. Locate the position and intensity of the bands in the electropherogram as prescribed in the monograph.

Variations to the Detailed Procedure (Subject to Validation)

Where reference to the general method on isoelectric focusing is made, variations in methodology or procedure may be made subject to validation. These include:

(1) the use of commercially available pre-cast gels and of commercial staining and destaining kits,
(2) the use of immobilized pH gradients,
(3) the use of rod gels,
(4) the use of gel cassettes of different dimensions, including ultra-thin (0.2 mm) gels,
(5) variations in the sample application procedure, including different sample volumes or the use of sample application masks or wicks other than paper,
(6) the use of alternate running conditions, including variations in the electric field depending on gel dimensions and equipment, and the use of fixed migration times rather than subjective interpretation of band stability,
(7) the inclusion of a pre-focusing step,
(8) the use of automated instrumentation,
(9) the use of agarose gels.

Validation of Iso-Electric Focusing Procedures

Where alternative methods to the detailed procedure are employed they must be validated. The following criteria may be used to validate the separation:

(1) formation of a stable pH gradient of desired characteristics, assessed for example using colored pH markers of known isoelectric points,
(2) comparison with the electropherogram provided with the chemical reference substance for the preparation to be examined,
(3) any other validation criteria as prescribed in the monograph.

Specified Variations to the General Method

Variations to the general method required for the analysis of specific substances may be specified in detail in monographs. These include:

(1) the addition of urea in the gel (3 mol/L concentration is often satisfactory to keep protein in solution but up to 8 mol/L can be used): some proteins precipitate at their isoelectric point. In this case, urea is included in the gel formulation to keep the protein in solution. If urea is used, only fresh solutions should be used to prevent carbamylation of the protein,
(2) the use of alternative staining methods,
(3) the use of gel additives such as non-ionic detergents (e.g. octylglucoside) or zwitterionic detergents (e.g., 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) or 3-[(3-Cholamidopropyl)dimethy lammonio]-2-hydroxy-1-propanesulfonate (CHAPSO)), and the addition of ampholyte to the sample, to prevent proteins from aggregating or precipitating.

Points to Consider

Samples can be applied to any area on the gel, but to protect the proteins from extreme pH environments samples should not be applied close to either electrode. During method development the analyst can try applying the protein in 3 positions on the gel (i.e. middle and both ends); the pattern of a protein applied at opposite ends of the gel may not be identical.

A phenomenon known as cathodic drift, where the pH gradient decays over time, may occur if a gel is focused too long. Although not well understood, electroendoosmosis and absorption of carbon dioxide may be factors that lead to cathodic drift. Cathodic drift is observed as focused protein migrating off the cathode end of the gel. Immobilized pH gradients may be used to address this problem.

Efficient cooling (approximately 4°C) of the bed that the gel lies on during focusing is important. High field strengths used during isoelectric focusing can lead to overheating and affect the quality of the focused gel.

Reagents and Solutions—

Fixing solution for isoelectric focusing in polyacrylamide gel Dissolve 35 g of 5-sulfosalicylic acid dihydrate and 100 g of trichloroacetic acid in water to make 1000 mL.

◆**Coomassie staining TS** Dissolve 125 mg of coomassie brilliant blue R-250 in 100 mL of a mixture of water, methanol and acetic acid (100) (5:4:1), and filter.

Destaining solution A mixture of water, methanol and acetic acid (100) (5:4:1).◆

Capillary Electrophoresis
⟨*G3-7-180*⟩

This test is harmonized with the European Pharmacopoeia and the U.S. Pharmacopeia.

Information on the harmonization with the European Pharmacopoeia and the U.S. Pharmacopeia is available on the website of the Pharmaceuticals and Medical Devices Agency.

1. General Principles

Capillary electrophoresis is a physical method of analysis based on the migration, inside a capillary, of charged analytes dissolved in an electrolyte solution, under the influence of a direct-current electric field.

The migration velocity of an analyte under an electric field of intensity E, is determined by the electrophoretic mobility of the analyte and the electroosmotic mobility of the buffer inside the capillary. The electrophoretic mobility of a solute (μ_{ep}) depends on the characteristics of the solute (electric charge, molecular size and shape) and those of the buffer in which the migration takes place (type and ionic strength of the electrolyte, pH, viscosity and additives). The electrophoretic velocity (υ_{ep}) of a solute, assuming a spherical shape, is given by the equation:

$$\upsilon_{ep} = \mu_{ep}E = \left(\frac{q}{6\pi\eta r}\right)\left(\frac{V}{L}\right)$$

q: Effective charge of the solute,
η: Viscosity of the electrolyte solution,
r: Stoke's radius of the solute,
V: Applied voltage,
L: Total length of the capillary.

When an electric field is applied through the capillary filled with buffer, a flow of solvent is generated inside the capillary, called electroosmotic flow. The velocity of the electroosmotic flow depends on the electroosmotic mobility (μ_{eo}) which in turn depends on the charge density on the capillary internal wall and the buffer characteristics. The electroosmotic velocity (υ_{eo}) is given by the equation:

$$\upsilon_{eo} = \mu_{eo}E = \left(\frac{\varepsilon\zeta}{\eta}\right)\left(\frac{V}{L}\right)$$

ε: Dielectric constant of the buffer,
ζ: Zeta potential of the capillary surface.

The velocity of the solute (υ) is given by:

$$\upsilon = \upsilon_{ep} + \upsilon_{eo}$$

The electrophoretic mobility of the analyte and the elec-

troosmotic mobility may act in the same direction or in opposite directions, depending on the charge of the solute. In normal capillary electrophoresis, anions will migrate in the opposite direction to the electroosmotic flow and their velocities will be smaller than the electroosmotic velocity. Cations will migrate in the same direction as the electroosmotic flow and their velocities will be greater than the electroosmotic velocity. Under conditions in which there is a fast electroosmotic velocity with respect to the electrophoretic velocity of the solutes, both cations and anions can be separated in the same run.

The time (t) taken by the solute to migrate the distance (l) from the injection end of the capillary to the detection point (capillary effective length) is given by the expression:

$$t = \frac{l}{v_{ep} + v_{eo}} = \frac{l \times L}{(\mu_{ep} + \mu_{eo})V}$$

In general, uncoated fused-silica capillaries above pH 3 have negative charge due to ionized silanol groups in the inner wall. Consequently, the electroosmotic flow is from anode to cathode. The electroosmotic flow must remain constant from run to run if good reproducibility is to be obtained in the migration velocity of the solutes. For some applications, it may be necessary to reduce or suppress the electroosmotic flow by modifying the inner wall of the capillary or by changing the concentration, composition and/or pH of the buffer solution.

After the introduction of the sample into the capillary, each analyte ion of the sample migrates within the background electrolyte as an independent zone, according to its electrophoretic mobility. Zone dispersion, that is the spreading of each solute band, results from different phenomena. Under ideal conditions the sole contribution to the solute-zone broadening is molecular diffusion of the solute along the capillary (longitudinal diffusion). In this ideal case the efficiency of the zone, expressed as the number of theoretical plates (N), is given by:

$$N = \frac{(\mu_{ep} + \mu_{eo}) \times V \times l}{2 \times D \times L}$$

D: Molecular diffusion coefficient of the solute in the buffer.

In practice, other phenomena such as heat dissipation, sample adsorption onto the capillary wall, mismatched conductivity between sample and buffer, length of the injection plug, detector cell size and unlevelled buffer reservoirs can also significantly contribute to band dispersion.

Separation between two bands (expressed as the resolution, R_S) can be obtained by modifying the electrophoretic mobility of the analytes, the electroosmotic mobility induced in the capillary and by increasing the efficiency for the band of each analyte, according to the equation:

$$R_S = \frac{\sqrt{N}(\mu_{epb} - \mu_{epa})}{4(\bar{\mu}_{ep} + \mu_{eo})}$$

μ_{epa} and μ_{epb}: Electrophoretic mobilities of the two analytes separated,

$\bar{\mu}_{ep}$: Mean electrophoretic mobility of the two analytes $\bar{\mu}_{ep} = \frac{1}{2}(\mu_{epa} + \mu_{epb})$.

2. Apparatus

An apparatus for capillary electrophoresis is composed of:

(1) a high-voltage, controllable direct-current power supply,

(2) two buffer reservoirs, held at the same level, containing the prescribed anodic and cathodic solutions,

(3) two electrode assemblies (the anode and the cathode), immersed in the buffer reservoirs and connected to the power supply,

(4) a separation capillary (usually made of fused-silica) which, when used with some specific types of detectors, has an optical viewing window aligned with the detector. The ends of the capillary are placed in the buffer reservoirs. The capillary is filled with the solution prescribed in the monograph,

(5) a suitable injection system,

(6) a detector able to monitor the amount of substances of interest passing through a segment of the separation capillary at a given time. It is usually based on absorption spectrophotometry (UV and visible) or fluorometry, but conductimetric, amperometric or mass spectrometric detection can be useful for specific applications. Indirect detection is an alternative method used to detect non-UV-absorbing and non-fluorescent compounds,

(7) a thermostatic system able to maintain a constant temperature inside the capillary is recommended to obtain a good separation reproducibility,

(8) a recorder and a suitable integrator or a computer.

The definition of the injection process and its automation are critical for precise quantitative analysis. Modes of injection include gravity, pressure or vacuum injection and electrokinetic injection. The amount of each sample component introduced electrokinetically depends on its electrophoretic mobility, leading to possible discrimination using this injection mode.

Use the capillary, the buffer solutions, the preconditioning method, the sample solution and the migration conditions prescribed in the monograph of the considered substance. The employed electrolytic solution is filtered to remove particles and degassed to avoid bubble formation that could interfere with the detection system or interrupt the electrical contact in the capillary during the separation run. A rigorous rinsing procedure should be developed for each analytical method to achieve reproducible migration times of the solutes.

3. Capillary Zone Electrophoresis

3.1. Principles

In capillary zone electrophoresis, analytes are separated in a capillary containing only buffer without any anticonvective medium. With this technique, separation takes place because the different components of the sample migrate as discrete bands with different velocities. The velocity of each band depends on the electrophoretic mobility of the solute and the electroosmotic flow in the capillary (see 1. General Principles). Coated capillaries can be used to increase the separation capacity of those substances adsorbing on fused-silica surfaces.

Using this mode of capillary electrophoresis, the analysis of both small ($M_r < 2000$) and large molecules ($2000 < M_r < 100{,}000$) can be accomplished. Due to the high efficiency achieved in capillary zone electrophoresis, separation of molecules having only minute differences in their charge-to-mass ratio can be effected. This separation mode also allows the separation of chiral compounds by addition of chiral selectors to the separation buffer.

3.2. Optimization

Optimization of the separation is a complex process where several separation parameters can play a major role. The main factors to be considered in the development of separations are instrumental and electrolytic solution parameters.

3.2.1. Instrumental parameters

(1) *Voltage*: A Joule heating plot is useful in optimizing

the applied voltage and column temperature. Separation time is inversely proportional to applied voltage. However, an increase in the voltage used can cause excessive heat production, giving rise to temperature and, as a result thereof, viscosity gradients in the buffer inside the capillary. This effect causes band broadening and decreases resolution.

(2) *Polarity*: Electrode polarity can be normal (anode at the inlet and cathode at the outlet) and the electroosmotic flow will move toward the cathode. If the electrode polarity is reversed, the electroosmotic flow is away from the outlet and only charged analytes with electroosmotic mobilities greater than the electroosmotic flow will pass to the outlet.

(3) *Temperature*: The main effect of temperature is observed on buffer viscosity and electrical conductivity, and therefore on migration velocity. In some cases, an increase in capillary temperature can cause a conformational change in proteins, modifying their migration time and the efficiency of the separation.

(4) *Capillary*: The dimensions of the capillary (length and internal diameter) contribute to analysis time, efficiency of separations and load capacity. Increasing both effective length and total length can decrease the electric fields (working at constant voltage), and increasing both effective length and total length increase migration time. For a given buffer and electric field, heat dissipation, and hence sample band-broadening, depend on the internal diameter of the capillary. The latter also affects the detection limit, depending on the sample volume injected and the detection system employed.

Since the adsorption of the sample components on the capillary wall limits efficiency, methods to avoid these interactions should be considered in the development of a separation method. In the specific case of proteins, several strategies have been devised to avoid adsorption on the capillary wall. Some of these strategies (use of extreme pH and adsorption of positively charged buffer additives) only require modification of the buffer composition to prevent protein adsorption. In other strategies, the internal wall of the capillary is coated with a polymer, covalently bonded to the silica, that prevents interaction between the proteins and the negatively charged silica surface. For this purpose, ready-to-use capillaries with coatings consisting of neutral-hydrophilic, cationic and anionic polymers are available.

3.2.2. Electrolytic solution parameters

(1) *Buffer type and concentration*: Suitable buffers for capillary electrophoresis have an appropriate buffer capacity in the pH range of choice and low mobility to minimize current generation.

Matching buffer-ion mobility to solute mobility, whenever possible, is important for minimizing band distortion. The type of sample solvent used is also important to achieve on-column sample focusing, which increases separation efficiency and improves detection.

An increase in buffer concentration (for a given pH) decreases electroosmotic flow and solute velocity.

(2) *Buffer pH*: The pH of the buffer can affect separation by modifying the charge of the analyte or additives, and by changing the electroosmotic flow. In protein and peptide separation, changing the pH of the buffer from above to below the isoelectric point (pI) changes the net charge of the solute from negative to positive. An increase in the buffer pH generally increases the electroosmotic flow.

(3) *Organic solvents*: Organic modifiers (methanol, acetonitrile, etc.) may be added to the aqueous buffer to increase the solubility of the solute or other additives and/or to affect the degree of ionization of the sample components. The addition of organic modifiers to the buffer generally causes a decrease in the electroosmotic flow.

(4) *Additives for chiral separations*: For the separation of enantiomers, a chiral selector is added to the separation buffer. The most commonly used chiral selectors are cyclodextrins, but crown ethers, polysaccharides and proteins may also be used. Since chiral recognition is governed by the different interactions between the chiral selector and each of the enantiomers, the resolution achieved for the chiral compounds depends largely on the type of chiral selector used. In this regard, for the development of a given separation it may be useful to test cyclodextrins having a different cavity size (α-, β-, or γ-cyclodextrin) or modified cyclodextrins with neutral (methyl, ethyl, hydroxyalkyl, etc.) or ionizable (aminomethyl, carboxymethyl, sulfobutyl ether, etc.) groups. When using modified cyclodextrins, batch-to-batch variations in the degree of substitution of the cyclodextrins must be taken into account since it will influence the selectivity. Other factors controlling the resolution in chiral separations are concentration of chiral selector, composition and pH of the buffer and temperature. The use of organic additives, such as methanol or urea can also modify the resolution achieved.

4. Capillary Gel Electrophoresis

4.1. Principles

In capillary gel electrophoresis, separation takes place inside a capillary filled with a gel that acts as a molecular sieve. Molecules with similar charge-to-mass ratios are separated according to molecular size since smaller molecules move more freely through the network of the gel and therefore migrate faster than larger molecules. Different biological macromolecules (for example, proteins and DNA fragments), which often have similar charge-to-mass ratios, can thus be separated according to their molecular mass by capillary gel electrophoresis.

4.2. Characteristics of Gels

Two types of gels are used in capillary electrophoresis: permanently coated gels and dynamically coated gels. Permanently coated gels, such as cross-linked polyacrylamide, are prepared inside the capillary by polymerization of the monomers. They are usually bonded to the fused-silica wall and cannot be removed without destroying the capillary. If the gels are used for protein analysis under reducing conditions, the separation buffer usually contains sodium dodecyl sulfate and the samples are denatured by heating a mixture of sodium dodecyl sulfate and 2-mercaptoethanol or dithiothreitol before injection. When non-reducing conditions are used (for example, analysis of an intact antibody), 2-mercaptoethanol and dithiothreitol are not used. Separation in cross-linked gels can be optimized by modifying the separation buffer (as indicated in the capillary zone electrophoresis section) and controlling the gel porosity during the gel preparation. For cross-linked polyacrylamide gels, the porosity can be modified by changing the concentration of acrylamide and/or the proportion of cross-linker. As a rule, a decrease in the porosity of the gel leads to a decrease in the mobility of the solutes. Due to the rigidity of these gels, only electrokinetic injection can be used.

Dynamically coated gels are hydrophilic polymers, such as linear polyacrylamide, cellulose derivatives, dextran, etc., which can be dissolved in aqueous separation buffers giving rise to a separation medium that also acts as a molecular sieve. These separation media are easier to prepare than cross-linked polymers. They can be prepared in a vial and filled by pressure in a wall-coated capillary (with no electroosmotic flow). Replacing the gel before every injection generally improves the separation reproducibility. The porosity of the gels can be increased by using polymers of higher

molecular mass (at a given polymer concentration) or by decreasing the polymer concentration (for a given polymer molecular mass). A reduction in the gel porosity leads to a decrease in the mobility of the solute for the same buffer. Since the dissolution of these polymers in the buffer gives low viscosity solutions, both hydrodynamic and electrokinetic injection techniques can be used.

5. Capillary Isoelectric Focusing

5.1. Principles

In isoelectric focusing, the molecules migrate under the influence of the electric field, so long as they are charged, in a pH gradient generated by ampholytes having pI values in a wide range (poly-aminocarboxylic acids), dissolved in the separation buffer.

The three basic steps of isoelectric focusing are loading, focusing and mobilization.

(1) Loading step: Two methods may be employed:

(i) loading in one step: the sample is mixed with ampholytes and introduced into the capillary either by pressure or vacuum;

(ii) sequential loading: a leading buffer, then the ampholytes, then the sample mixed with ampholytes, again ampholytes alone and finally the terminating buffer are introduced into the capillary. The volume of the sample must be small enough not to modify the pH gradient.

(2) Focusing step: When the voltage is applied, ampholytes migrate toward the cathode or the anode, according to their net charge, thus creating a pH gradient from anode (lower pH) to cathode (higher pH). During this step the components to be separated migrate until they reach a pH corresponding to their isoelectric point (pI) and the current drops to very low values.

(3) Mobilization step: If mobilization is required for detection, use one of the following methods. Three methods are available:

(i) mobilization is accomplished during the focusing step under the effect of the electroosmotic flow; the electroosmotic flow must be small enough to allow the focusing of the components;

(ii) mobilization is accomplished by applying positive pressure after the focusing step;

(iii) mobilization is achieved after the focusing step by adding salts to the cathode reservoir or the anode reservoir (depending on the direction chosen for mobilization) in order to alter the pH in the capillary when the voltage is applied. As the pH is changed, the proteins and ampholytes are mobilized in the direction of the reservoir which contains the added salts and pass the detector.

The separation achieved, expressed as ΔpI, depends on the pH gradient (dpH/dx), the number of ampholytes having different pI values, the molecular diffusion coefficient (D), the intensity of the electric field (E) and the variation of the electrophoretic mobility of the analyte with the pH ($-d\mu/dpH$):

$$\Delta pI = 3\sqrt{\frac{D(dpH/dx)}{E(-d\mu/dpH)}}$$

5.2. Optimization

The main parameters to be considered in the development of separations are:

(1) Voltage: Capillary isoelectric focusing utilises very high electric fields, 300 V/cm to 1000 V/cm in the focusing step.

(2) Capillary: The electroosmotic flow must be reduced or suppressed depending on the mobilization strategy (see above). Coated capillaries tend to reduce the electroosmotic flow.

(3) Solutions: The anode buffer reservoir is filled with a solution with a pH lower than the pI of the most acidic ampholyte and the cathode reservoir is filled with a solution with a pH higher than the pI of the most basic ampholyte. Phosphoric acid for the anode and sodium hydroxide for the cathode are frequently used.

Addition of a polymer, such as methylcellulose, in the ampholyte solution tends to suppress convective forces (if any) and electroosmotic flow by increasing the viscosity. Commercial ampholytes are available covering many pH ranges and may be mixed if necessary to obtain an expanded pH range. Broad pH ranges are used to estimate the isoelectric point whereas narrower ranges are employed to improve accuracy. Calibration can be done by correlating migration time with isoelectric point for a series of protein markers.

During the focusing step precipitation of proteins at their isoelectric point can be prevented, if necessary, using buffer additives such as glycerol, surfactants, urea or zwitterionic buffers. However, depending on the concentration, urea denatures proteins.

6. Micellar Electrokinetic Chromatography (MEKC)

6.1. Principles

In micellar electrokinetic chromatography, separation takes place in an electrolyte solution which contains a surfactant at a concentration above the critical micellar concentration (*cmc*). The solute molecules are distributed between the aqueous buffer and the pseudo-stationary phase composed of micelles, according to the partition coefficient of the solute. The technique can therefore be considered as a hybrid of electrophoresis and chromatography. It is a technique that can be used for the separation of both neutral and charged solutes, maintaining the efficiency, speed and instrumental suitability of capillary electrophoresis. One of the most widely used surfactants in MEKC is the anionic surfactant sodium dodecyl sulfate, although other surfactants, for example cationic surfactants such as cetyltrimethylammonium salts, are also used.

The separation mechanism is as follows. At neutral and alkaline pH, a strong electroosmotic flow is generated and moves the separation buffer ions in the direction of the cathode. If sodium dodecyl sulfate is employed as the surfactant, the electrophoretic migration of the anionic micelle is in the opposite direction, towards the anode. As a result, the overall micelle migration velocity is slowed down compared to the bulk flow of the electrolytic solution. In the case of neutral solutes, since the analyte can partition between the micelle and the aqueous buffer, and has no electrophoretic mobility, the analyte migration velocity will depend only on the partition coefficient between the micelle and the aqueous buffer. In the electropherogram, the peaks corresponding to each uncharged solute are always between that of the electroosmotic flow marker and that of the micelle (the time elapsed between these two peaks is called the separation window). For electrically charged solutes, the migration velocity depends on both the partition coefficient of the solute between the micelle and the aqueous buffer, and on the electrophoretic mobility of the solute in the absence of micelle.

Since the mechanism in MEKC of neutral and weakly ionized solutes is essentially chromatographic, migration of the solute and resolution can be rationalized in terms of the retention factor (k'), also referred to as mass distribution ratio (D_m), which is the ratio of the number of moles of solute in the micelle to those in the mobile phase. For a neutral

compound, k' is given by:

$$k' = \frac{t_R - t_0}{t_0\left(1 - \frac{t_R}{t_{mc}}\right)} = K\frac{V_S}{V_M}$$

t_R: Migration time of the solute,
t_0: Analysis time of an unretained solute (determined by injecting an electroosmotic flow marker which does not enter the micelle, for instance methanol),
t_{mc}: Micelle migration time (measured by injecting a micelle marker, such as Sudan III, which migrates while continuously associated in the micelle),
K: Partition coefficient of the solute,
V_S: Volume of the micellar phase,
V_M: Volume of the mobile phase.

Likewise, the resolution between two closely-migrating solutes (R_S) is given by:

$$R_S = \frac{\sqrt{N}}{4} \times \frac{\alpha - 1}{\alpha} \times \frac{k'_b}{k'_b + 1} \times \frac{1 - \left(\frac{t_0}{t_{mc}}\right)}{1 + \left(\frac{t_0}{t_{mc}}\right)k'_a}$$

N: Number of theoretical plates for one of the solutes,
α: Selectivity,
k'_a and k'_b: Retention factors for both solutes, respectively ($k'_b > k'_a$).

Similar, but not identical, equations give k' and R_S values for electrically charged solutes.

6.2. Optimization

The main parameters to be considered in the development of separations by MEKC are instrumental and electrolytic solution parameters.

6.2.1. Instrumental parameters

(1) *Voltage*: Separation time is inversely proportional to applied voltage. However, an increase in voltage can cause excessive heat production that gives rise to temperature gradients and viscosity gradients of the buffer in the cross-section of the capillary. This effect can be significant with high conductivity buffers such as those containing micelles. Poor heat dissipation causes band broadening and decreases resolution.

(2) *Temperature*: Variations in capillary temperature affect the partition coefficient of the solute between the buffer and the micelles, the critical micellar concentration and the viscosity of the buffer. These parameters contribute to the migration time of the solutes. The use of a good cooling system improves the reproducibility of the migration time for the solutes.

(3) *Capillary*: As in capillary zone electrophoresis, the dimensions of the capillary (length and internal diameter) contribute to analysis time and efficiency of separations. Increasing both effective length and total length can decrease the electric fields (working at constant voltage), increase migration time and improve the separation efficiency. The internal diameter controls heat dissipation (for a given buffer and electric field) and consequently the sample band broadening.

6.2.2. Electrolytic solution parameters

(1) *Surfactant type and concentration*: The type of surfactant, in the same way as the stationary phase in chromatography, affects the resolution since it modifies separation selectivity. Also, the log k' of a neutral compound increases linearly with the concentration of surfactant in the mobile phase. Since resolution in MEKC reaches a maximum when k' approaches the value of $\sqrt{t_m/t_0}$, modifying the concentration of surfactant in the mobile phase changes the resolution obtained.

(2) *Buffer pH*: Although pH does not modify the partition coefficient of non-ionized solutes, it can modify the electroosmotic flow in uncoated capillaries. A decrease in the buffer pH decreases the electroosmotic flow and therefore increases the resolution of the neutral solutes in MEKC, resulting in a longer analysis time.

(3) *Organic solvents*: To improve MEKC separation of hydrophobic compounds, organic modifiers (methanol, propanol, acetonitrile, etc.) can be added to the electrolytic solution. The addition of these modifiers usually decreases migration time and the selectivity of the separation. Since the addition of organic modifiers affects the critical micellar concentration, a given surfactant concentration can be used only within a certain percentage of organic modifier before the micellization is inhibited or adversely affected, resulting in the absence of micelles and, therefore, in the absence of partition. The dissociation of micelles in the presence of a high content of organic solvent does not always mean that the separation will no longer be possible; in some cases the hydrophobic interaction between the ionic surfactant monomer and the neutral solutes forms solvophobic complexes that can be separated electrophoretically.

(4) *Additives for chiral separations*: For the separation of enantiomers using MEKC, a chiral selector is included in the micellar system, either covalently bound to the surfactant or added to the micellar separation electrolyte. Micelles that have a moiety with chiral discrimination properties include salts of *N*-dodecanoyl-L-amino acids, bile salts, etc. Chiral resolution can also be achieved using chiral discriminators, such as cyclodextrins, added to the electrolytic solutions which contain micellized achiral surfactants.

(5) *Other additives*: Several strategies can be carried out to modify selectivity, by adding chemicals to the buffer. The addition of several types of cyclodextrins to the buffer can also be used to reduce the interaction of hydrophobic solutes with the micelle, thus increasing the selectivity for this type of compound.

The addition of substances able to modify solute-micelle interactions by adsorption on the latter, is used to improve the selectivity of the separations in MEKC. These additives may be a second surfactant (ionic or non-ionic) which gives rise to mixed micelles or metallic cations which dissolve in the micelle and form co-ordination complexes with the solutes.

7. Quantification

Peak areas must be divided by the corresponding migration time to give the corrected peak area in order to:

(1) compensate for the shift in migration time from run to run, thus reducing the variation of the response,
(2) compensate for the different responses of sample constituents with different migration times.

Where an internal standard is used, verify that no peak of the substance to be examined is masked by that of the internal standard.

7.1. Calculations

From the values obtained, calculate the content of the component or components being examined. When prescribed, the percentage content of one or more components of the sample to be examined is calculated by determining the corrected area(s) of the peak(s) as a percentage of the total of the corrected areas of all peaks, excluding those due to solvents or any added reagents (normalization procedure). The use of an automatic integration system (integrator or data acquisition and processing system) is recommended.

8. System Suitability

In order to check the behavior of the capillary electrophoresis system, system suitability parameters are used. The choice of these parameters depends on the mode of capillary electrophoresis used. They are retention factor (k') (only for micellar electrokinetic chromatography), apparent number of theoretical plates (N), symmetry factor (A_S) and resolution (R_S). In previous sections, the theoretical expressions for N and R_S have been described, but more practical equations that allow these parameters to be calculated from the electropherograms are given below.

Apparent Number of Theoretical Plates

The apparent number of theoretical plates (N) may be calculated using the expression:

$$N = 5.54\left(\frac{t_R}{w_h}\right)^2$$

t_R: Migration time or distance along the baseline from the point of injection to the perpendicular dropped from the maximum of the peak corresponding to the component,

w_h: Width of the peak at half-height.

Resolution

The resolution (R_S) between peaks of similar height of two components may be calculated using the expression:

$$R_S = \frac{1.18(t_{R2} - t_{R1})}{w_{h1} + w_{h2}}$$

$$t_{R2} > t_{R1}$$

t_{R1} and t_{R2}: Migration times or distances along the baseline from the point of injection to the perpendiculars dropped from the maxima of two adjacent peaks,

w_{h1} and w_{h2}: Peak widths at half-height.

When appropriate, the resolution may be calculated by measuring the height of the valley (H_v) between two partly resolved peaks in a standard preparation and the height of the smaller peak (H_p) and calculating the peak-to-valley ratio (ρ/υ):

$$p/\upsilon = \frac{H_p}{H_v}$$

Symmetry Factor

The symmetry factor (A_S) of a peak may be calculated using the expression:

$$A_S = \frac{w_{0.05}}{2d}$$

$w_{0.05}$: Width of the peak at one-twentieth of the peak height,

d: Distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at one-twentieth of the peak height.

Tests for peak area repeatability (standard deviation of areas or of the area/migration-time ratio) and for migration time repeatability (standard deviation of migration time) are introduced as suitability parameters. Migration time repeatability provides a test for the suitability of the capillary washing procedures. An alternative practice to avoid the lack of repeatability of the migration time is to use migration time relative to an internal standard.

A test for the verification of the signal-to-noise ratio for a standard preparation (or the determination of the limit of quantification) may also be useful for the determination of related substances.

Signal-to-noise Ratio (S/N)

The detection limit and the quantification limit are equivalent to signal-to-noise ratios of 3 and 10, respectively. The signal-to-noise ratio (S/N) is calculated using the expression:

$$S/N = \frac{2H}{h}$$

H: Height of the peak corresponding to the component concerned, in the electropherogram obtained with the prescribed reference solution, measured from the maximum of the peak to the extrapolated baseline of the signal observed over a distance equal to twenty times the width at half-height,

h: Range of the background in an electropherogram obtained after injection of a blank, observed over a distance equal to twenty times the width at the half-height of the peak in the electropherogram obtained with the prescribed reference solution and, if possible, situated equally around the place where this peak would be found.

SDS-Polyacrylamide Gel Electrophoresis <*G3-8-170*>

This test is harmonized with the European Pharmacopoeia and the U.S. Pharmacopeia.

Information on the harmonization with the European Pharmacopoeia and the U.S. Pharmacopeia is available on the website of the Pharmaceuticals and Medical Devices Agency.

Polyacrylamide gel electrophoresis is used for the qualitative characterisation of proteins in biological preparations, for control of purity and for quantitative determinations.

Analytical gel electrophoresis is an appropriate method with which to identify and to assess the homogeneity of proteins in pharmaceutical preparations. The method is routinely used for the estimation of protein subunit molecular masses and for determination of the subunit compositions of purified proteins.

Ready-to-use gels and reagents are commercially available and can be used instead of those described in this text, provided that they give equivalent results and that they meet the validity requirements given below under Validation of the test.

1. Characteristics of Polyacrylamide Gels

The sieving properties of polyacrylamide gels are established by the three-dimensional network of fibres and pores which is formed as the bifunctional bisacrylamide cross-links adjacent polyacrylamide chains. Polymerisation is usually catalysed by a free radical-generating system composed of ammonium persulfate and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED).

As the acrylamide concentration of a gel increases, its effective pore size decreases. The effective pore size of a gel is operationally defined by its sieving properties; that is, by the resistance it imparts to the migration of macromolecules. There are limits on the acrylamide concentrations that can be used. At high acrylamide concentrations, gels break much more easily and are difficult to handle. As the pore size of a gel decreases, the migration rate of a protein through the gel decreases. By adjusting the pore size of a gel, through manipulating the acrylamide concentration, the resolution of

the method can be optimised for a given protein product. Thus, a given gel is physically characterised by its respective composition of acrylamide and bisacrylamide.

In addition to the composition of the gel, the state of the protein is an important component to the electrophoretic mobility. In the case of proteins, the electrophoretic mobility is dependent on the pK value of the charged groups and the size of the molecule. It is influenced by the type, the concentration and the pH of the buffer, by the temperature and the field strength, and by the nature of the support material.

2. Denaturing Polyacrylamide Gel Electrophoresis

The method cited as an example is limited to the analysis of monomeric polypeptides with a mass range of 14,000 to 100,000 daltons. It is possible to extend this mass range by various techniques (e.g. gradient gels, particular buffer system). For instance, tricine sodium dodecyl sulfate (SDS) gels, using tricine as the trailing ion in the electrophoresis running buffer (instead of glycine as in the method described here), can separate very small proteins and peptides under 10,000-15,000 daltons.

Denaturing polyacrylamide gel electrophoresis using glycine SDS (SDS-PAGE) is the most common mode of electrophoresis used in assessing the pharmaceutical quality of protein products and will be the focus of the example method. Typically, analytical electrophoresis of proteins is carried out in polyacrylamide gels under conditions that ensure dissociation of the proteins into their individual polypeptide subunits and that minimize aggregation. Most commonly, the strongly anionic detergent SDS is used in combination with heat to dissociate the proteins before they are loaded on the gel. The denatured polypeptides bind to SDS, become negatively charged and exhibit a consistent charge-to-mass ratio regardless of protein type. Because the amount of SDS bound is almost always proportional to the molecular mass of the polypeptide and is independent of its sequence, SDS-polypeptide complexes migrate through polyacrylamide gels with mobilities dependent on the size of the polypeptide.

The electrophoretic mobilities of the resultant detergent-polypeptide complexes all assume the same functional relationship to their molecular masses. SDS complexes will migrate toward the anode in a predictable manner, with low molecular mass complexes migrating faster than larger ones. The molecular mass of a protein can therefore be estimated from its relative mobility in calibrated SDS-PAGE and the intensity of a single band relative to other undesired bands in such a gel can be a measure of purity.

Modifications to the polypeptide backbone, such as N- or O-linked glycosylation, can change the apparent molecular mass of a protein since SDS does not bind to a carbohydrate moiety in a manner similar to a polypeptide; therefore, a consistent charge-to-mass ratio is not maintained.

Depending on the extent of glycosylation and other post-translational modifications, the apparent molecular mass of proteins may not be a true reflection of the mass of the polypeptide chain.

2.1. Reducing conditions

Polypeptide subunits and three-dimensional structure are often maintained in proteins by the presence of disulfide bonds. A goal of SDS-PAGE analysis under reducing conditions is to disrupt this structure by reducing disulfide bonds. Complete denaturation and dissociation of proteins by treatment with 2-mercaptoethanol (2-ME) or dithiothreitol (DTT) will result in unfolding of the polypeptide backbone and subsequent complexation with SDS. Using these conditions, the molecular mass of the polypeptide subunits can reasonably be calculated by linear regression (or, more closely, by non linear regression) in the presence of suitable molecular mass standards.

2.2. Non-reducing conditions

For some analyses, complete dissociation of the protein into subunit peptides is not desirable. In the absence of treatment with reducing agents such as 2-ME or DTT, disulfide covalent bonds remain intact, preserving the oligomeric form of the protein. Oligomeric SDS-protein complexes migrate more slowly than their SDS-polypeptide subunits. In addition, non-reduced proteins may not be completely saturated with SDS and, hence, may not bind the detergent in a constant mass ratio. Moreover, intra-chain disulphide bonds constrain the molecular shape, usually in such a way as to reduce the Stokes radius of the molecule, thereby reducing the apparent molecular mass M_r. This makes molecular mass determinations of these molecules by SDS-PAGE less straightforward than analyses of fully denatured polypeptides, since it is necessary that both standards and unknown proteins be in similar configurations for valid comparisons.

3. Characteristics of Discontinuous Buffer System Gel Electrophoresis

The most popular electrophoretic method for the characterisation of complex mixtures of proteins uses a discontinuous buffer system involving two contiguous, but distinct gels: a resolving or separating (lower) gel and a stacking (upper) gel. The two gels are cast with different porosities, pH, and ionic strengths. In addition, different mobile ions are used in the gel and electrode buffers. The buffer discontinuity acts to concentrate large volume samples in the stacking gel, resulting in improved resolution. When power is applied, a voltage drop develops across the sample solution which drives the proteins into the stacking gel. Glycinate ions from the electrode buffer follow the proteins into the stacking gel. A moving boundary region is rapidly formed with the highly mobile chloride ions in the front and the relatively slow glycinate ions in the rear. A localised high-voltage gradient forms between the leading and trailing ion fronts, causing the SDS-protein complexes to form into a thin zone (stack) and migrate between the chloride and glycinate phases. Within broad limits, regardless of the height of the applied sample, all SDS-proteins condense into a very narrow region and enter the resolving gel as a well-defined, thin zone of high protein density. The large-pore stacking gel does not retard the migration of most proteins and serves mainly as an anti-convective medium. At the interface of the stacking and resolving gels, the proteins undergo a sharp increase in retardation due to the restrictive pore size of the resolving gel and the buffer discontinuity, which also contributes to focusing of the proteins. Once in the resolving gel, proteins continue to be slowed by the sieving of the matrix. The glycinate ions overtake the proteins, which then move in a space of uniform pH formed by the 2-amino-2-hydroxymethyl-1,3-propanediol and glycine. Molecular sieving causes the SDS-polypeptide complexes to separate on the basis of their molecular masses.

4. Preparing Vertical Discontinuous Buffer SDS Polyacrylamide Gels

This section describes the preparation of gels using particular instrumentation. This does not apply to pre-cast gels. For pre-cast gels or any other commercially available equipment, the manufacturer's instructions must be used for guidance.

The use of commercial reagents that have been purified in solution is recommended. When this is not the case and where the purity of the reagents used is not sufficient, a pre-

treatment is applied. For instance, any solution sufficiently impure to require filtration must also be deionized with a mixed bed (anion/cation exchange) resin to remove acrylic acid and other charged degradation products. When stored according to recommendations, acrylamide/bisacrylamide solutions and solid persulfate are stable for long periods.

4.1. Assembling the gel moulding cassette

Clean the two glass plates (size: e.g. 10 cm × 8 cm), the polytetrafluoroethylene comb, the two spacers and the silicone rubber tubing (diameter e.g. 0.6 mm × 35 cm) with mild detergent and rinse extensively with water, followed by dehydrated alcohol, and allow the plates to dry at room temperature. Lubricate the spacers and the tubing with non-silicone grease. Apply the spacers along each of the two short sides of the glass plate 2 mm away from the edges and 2 mm away from the long side corresponding to the bottom of the gel. Begin to lay the tubing on the glass plate by using one spacer as a guide. Carefully twist the tubing at the bottom of the spacer and follow the long side of the glass plate. While holding the tubing with one finger along the long side twist again the tubing and lay it on the second short side of the glass plate, using the spacer as a guide. Place the second glass plate in perfect alignment and hold the mould together by hand pressure. Apply two clamps on each of the two short sides of the mould. Carefully apply four clamps on the longer side of the gel mould thus forming the bottom of the gel mould. Verify that the tubing is running along the edge of the glass plates and has not been extruded while placing the clamps. The gel mould is now ready for pouring the gel.

4.2. Preparation of the gel

In a discontinuous buffer SDS polyacrylamide gel, it is recommended to pour the resolving gel, let the gel set, and then pour the stacking gel since the composition of the two gels in acrylamide-bisacrylamide, buffer and pH are different.

4.2.1. Preparation of the resolving gel

In a conical flask, prepare the appropriate volume of solution containing the desired concentration of acrylamide for the resolving gel, using the values given in Table 1. Mix the components in the order shown. Where appropriate, before adding the ammonium persulfate solution and the TEMED, filter the solution if necessary under vacuum through a cellulose acetate membrane (pore diameter 0.45 μm). Keep the solution under vacuum, while swirling the filtration unit, until no more bubbles are formed in the solution. Add appropriate amounts of ammonium persulfate solution and TEMED as indicated in Table 1, swirl and pour immediately into the gap between the two glass plates of the mould. Leave sufficient space for the stacking gel (the length of the teeth of the comb plus 1 cm). Using a tapered glass pipette, carefully overlay the solution with water-saturated isobutanol. Leave the gel in a vertical position at room temperature to allow polymerization.

4.2.2. Preparation of the stacking gel

After polymerization is complete (about 30 minutes), pour off the isobutanol and wash the top of the gel several times with water to remove the isobutanol overlay and any unpolymerized acrylamide. Drain as much fluid as possible from the top of the gel, and then remove any remaining water with the edge of a paper towel.

In a conical flask, prepare the appropriate volume of solution containing the desired concentration of acrylamide, using the values given in Table 2. Mix the components in the order shown. Where appropriate, before adding the ammonium persulfate solution and the TEMED, filter the solution if necessary under vacuum through a cellulose acetate membrane (pore diameter: 0.45 μm). Keep the solution under vacuum, while swirling the filtration unit, until no more bubbles are formed in the solution. Add appropriate amounts of ammonium persulfate solution and TEMED as indicated in Table 2. Swirl and pour immediately into the gap between the two glass plates of the mould directly onto the surface of the polymerized resolving gel. Immediately insert a clean polytetrafluoroethylene comb into the stacking gel solution, being careful to avoid trapping air bubbles. Add more stacking gel solution to fill the spaces of the comb completely. Leave the gel in a vertical position and allow to polymerize at room temperature.

4.3. Preparation of the sample

Unless otherwise specified in the specific monograph the samples can be prepared as follows:

Sample solution (non-reducing conditions). Mix equal volumes of: a mixture comprising water plus the preparation or the reference solutions, and concentrated SDS-PAGE sample buffer.

Sample solution (reducing conditions). Mix equal volumes of: a mixture comprising water plus the preparation or the reference solutions, and concentrated SDS-PAGE sample buffer for reducing conditions containing 2-ME (or DTT) as reducing agent.

The concentration prescribed in the monograph can vary depending on the protein and staining method.

Sample treatment: keep for 5 minutes in a boiling water bath or in a block heater set at 100°C, then chill. (Note that temperature and time may vary in the monograph since protein cleavage may occur during the heat treatment.)

4.4. Mounting the gel in the electrophoresis apparatus and electrophoretic separation

After polymerization is complete (about 30 minutes), remove the polytetrafluoroethylene comb carefully. Rinse the wells immediately with water or with the SDS-PAGE running buffer to remove any unpolymerized acrylamide. If necessary, straighten the teeth of the stacking gel with a blunt hypodermic needle attached to a syringe. Remove the clamps on one short side, carefully pull out the tubing and replace the clamps. Proceed similarly on the other short side. Remove the tubing from the bottom part of the gel. Mount the gel in the electrophoresis apparatus. Add the electrophoresis buffers to the top and bottom reservoirs. Remove any bubbles that become trapped at the bottom of the gel between the glass plates. This is best done with a bent hypodermic needle attached to a syringe. Never pre-run the gel before loading the samples, since this will destroy the discontinuity of the buffer systems. Before loading the sample carefully rinse each well with SDS-PAGE running buffer. Prepare the test and reference solutions in the recommended sample buffer and treat as specified in the individual monograph. Apply the appropriate volume of each solution to the stacking gel wells. Start the electrophoresis using the conditions recommended by the manufacturer of the equipment. Manufacturers of SDS-PAGE equipment may provide gels of different surface area and thickness and electrophoresis running time and current/voltage may vary in order to achieve optimal separation. Check that the dye front is moving into the resolving gel. When the dye is near the bottom of the gel, stop the electrophoresis. Remove the gel assembly from the apparatus and carefully separate the glass plates. Remove the spacers, cut off and discard the stacking gel and immediately proceed with staining.

4.5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)—Gradient concentration gels

Gradient gels (resolving gels) are prepared with an increasing concentration of acrylamide from the top to the bottom. Preparation of gradient gels requires a gradient forming ap-

paratus. Ready-to-use gradient gels are commercially available with specific recommended protocols. Gradient gels offer some advantages over fixed concentration gels. Some proteins which co-migrate on fixed concentration gels can be resolved within gradient gels. During electrophoresis the proteins migrate until the pore size stops further progress and therefore a stacking effect occurs, resulting in sharper bands. Per the table below, gradient gels also allow separation of a wider range of proteins molecular masses than on a single fixed concentration gel.

The table below gives suggested compositions of the linear gradient, relating the range of acrylamide concentrations to the appropriate protein molecular ranges. Note that other gradient shapes (e.g. concave) can be prepared for specific applications.

Acrylamide (%)	Protein range (kDa)
5–15	20–250
5–20	10–200
10–20	10–150
8–20	8–150

Gradient gels are also used for molecular mass determination and protein purity determination.

4.6. Detection of proteins in gels

Coomassie and silver staining are the most common protein staining methods and are described in more detail below. Several other commercial stains, detection methods and commercial kits are available. For example, fluorescent stains are visualised using a fluorescent imager and often provide a linear response over a wide range of protein concentrations, often several orders of magnitude depending on the protein.

Coomassie staining has a protein detection level of approximately 1 to 10 μg of protein per band. Silver staining is the most sensitive method for staining proteins in gels and a band containing 10 ng to 100 ng can be detected. These figures are considered robust in the context of these gels. Improved sensitivity of one or two orders of magnitude has sometimes been reported in the literature.

Coomassie staining responds in a more linear manner than silver staining; however the response and range depend on the protein and development time. Both Coomassie and silver staining can be less reproducible if staining is stopped in a subjective manner, i.e. when the staining is deemed satisfactory. Wide dynamic ranges of reference proteins are very important to use since they help assess the intra-experimental sensitivity and linearity. All gel staining steps are done while wearing gloves, at room temperature, with gentle shaking (e.g. on an orbital shaker platform) and using any convenient container.

4.6.1. Coomassie staining

Immerse the gel in a large excess of Coomassie staining solution and allow to stand for at least 1 hour. Remove the staining solution.

Destain the gel with a large excess of destaining solution. Change the destaining solution several times, until the stained protein bands are clearly distinguishable on a clear background. The more thoroughly the gel is destained, the smaller is the amount of protein that can be detected by the method. Destaining can be speeded up by including a few grams of anion-exchange resin or a small sponge in the destaining solution.

The acid-alcohol solutions used in this procedure do not completely fix proteins in the gel. This can lead to losses of some low-molecular-mass proteins during the staining and destaining of thin gels. Permanent fixation is obtainable by allowing the gel to stand in a mixture of 1 volume of trichloroacetic acid, 4 volumes of methanol and 5 volumes of water for 1 hour before it is immersed in the Coomassie staining solution.

4.6.2. Silver staining

Immerse the gel in a large excess of fixing solution and allow to stand for 1 hour. Remove the fixing solution, add fresh fixing solution and incubate either for at least 1 hour or overnight, if convenient. Discard the fixing solution and wash the gel in a large excess of water for 1 hour. Soak the gel for 15 minutes in a 1 vol% solution of glutaraldehyde. Wash the gel twice for 15 minutes in a large excess of water. Soak the gel in fresh silver nitrate reagent for 15 minutes, in darkness. Wash the gel three times for 5 minutes in a large excess of water. Immerse the gel for about 1 minute in developer solution until satisfactory staining has been obtained. Stop the development by incubation in the blocking solution for 15 minutes. Rinse the gel with water.

4.7. Recording of the results

Gels are photographed or scanned while they are still wet or after an appropriate drying procedure. Currently, ''gel scanning'' systems with data analysis software are commercially available to photograph and analyze the wet gel immediately.

Depending on the staining method used, gels are treated in a slightly different way. For Coomassie staining, after the destaining step, allow the gel to stand in a 100 g/L solution of glycerol for at least 2 hours (overnight incubation is possible).

For silver staining, add to the final rinsing a step of 5 minutes in a 20 g/L solution of glycerol.

Drying of stained SDS Polyacrylamide gels is one of the methods to have permanent documentation. This method frequently results in the ''cracking of gel'' during drying between cellulose films.

Immerse two sheets of porous cellulose film in water and incubate for 5 minutes to 10 minutes. Place one of the sheets on a drying frame. Carefully lift the gel and place it on the cellulose film. Remove any trapped air bubbles and pour a few millilitres of water around the edges of the gel. Place the second sheet on top and remove any trapped air bubbles. Complete the assembly of the drying frame. Place in an oven or leave at room temperature until dry.

4.8. Molecular mass determination

Molecular masses of proteins are determined by comparison of their mobilities with those of several marker proteins of known molecular weight. Mixtures of pre-stained and unstained proteins with precisely known molecular masses blended for uniform staining are available for calibrating gels. They are available in various molecular mass ranges. Concentrated stock solutions of proteins of known molecular mass are diluted in the appropriate sample buffer and loaded on the same gel as the protein sample to be studied.

Immediately after the gel has been run, the position of the bromophenol blue tracking dye is marked to identify the leading edge of the electrophoretic ion front. This can be done by cutting notches in the edges of the gel or by inserting a needle soaked in India ink into the gel at the dye front. After staining, measure the migration distances of each protein band (markers and unknowns) from the top of the resolving gel. Divide the migration distance of each protein by the distance travelled by the tracking dye. The normalized migration distances are referred to as the relative mobilities of the proteins (relative to the dye front), or Rf. Construct a plot of the logarithm of the relative molecular masses (M_r)

Table 1

Solution components	Component volumes (mL) per gel mould volume of							
	5 mL	10 mL	15 mL	20 mL	25 mL	30 mL	40 mL	50 mL
6% Acrylamide								
Water	2.6	5.3	7.9	10.6	13.2	15.9	21.2	26.5
Acrylamide solution[1]	1.0	2.0	3.0	4.0	5.0	6.0	8.0	10.0
1.5 M Tris (pH 8.8)[2]	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
100 g/L SDS[3]	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
100 g/L APS[4]	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED[5]	0.004	0.008	0.012	0.016	0.02	0.024	0.032	0.04
8% Acrylamide								
Water	2.3	4.6	6.9	9.3	11.5	13.9	18.5	23.2
Acrylamide solution[1]	1.3	2.7	4.0	5.3	6.7	8.0	10.7	13.3
1.5 M Tris (pH 8.8)[2]	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
100 g/L SDS[3]	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
100 g/L APS[4]	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED[5]	0.003	0.006	0.009	0.012	0.015	0.018	0.024	0.03
10% Acrylamide								
Water	1.9	4.0	5.9	7.9	9.9	11.9	15.9	19.8
Acrylamide solution[1]	1.7	3.3	5.0	6.7	8.3	10.0	13.3	16.7
1.5 M Tris (pH 8.8)[2]	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
100 g/L SDS[3]	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
100 g/L APS[4]	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED[5]	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02
12% Acrylamide								
Water	1.6	3.3	4.9	6.6	8.2	9.9	13.2	16.5
Acrylamide solution[1]	2.0	4.0	6.0	8.0	10.0	12.0	16.0	20.0
1.5 M Tris (pH 8.8)[2]	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
100 g/L SDS[3]	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
100 g/L APS[4]	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED[5]	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02
14% Acrylamide								
Water	1.4	2.7	3.9	5.3	6.6	8.0	10.6	13.8
Acrylamide solution[1]	2.3	4.6	7.0	9.3	11.6	13.9	18.6	23.2
1.5 M Tris (pH 8.8)[2]	1.2	2.5	3.6	5.0	6.3	7.5	10.0	12.5
100 g/L SDS[3]	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
100 g/L APS[4]	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED[5]	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02
15% Acrylamide								
Water	1.1	2.3	3.4	4.6	5.7	6.9	9.2	11.5
Acrylamide solution[1]	2.5	5.0	7.5	10.0	12.5	15.0	20.0	25.0
1.5 M Tris (pH 8.8)[2]	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
100 g/L SDS[3]	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
100 g/L APS[4]	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED[5]	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02

(1) Acrylamide solution: 30% acrylamide/bisacrylamide (29:1) solution.
(2) 1.5 M Tris (pH 8.8): 1.5 M tris-hydrochloride buffer solution (pH 8.8).
(3) 100 g/L SDS: a 100 g/L solution of sodium dodecyl sulfate.
(4) 100 g/L APS: a 100 g/L solution of ammonium persulfate. Ammonium persulfate provides the free radicals that drive polymerization of acrylamide and bisacrylamide. Since ammonium persulfate solution decomposes rapidly, fresh solutions must be prepared daily.
(5) TEMED: *N*,*N*,*N*′,*N*′-tetramethylethylenediamine.

of the protein standards as a function of the *R*f values. Unknown molecular masses can be estimated by linear regression analysis (more accurately by non-linear regression analysis) or interpolation from the curves of log M_r against *R*f if the values obtained for the unknown samples are positioned along the approximately linear part of the graph.

4.9. Validation of the test

The test is not valid unless the target resolution range of the gel has been demonstrated by the distribution of appropriate molecular mass markers e.g. across 80% of the length of the gel. The separation obtained for the expected proteins must show a linear relationship between the logarithm of the molecular mass and the *R*f. If the plot has a sigmoidal shape then only data from the linear region of the curve can be used in the calculations. Additional validation requirements with respect to the test sample may be specified in individual monographs.

Sensitivity must also be validated. A reference protein control corresponding to the desired concentration limit that is run in parallel with the test samples can serve as a system

Table 2 Preparation of stacking gel

Solution components	Component volumes (mL) per gel mould volume of							
	1 mL	2 mL	3 mL	4 mL	5 mL	6 mL	8 mL	10 mL
Water	0.68	1.4	2.1	2.7	3.4	4.1	5.5	6.8
Acrylamide solution(1)	0.17	0.33	0.5	0.67	0.83	1.0	1.3	1.7
1.0 M Tris (pH 6.8)(2)	0.13	0.25	0.38	0.5	0.63	0.75	1.0	1.25
100 g/L SDS(3)	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
100 g/L APS(4)	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
TEMED(5)	0.001	0.002	0.003	0.004	0.005	0.006	0.008	0.01

(1) Acrylamide solution: 30% acrylamide/bisacrylamide (29:1) solution.
(2) 1.0 M Tris (pH 6.8): 1 M tris-hydrochloride buffer solution (pH 6.8).
(3) 100 g/L SDS: a 100 g/L solution of sodium dodecyl sulfate.
(4) 100 g/L APS: a 100 g/L solution of ammonium persulfate. Ammonium persulfate provides the free radicals that drive polymerization of acrylamide and bisacrylamide. Since ammonium persulfate solution decomposes rapidly, fresh solutions must be prepared daily.
(5) TEMED: *N*,*N*,*N*′,*N*′-tetramethylethylenediamine.

suitability of the experiment.

4.10. Quantification of impurities

SDS-PAGE is often used as a limit test for impurities. When impurities are quantified by normalization to the main band using an integrating densitometer or image analysis, the responses must be validated for linearity. Note that depending on the detection method and protein as described in the introduction of the section ''Detection of proteins in gels'' the linear range can vary but can be assessed within each run by using one or more control samples containing an appropriate range of protein concentration.

Where the impurity limit is specified in the individual monograph, a reference solution corresponding to that level of impurity should be prepared by diluting the test solution. For example, where the limit is 5%, a reference solution would be a 1:20 dilution of the test solution. No impurity (any band other than the main band) in the electropherogram obtained with the test solution may be more intense than the main band obtained with the reference solution.

Under validated conditions impurities may be quantified by normalization to the main band using an integrating densitometer or by image analysis.

5. Reagents

(i) 30% acrylamide/bisacrylamide (29:1) solution: Prepare a solution containing 290 g of acrylamide and 10 g of methylenebisacrylamide per litre of water. Filter.

(ii) SDS-PAGE running buffer: Dissolve 151.4 g of 2-amino-2-hydroxymethyl-1,3-propanediol, 721.0 g of glycine and 50.0 g of sodium dodecyl sulfate in water and dilute to 5000 mL with the same solvent. Immediately before use, dilute to 10 times its volume with water and mix. Measure the pH of the diluted solution. The pH is between 8.1 and 8.8.

(iii) SDS-PAGE sample buffer (concentrated): Dissolve 1.89 g of 2-amino-2-hydroxymethyl-1,3-propanediol, 5.0 g of sodium dodecyl sulfate and 50 mg of bromophenol blue in water. Add 25.0 mL of glycerol and dilute to 100 mL with water. Adjust the pH to 6.8 with hydrochloric acid, and dilute to 125 mL with water.

(iv) SDS-PAGE sample buffer for reducing conditions (concentrated): Dissolve 3.78 g of 2-amino-2-hydroxymethyl-1,3-propanediol, 10.0 g of sodium dodecyl sulfate and 100 mg of bromophenol blue in water. Add 50.0 mL of glycerol and dilute to 200 mL with water. Add 25.0 mL of 2-mercaptoethanol. Adjust to pH 6.8 with hydrochloric acid, and dilute to 250.0 mL with water. Alternatively, dithiothreitol may be used as reducing agent instead of 2-mercaptoethanol. In this case prepare the sample buffer as follows: dissolve 3.78 g of 2-amino-2-hydroxymethyl-1,3-propanediol, 10.0 g of sodium dodecyl sulfate and 100 mg of bromophenol blue in water. Add 50.0 mL of glycerol and dilute to 200 mL with water. Adjust to pH 6.8 with hydrochloric acid, and dilute to 250.0 mL with water. Immediately before use, add dithiothreitol to a final concentration of 100 mM.

(v) Coomassie staining solution: A 1.25 g/L solution of acid blue 83 in a mixture consisting of 1 volume of glacial acetic acid, 4 volumes of methanol and 5 volumes of water. Filter.

(vi) Developer solution: Dilute 2.5 mL of a 20 g/L solution of citric acid and 0.27 mL of formaldehyde to 500.0 mL with water.

(vii) Fixing solution: To 250 mL of methanol, add 0.27 mL of formaldehyde and dilute to 500.0 mL with water.

(viii) Silver nitrate reagent: To a mixture of 3 mL of concentrated ammonia and 40 mL of 1 M sodium hydroxide, add 8 mL of a 200 g/L solution of silver nitrate, dropwise, with stirring. Dilute to 200 mL with water.

(ix) Destaining solution: A mixture consisting of 1 volume of glacial acetic acid, 4 volumes of methanol and 5 volumes of water.

(x) 1.5 M tris-hydrochloride buffer solution (pH 8.8): Dissolve 90.8 g of 2-amino-2-hydroxymethyl-1,3-propanediol in 400 mL of water. Adjust the pH with hydrochloric acid and dilute to 500.0 mL with water.

(xi) Blocking solution: A 10 vol% solution of acetic acid.

Host Cell Protein Assay ⟨*G3-9-172*⟩

Host cell protein (HCP) is a general term for proteins derived from host cells used for the production of pharmaceutical products. This general information describes HCP assays for therapeutic proteins produced by recombinant DNA technology (recombinant therapeutic proteins).

Residual HCP in recombinant therapeutic proteins has potential to elicit immune responses against itself and may also act as adjuvants to induce anti-drug antibodies. Therefore, in order to ensure the efficacy and safety of recombinant therapeutic proteins, it is necessary to establish a purification process to reduce HCP to a level that does not affect safety. In addition, residual levels of HCP must be ap-

propriately controlled by verifying that in-process tests can consistently eliminate HCP or by establishing purity testing of drug substance.

1. Selection of Test Methods for HCP assay

HCP assay is usually performed with a sandwich immunoassay using antibodies against HCP (anti-HCP antibodies) and detection systems including enzyme-linked immunosorbent assay (ELISA), electrochemiluminescent immunoassay (ECLIA), and time-resolved fluorescent immunoassay (TRFIA). This general information addresses the sandwich immunoassay, but does not discourage other assays.

Residual HCP from the manufacturing process of recombinant therapeutic proteins consists a large number of proteins and may have profiles that vary from one host cell to another or depending on manufacturing conditions. Based on differences in its intended use or differences in the concept of preparation of anti-HCP antibodies used for testing, HCP assay is classified into generic assay, product-specific assay, and platform assay. Generic assay is intended for wide use in pharmaceutical products manufactured using similar host cells (e.g., CHO-K1 or CHO-DG44 cells derived from CHO [Chinese hamster ovary] cells) and is a test method established using anti-HCP antibodies that are prepared with proteins from all components of the host cells (cell lysate or culture supernatant) as immunogens. Commercially available reagents or kits for an HCP assay commonly referred to as generic assay and need to be validated before use. Product-specific assay is intended to control HCP in a specific product and developed in consideration of characteristics of the manufacturing process of the product. Platform assay is developed for the application to recombinant therapeutic proteins (e.g., monoclonal antibodies with adequate experience) produced by a manufacturing platform.

Generic assay is intended to comprehensively obtain antibodies against a wide range of HCP by using proteins from all components of the host cells as antigens. However, it should be noted that it is difficult to obtain antibodies against all HCPs because of differences in proportions or immunogenicity of individual proteins used as antigens and that residual HCP from actual manufacturing processes may be inadequately covered because of potential different profiles of residual HCP from different manufacturing processes. In contrast, product-specific assay is expected to prepare anti-HCP antibodies that can detect residual HCP from actual manufacturing processes compared with generic assay because it uses potentially residual HCP as antigens. However, it should be noted that profiles of residual HCP may vary with modifications to manufacturing processes. Platform assay has both aspects, namely, the assay has an advantage of being able to be applied to various products prepared by a manufacturing platform; however, the assay may involve issues as generic or product-specific assay, depending on the method of preparation of antigens used for preparing anti-HCP antibodies.

In some products, it is possible that specific HCPs binding to desired products are present or that HCPs markedly increase in production amount with expression of desired products. If the residue of these HCPs is found, the need to establish other test methods for the HCPs is considered.

In light of these characteristics of the test methods for HCP assays and in consideration of properties of host cells, characteristics of manufacturing processes, knowledge about immunogenicity of HCPs, stages of development of products, etc., an appropriate test method is selected.

2. Preparation and Characterization of Reagents

2.1. HCP Antigens/HCP Reference Materials

For antigens to produce antibodies that specifically detect HCPs in products, it is necessary to prepare HCP not containing desired products. Usually, null cells are used for preparation of HCP antigen while keeping in mind that HCP appropriate to the purpose of the HCP assay are comprehensively contained. In addition, HCP are used not only as antigens but also as reference materials for HCP assay and may also be used as ligands for purification of anti-HCP antibodies by affinity chromatography.

2.1.1. Preparation of HCP Antigens/HCP Reference Materials

The method of preparation of HCP antigens/HCP reference materials varies widely depending on the type of test methods. The method of preparation of HCP used as antigens for preparation of anti-HCP antibodies or as HCP reference materials in the test methods is shown below along with points to note.

HCP used for generic assays are prepared from culture supernatant or lysed or disrupted null cells using minimal procedures such as concentration and dialysis and keeping preservation of component proteins in mind. It should be noted that these HCPs show profiles that are different from those of residual HCP in products because of preparation under conditions different from culture processes at commercial scale.

HCP used for product-specific assays are prepared from null cells using manufacturing processes of products. Usually, the application of the purification process is minimized to obtain a wide range of HCP. However, if a suitable antibody to detect the HCP is not obtained, it may be necessary to prepare appropriate HCP antigens by exploring conditions of preparation of HCP or excluding specific HCP.

HCP used for the platform assays are prepared from null cells using a manufacturing platform that can be used in multiple products. Usually, as with the other test methods, the application of the purification process is minimized to obtain a wide range and an enough amount of HCP. In addition, a mixture of HCPs prepared under multiple conditions can be used to address differences in HCP spectra due to slight differences in manufacturing conditions.

The use of mock cells as null cells used for preparation of HCP for product-specific and platform assays has advantages such as the presence of proteins expressed as selection markers in antigens and ability to culture the cells under similar culture conditions in actual manufacturing processes. In contrast, it should be noted that even a same cell line does not show consistent properties (such as cell growth rate) among different clones and that differences such as the presence or absence of production of desired products may cause different profiles of HCP.

2.1.2. Characterization of HCP Antigens/HCP Reference Materials

Prepared HCPs are analyzed for the following items.

1) Protein Concentrations

Protein concentrations are determined by a suitable measurement method keeping in mind that host cell nucleic acids or culture medium components may be contained depending on the method of preparation of HCP. For information on detailed measurement methods and points to consider, "Total Protein Assay *<G3-12-172>*" in General Information would be helpful.

2) HCP Profiles

Usually, one-dimensional electrophoresis (SDS-PAGE) or two-dimensional electrophoresis is used to confirm that prepared HCP include HCP species that are considered to

remain in manufacturing processes or drug substances. Identification of HCP species by mass spectrometry is also a helpful approach.

2.2. Anti-HCP Antibodies

2.2.1. Preparation of Anti-HCP Antibodies

Since HCPs represent a heterogeneous variety of different proteins, polyclonal antibodies are obtained as anti-HCP antibodies used for the assay to comprehensively detect HCPs. The rabbit, goat, and sheep are commonly used animal species for immunization. For immunization, it is useful to enhance immune response with adjuvants. Due to different degrees of immunogenicity of individual proteins comprising HCPs, the timing of induction of antibodies or the amount of antibodies produced is not constant, regardless of the amount of proteins as antigens. In addition, inter-individual variability in animals used for immunization makes the profile of induced antibodies inconsistent. Several rounds of immunization are usually required and, after determining the reactivity of induced antibodies with HCPs by Western blotting using antisera in each period of immunization, whole serum is collected. Mixing of anti-HCP antibodies derived from multiple individuals is intended to obtain adequate amounts of antibodies and is also expected to contribute to the elimination of imbalance of HCP profiles.

Anti-HCP antibodies are purified from the obtained antiserum by Protein A or Protein G chromatography. In either case, aggregates may be formed from some antibodies due to use of acid conditions for antibody elution from the columns. It is useful to remove antibody aggregates by a suitable method because they may cause interference with measurement.

Anti-HCP antibodies can also be purified by affinity chromatography using HCP as ligands. This purification is expected to eliminate non-specific reactions because of concentration of antibodies specific to HCPs, however, it should be noted that anti-HCP antibodies may get less diverse due to less adsorption of low-affinity antibodies or less elution of very high-affinity antibodies.

2.2.2. Suitability of Anti-HCP Antibodies

Anti-HCP antibodies have to be able to comprehensively recognize HCPs with wide ranges of electric charges and molecular masses that potentially remain in manufacturing processes or drug substances. However, because differences in immunogenicity of each HCP species may make antibodies against some HCPs less likely to be induced, obtained anti-HCP antibodies have to be qualified, usually as measured by antigen coverage. A specific example of assessment methods is shown below. After separation of HCPs by two-dimensional electrophoresis, total protein on the gel is stained. After performing two-dimensional electrophoresis in the same manner, Western blotting using anti-HCP antibodies is performed. Spot patterns obtained from each staining are compared and the proportion of spots detected by Western blotting vs spots obtained in total protein staining is determined as antigen coverage.

2.3. Storage of Reagents

HCP reference materials and anti-HCP antibodies are stored with attention to stability. The stability of these reagents can be confirmed by continuously monitoring parameters of dose-response curves of reference materials.

3. Validation of HCP Assay

When a sandwich immunoassay is used for HCP assay, ''Validation of Analytical Procedures *<G1-1-130>*'' in General Information would be helpful for information on basic requirements for validation. However, unlike a conventional sandwich immunoassay to determine a single antigen, HCP assay is an approach to use antibodies prepared with a mixture of various HCP species as antigens to determine the HCP species simultaneously. Therefore, changes in concentrations as a function of dilution ratios of samples (dilution linearity) may not be observed in highly purified samples even within the quantitation limits in which linearity has been obtained for HCP reference materials. This phenomenon is likely attributed to insufficient amounts of antibodies to some HCPs that are found in increased proportions in samples for measurement due to the difference of removal rates of individual HCPs in the purification process and may lead to underestimation of HCP concentrations.

Therefore, HCP assay should be validated for accuracy, precision, specificity, standard curve, quantitative range, and dilution linearity.

(1) Accuracy and Precision

Accuracy and precision are expressed as coefficients of variation of recovery rates and quantitative values of HCP reference materials, respectively, by performing spike and recovery tests of HCP reference materials in purification process pools or drug substances to be measured.

(2) Specificity

Because HCP assay involves measurement of trace amounts of HCPs remaining in samples containing large quantities of desired products, it should be confirmed that there is no interference of desired substances or ingredients in sample solutions.

(3) Standard Curve and Quantitative Range

A standard curve is generated using serially diluted HCP reference materials to obtain a regression expression, and validity is expressed in terms of determination coefficient, etc. Quantitative values of reference materials at each concentration level are determined from the regression expression and a range of concentrations with acceptable levels of accuracy and precision is defined as quantitative range with the lowest concentration within the range being defined as the minimum limit of quantitation.

(4) Dilution Linearity

Purification process pools or drug substances to be measured are examined for the range of dilution ratios of samples in which quantitative values of samples diluted within the quantitative range of a standard curve are linear.

4. Establishment of HCP Assay

HCP assay is used for confirmation of the status of removal of HCP in manufacturing processes or as a purity test of drug substances. For information on basic concepts of procedures or data analysis in HCP assay, ''Enzyme-linked Immunosorbent Assay *<G3-11-171>*'' in General Information would be helpful.

Results from HCP assay are usually presented as contents in total protein or desired products. Contents of HCP per total protein or desired products can be determined by separately determining concentrations of the proteins in samples. For example, when total protein concentration is 2 mg/mL and an HCP concentration is 20 ng/mL, the content of HCP should be indicated as 10 ng/mg.

5. Others

5.1. Considerations for Modifications to Manufacturing Processes

Since any modifications made to manufacturing processes for recombinant therapeutic proteins may affect profiles of residual HCP, it is necessary to confirm that appropriate measurement of HCP is performed after modifications to manufacturing processes. If HCP profiles are altered and it is considered inappropriate to apply a test method for HCP

assay before modifications to manufacturing processes, a test method for HCP assay has to be established again. Changes in HCP profiles can be assessed by an analytical procedure such as two-dimensional electrophoresis or mass spectrometry.

5.2. Considerations for Modifications to Reagents for Tests

It is desirable to secure sufficient quantities of HCP antigens/HCP reference materials and anti-HCP antibodies, all important reagents, whenever possible, in consideration of the life cycle of products. When HCP antigens/HCP reference materials or anti-HCP antibodies are newly prepared, it should be confirmed that their characteristics are comparable before and after renewal by analytical procedures such as two-dimensional electrophoresis, Western blotting, and mass spectrometry. In addition, test methods should be validated again for necessary items and newly prepared reagents should be used after confirming their consistency with the reagents and test methods before renewal.

When generic assay is employed, qualified commercially available kits can also be used. However, availability of information on lot renewal of reagents, etc. is necessary to ensure consistency and quality of tests using commercially available kits, and therefore characterization of important reagents and method validation should be performed as needed.

6. Terms

Mock cell: A cell line established from the host cell line by transferring expression vectors that do not contain genes encoding desired products.

Null cell: A host cell that does not express desired products. It includes parent cells or mock cells.

Antigen coverage: The rate of detection of proteins comprising HCPs by anti-HCP antibodies. For example, an antigen coverage can be calculated from the number of spots obtained in total protein staining and the number of spots obtained in Western blotting using anti-HCP antibodies after separation of HCPs by two-dimensional electrophoresis.

Surface Plasmon Resonance
<G3-10-170>

Surface plasmon resonance (SPR) optical detection is a method for detecting changes in mass on a sensor chip as changes in the angle at which the reflected light disappears by SPR. This method is used to analyze the binding specificity and binding affinity between substances, and to measure the concentration of analytes in samples.

The devices designed to measure interactions between substances by means of surface plasmon resonance usually adopt a prism-based Kretschmann configuration (Fig. 1). If polarized light is applied in a manner allowing total reflection on the metallic film surface of the sensor chip, an SPR signal (a reduction in intensity in a portion of the reflected light) is observed. The angle at which the SPR signal is produced varies depending on the mass placed on the sensor chip. Thus, the angle at which the SPR signal is produced is changed by binding or dissociation between the molecule immobilized on the sensor chip (the ligand) and the molecule added (the analyte) (Fig. 1). The results of measurement are in the form of a sensorgram presenting changes over time in the SPR signal-producing angle or the response unit (RU) converted from changes in the angle. If the thus-obtained binding and dissociation sensorgram is fitted to the theoretical curve, the ligand/analyte binding rate constant (k_a), dissociation rate constant (k_d) and dissociation constant ($K_D = k_d/k_a$) can be determined. If the response of the analyte in a given sample is compared to the response of the analyte of known concentration, the analyte concentration in a given sample can be determined.

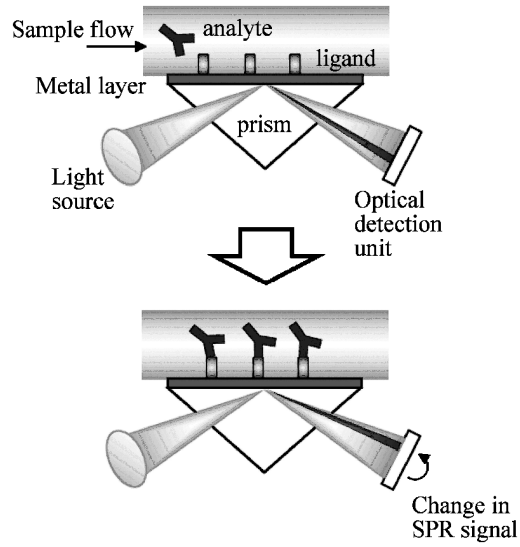


Fig. 1 Principle of SPR measurement (Kretschmann configuration)

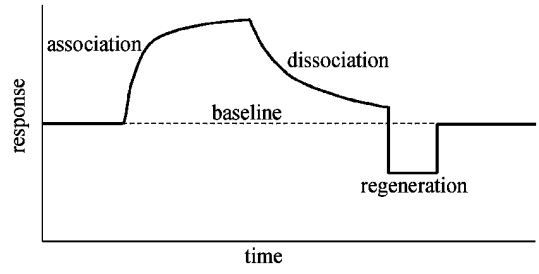


Fig. 2 Example of an SPR sensorgram

1. Instrument

The instrument usually used for SPR optical detection (a continuous flow system) consists of a light source, an optical detector, a fluid delivery system, a sensor chip insertion port and a data accumulating unit. A sensor chip conjugated with carboxymethyl dextran is usually employed. An appropriate sensor chip tailored to the characteristics of the molecule to be immobilized should be selected. If the sensor chip is set on the device, multiple flow cells are formed on the sensor chip surface, allowing the ligand to be immobilized on each flow cell.

2. Measurement

SPR optical detection is used for testing the binding specificity between the ligand and the analyte, analyzing the binding affinity between the ligand and the analyte, or measuring the analyte concentration. Usually, SPR signals are observed over time while applying the buffer solution to the flow cell and injecting the analyte, and a sensorgram illustrating the binding of the analyte to the ligand immobilized on the sensor chip is obtained. In kinetic analysis designed to analyze the binding affinity from the shape of the sensorgram, a running buffer free of the analyte is applied after the end of analyte injection in order to obtain a dissociation sensorgram. After measurement, a regeneration buffer is applied so that the analyte bound to the ligand can be removed

completely (Fig. 2).

2.1. Sample and buffer solution

(1) Analyte solution

Depending on the objective of analysis and the affinity between the molecules to be measured, the sample is diluted to an optimal concentration with the running buffer to yield an analyte solution. If the sample contains insoluble contaminants, they need to be removed by an appropriate method such as centrifugation or filtration using a low protein adsorptive filter.

(2) Running buffer

A buffer appropriate for the ligand and analyte is selected. Addition of salts or detergents to the buffer can be useful in stabilizing the ligand and the analyte. If appropriate, the buffer is filtrated and degassed prior to use. If non-specific binding to the reference flow cell is observed at the time of analyte injection, the buffer needs to be optimized by changing its pH, ionic strength, or other conditions.

(3) Regeneration buffer

As regeneration solution, a buffer with low pH, high pH, or high ionic strength, or a buffer containing surfactants, nonpolar reagents, or chelating agents can be used. The type of buffer to be used varies depending on the material of the flow path of the device. Thus, it is necessary to check the chemical resistance of the device. The ideal setting for regeneration is the one under which complete dissociation of the bound analyte can be achieved without altering the nature of the ligand on the sensor chip surface. If the regeneration buffer is suitable, the baseline after regeneration returns to the baseline recorded before addition of the analyte and it is possible to avoid a reduction in the binding response during repeated measurement. If the setting for regeneration is inappropriate, the level of binding to the ligand decreases during the cycles of measurement, thus affecting the reproducibility of measurement. If the dissociation rate is sufficiently high, the flow of the buffer allows dissociation of the analyte from the ligand, thus making it unnecessary to apply the buffer for regeneration.

2.2. Preparation of the sensor chip used for the measurement

Two methods of binding the ligand to the sensor chip are available: a direct method (direct immobilization of the ligand), and a capture method (capture immobilization of the ligand). In both methods, it is essential to immobilize the ligand while retaining its biological activity and minimizing the impact on binding to the analyte. A ligand of high purity level must be used for the immobilization.

The amount of the ligand to be immobilized is determined with reference to the equation given below.

$$\text{Ligand immobilization quantity} = \frac{\text{Required } R_{\max}}{\text{Valency of the ligand}} \times \frac{\text{molecular weight of the ligand}}{\text{molecular weight of the analyte}}$$

The $R_{\max}$ (response in the case of maximal binding of the analyte to the ligand) needed for measurement is determined depending on the sensitivity of the instrument used. For analysis of binding affinity, $R_{\max}$ needs to be low to avoid steric effects, aggregation and mass transport limitation (a condition under which the amount of analyte supplied serves as a rate-limiting factor for changes in the binding amount due to a shortage in the amount of analyte against the excess ligand). For measurement of the analyte concentration, high $R_{\max}$ is desirable to induce a mass transport limitation which increases the dependency of the analyte-binding amount on the concentration and improves the linearity of the calibration curve.

Usually, a control flow cell free of bound ligand is prepared on the sensor chip and is used to detect nonspecific bindings. The following can serve as the control flow cell: (1) an untreated flow cell, (2) a flow cell having undergone a chemical treatment identical to that for ligand immobilization, and (3) a flow cell having undergone immobilization of a ligand-like molecule having no potential for binding to the analyte. In the case that the ligand is immobilized by the capture method, a flow cell having undergone immobilization of the capturing molecule serves as the control flow cell.

If the immobilized ligand is stable, it is possible to store the sensor chip apart from the device. Such storage is used under conditions such as dry environments or immersion in a buffer solution at low temperature.

Ligand immobilization methods

(1) Direct method

The ligand is immobilized directly via the amino group, thiol group, carboxyl group, aldehyde group or hydroxyl group of the ligand, or via the hydrophobicity of the ligand. The sensor chip usually possesses a layer containing carboxyl groups, which can be used for immobilization. Thus, the ligand is immobilized by the covalent bond. In the case of direct immobilization, the surface often becomes inhomogeneous due to the lack of a uniform direction in the ligand.

(2) Capture method

A capturing molecule having the potential to bind to the ligand is immobilized on the sensor chip so that the ligand can be captured on the chip by binding to the capturing molecule. Capturing molecules include the antibody to the ligand, and antibody to the specific tag sequence allocated to the ligand. If the ligand is an antibody drug, protein A and protein G serve as capturing molecules. If the ligand is a biotinylated molecule, streptavidin is used as a capturing molecule. By the capture method, the direction in the ligand is likely to become uniform. It is important that dissociation of the ligand from the capturing molecule does not occur during measurement. If ligand capturing is performed at each cycle of measurement, there is no need to determine the conditions of regeneration for individual ligands, thus making it easy to set the conditions for measurement.

2.3. Setting the conditions for measurement

(1) Checking the baseline

Before starting the measurement, the stability of the baseline needs to be confirmed. If the baseline is not stable, stabilization should be attempted by the following procedures: administration of several infusions of buffer solution, high ion intensity solution or surfactant solution, application of buffer at a high flow rate, and repetition of the sequence of analyte binding and regeneration.

(2) Flow rate

For analysis of the binding affinity, it is necessary to set the flow rate high in order to suppress the mass transport limitation. For measurement of the analyte concentration, the flow rate must be set low to facilitate the mass transport limitation.

(3) Duration of the analysis

The time needed for analysis in each step (binding, dissociation, etc.) varies depending on the type of measurement. When specific binding is to be tested, the time for binding is set as the time allowing sufficient observation of changes in response. In the case of binding affinity analysis by means of kinetic analysis, a sufficient amount of time should be allowed for dissociation if the response involves slow dissociation. In the case of affinity analysis by means of steady state analysis, the time sufficient for the binding level to reach the equilibrium must be set. In the case of concentration meas-

urement, the time will suffice if it covers the points of measurement capable of yielding an appropriate calibration curve.

(4) Checking R_{max}

If the R_{max} measured exceeds the theoretical R_{max} calculated from the molecular weight of the ligand and analyte together with the ligand immobilization quantity and the ligand's binding valency, the following reasons can be considered: inappropriate binding valency, analyte aggregation, or non-specific binding. In such cases, the conditions for measurement or analysis need to be modified.

(5) Checking the reproducibility of measurement

The reproducibility of measurement may be affected if the conditions for measurement are not optimal, and if the ligand is inactivated during repetition of the measuring cycle. Furthermore, if a sensor chip that has been kept stored is used, the reproducibility may be affected by the storage. When the conditions for measurement are set, close attention needs to be paid to reproducibility. The acceptable repeated number of measurements and the acceptable storage period should be set in advance.

2.4. Methods of measurement

2.4.1. Analysis of binding specificity

The analyte is added, and its binding to the ligand is tested on the basis of the binding responses. An appropriate control experiment should be carried out (e.g., demonstrating lack of binding of other analytes to the immobilized ligand), to confirm that the binding observed in the measurement was specific to the analyte.

2.4.2. Analysis of binding affinity

(1) Kinetic analysis

The analyte is injected and its binding is measured. Then, fluid free of the analyte is applied and dissociation of the analyte is measured. Thereafter, complete dissociation of the analyte is achieved by the regeneration step, followed by measurement of the next analyte solution. Another method is analysis of the binding affinity through successive application of analyte solutions at varying concentration levels without interposing a regeneration step. Usually, measurement is performed using the analyte at 5 or more concentrations (between 1/10 of K_D and $10 \times K_D$).

(2) Steady state analysis

If binding and dissociation take place rapidly, making kinetic analysis or model fitting difficult, steady state analysis is performed. The analyte injection is continued for a period of time until the analyte binding reaches equilibrium. The responses upon reaching equilibrium are recorded. Dissociation of the bound analyte is achieved by regeneration and the next analyte solution is measured. With this method, K_D is calculated as an analyte concentration which yields $1/2\ R_{max}$. Thus, the analyte concentration needs to be set so that binding to the ligand at the highest analyte concentration is close to saturation.

2.4.3. Measurement of concentration

If measurement is done under the conditions facilitating mass transport limitation, the linearity of the calibration curve is improved, allowing increased accuracy of measurement over a wide range. For this reason, the analyte is injected into the flow cell immobilized with a large amount of ligand, and binding is measured under this setting. Then, dissociation of the analyte is achieved by regeneration, and the next analyte solution is measured. A calibration curve is prepared from the results of measurement of the analyte at known concentration levels. Then, the analyte concentration is calculated. Another available method attempts to calculate the analyte concentration by making use of the proportional relationship between the analyte concentration and diffusion rate, without using a calibration curve.

3. Data analysis

When analysis is performed, the unnecessary part of the sensorgram (e.g., corresponding to capture of the ligand by the capturing molecule, and the regeneration step) is removed, and the response of the control flow cells is subtracted from the response of the ligand-bound flow cells. In addition, the sensorgram baseline is adjusted to 0. As needed, the sensorgram yielded by injection of the buffer for measurement alone is subtracted from the sensorgram yielded by analyte injection.

3.1. Analysis of binding affinity

(1) Kinetic analysis

Kinetic analysis is intended to calculate the parameters for the approximate formula (k_a, k_d, K_D, R_{max}, etc.) from the sensorgram with the use of the reaction rate equation derived from the ligand/analyte binding model. If the ligand binds to the analyte at a ratio of 1:1, the reaction rate equation for association phase is as follows:

$$dR/dt = k_a \times C \times (R_{max} - R) - k_d \times R$$

The reaction rate equation for the dissociation phase is as follows:

$$dR/dt = -k_d \times R$$

(C: analyte concentration; R: response).

A reaction rate equation involving a term corresponding to mass transport limitation or fluid effect can also be used.

The dissociation constant (K_D) serving as an indicator of binding affinity is defined as follows.

$$K_D = k_d/k_a$$

The reaction models employed for analysis of binding affinity include: ① a model of 1:1 ligand/analyte binding, ② a model of 2:1 ligand/analyte binding, like antigen/antibody binding, ③ a model of competitive binding of two analytes to the ligand, ④ a model of one analyte to the ligand possessing two binding sites of different affinity levels, and ⑤ a model of conformational change after 1:1 complex formation. A model theoretically considered as appropriate should be selected, with the results of other biochemical experiments being taken into account.

After the kinetic analysis, an evaluation is needed to determine the appropriateness of the fitting performed. This is accomplished by evaluating the residual plot between the sensorgram obtained and the theoretical curve, or statistical parameters such as χ^2 (mean squared residual, demonstrating the difference between the measured data and the calculated theoretical curve).

Poor fitting to the theoretical curve may be attributable to the following factors: (1) low purity of the reagent, (2) inappropriate method or density of immobilization, (3) inappropriate analyte concentration, (4) nonspecific binding, (5) reduced ligand activity, and (6) inappropriate selection of a reaction model. Thus, the conditions for measurement and the reaction model need to be reviewed. If the RI (refractive index) calculated as a response of the buffer components in the sample is excessively high during analysis of the data on reactions involving rapid binding/dissociation, fitting is performed by fixing the RI to 0. In the case of a poor fitting, the fit might be improved by setting the initial values close to the anticipated values of k_a and k_d.

(2) Steady state analysis

Steady state analysis is as follows. The response reaching equilibrium at each analyte concentration (Y axis) is plotted against the analyte concentration (X axis). Then, regression

is performed using the following equation:

Steady state response equation:

$$\text{Equilibrium level at analyte concentration} = \text{analyte concentration} \times \frac{R_{max}}{\text{analyte concentration} + K_D}$$

In this way, the K_D shown by the response of 1/2 R_{max} is determined. The K_D calculated with this equation is the value when 1:1 ligand/analyte binding is assumed. If the actually measured response converges at R_{max}, good analysis is possible. However, if it is in a range lower than R_{max}, the analytical data are less reliable and it is desirable to repeat the measurement by expanding the range of measured concentrations to cover the higher concentration levels.

3.2. Measurement of concentration

From the sensorgram derived by injecting an analyte of known concentration, the slope of the sensorgram near the start of injection or the response at a certain time after starting the injection is determined and plotted against the analyte concentration. A calibration curve is prepared with an appropriate formula for approximation (e.g., the formula for 4-parameter logistic regression, linear regression). Then, the slope or the response is determined from measurement of the sample as an analyte, followed by calculation of the sample concentration from the calibration curve.

4. Application to various tests

4.1. Example of application to identification test

This test is aimed at confirming binding of the sample to the ligand by testing the specific binding as described in 2.4.1. To evaluate the system performance, measurement is performed on the reference material and a negative control (a substance distinguishable from the ligand in terms of the ligand-binding activity) to confirm the specificity of the binding.

4.2. Example of application to binding affinity test

This test is aimed at determining the K_D of the reference material and the sample, making use of the binding affinity analysis described in section 2.4.2. The criterion value related to the binding affinity may be set as the K_D or relative K_D (sample K_D/reference material K_D).

Regarding system suitability, the system performance and system repeatability are set. For example, concerning the system performance, it is confirmed that the ligand immobilization quantity is within the predetermined range; that the calculated K_D of known ligand-binding affinity samples is consistent with the order of affinity levels; and that χ^2 is not more than the predetermined level. System repeatability is confirmed by checking that the relative standard deviation for K_D during repeated measurement is not more than the predetermined level.

4.3. Example of application to measurement of specific activity based on the binding quantity to the target molecule

When specific activity is calculated with the quantity of binding to the target molecule, the measurement is performed using the concentration measuring method described in 2.4.3. On the basis of the calibration curve prepared from the reference material, the relative potency to the reference material is calculated using the data on the response of the sample solution, and then the potency is divided by the protein concentration to yield the specific activity.

Regarding system suitability, the system performance and system repeatability are confirmed. For example, concerning system performance, it is confirmed that the ligand immobilization quantity is within the predetermined range; and that the correlation coefficient or determination coefficient of the calibration curve is not less than the predetermined level. System repeatability is confirmed by checking that the relative standard deviation of the response during repeated measurement is not more than the predetermined level.

Enzyme-linked Immunosorbent Assay (ELISA) <*G3-11-171*>

ELISA (Enzyme-linked immunosorbent assay) is one of the immunological assay methods to detect analytes by antigen-antibody reaction, in which an enzyme-labeled reagent is used as a detection reagent. In general, 96-well-plates and such are used, on which capture molecules specifically bound to the analyte are immobilized. A test sample, an enzyme-labeled reagent and other required reagents are sequentially added and washed to have the enzyme-labeled reagent bind onto a plate. After reaction by adding a substrate for the labeled enzyme, the response (e.g., absorbance) by the enzymatic reaction is measured to determine the concentration or binding activity of the analyte in the test sample. ELISA is also used as a qualitative test to detect the presence or absence of binding of the analyte with specific molecules.

In tests for biotechnological/biological products, ELISA is mainly used for two different purposes. One is to quantitate the target product or process-related impurities usually by measuring the concentration of the analyte with antibodies which specifically bind to the analyte. Another is to evaluate the biological activity of products such as therapeutic antibodies. For the latter purpose, ELISA is used to evaluate the binding activity of the target product with molecules related to its pharmacological action, or to evaluate the cell response based on the amount of the endogenous protein secreted from the cells treated with the test samples containing the target product.

1. Analytical methods

ELISA is broadly classified into competitive and noncompetitive methods, and also classified into direct and indirect detection methods based on the detection procedures (Fig. 1). In addition, ELISA is also classified into direct and indirect immobilization methods by the method for immobilizing capture molecules (Fig. 2).

An analyte bound to a solid phase is detected by the antibody against the analyte or other reagents (Fig. 1). In the direct detection method, an enzyme-labelled antibody against the analyte is used. In the indirect detection method, a molecule indirectly bound to the analyte such as an antibody (secondary antibody) against the antibody binding to the analyte (primary antibody), is used. The procedure of the direct detection method is simple, but the enzyme-labeled antibody against the analyte is required for each analyte. Compared to the direct detection method, the procedure of the indirect detection method is more complex, however, it allows for using a common secondary antibody such as an anti-IgG antibody even if the analyte is different.

When ELISA is used for measuring analyte concentration, an antibody against the analyte is typically used as a capture molecule. When ELISA is used to evaluate biological activity by measuring binding activity, the target molecule of a drug involved in its pharmacologic action is used as a capture molecule.

1.1. Noncompetitive method

In the noncompetitive method, an analyte is bound to a capture molecule without competing with other molecules (Fig. 1). This method can be used when the analyte possesses

rather high molecular mass and has binding sites for the capture molecule as well as for the molecule used for detection.

1.2. Competitive method

The competitive method has two approaches: the first is to immobilize a capture molecule, then has an analyte and an enzyme-labeled antibody compete to each other for binding with the capture molecule (Fig. 1a), and the second is to use the analyte prepared as the reagent which is immobilized onto a plate, then have the immobilized analyte and the analyte in test samples compete with each other for binding with an enzyme-labeled antibody (Fig. 1b). The competitive method is used when the molecular mass of the analyte is rather low, and it is difficult to prepare two molecules which bind to the analyte specifically.

2. Analytical procedures

2.1. Procedure

General procedures for both noncompetitive and competitive methods are shown below. As for a quantitative test, prepare reference material solutions diluted serially in order to obtain a dose-response curve or a calibration curve.

2.1.1. Noncompetitive method

1) Add a solution containing capture molecules onto a plate, and incubate to immobilize the capture molecules on a solid phase, then wash off the unbound capture molecules.
2) Add a blocking reagent, and have the reagent bind on the surface not occupied by the capture molecules. Wash off the unbound blocking reagent.
3) Add a reference material or a test sample onto each well of the plate, and have the analyte bind on the solid phase. Wash off the unbound analyte.
4) When the direct detection method is used, add an enzyme-labeled antibody to bind to the analyte. When the indirect detection method is used, add an antibody against the analyte, then wash and add the enzyme-labeled antibody which binds to the antibody against the analyte in order to bind it to the solid phase. Wash off the unbound enzyme-labeled antibody.
5) Add a substrate solution, incubate and add a stopping solution if required. Then measure the absorbance, luminescent intensity, or fluorescent intensity, which reflects the amount of the substrate converted by the enzyme reaction.
6) Determine the binding activity or concentration of the analyte with reference to the dose-response curve (calibration curve) of the reference material.

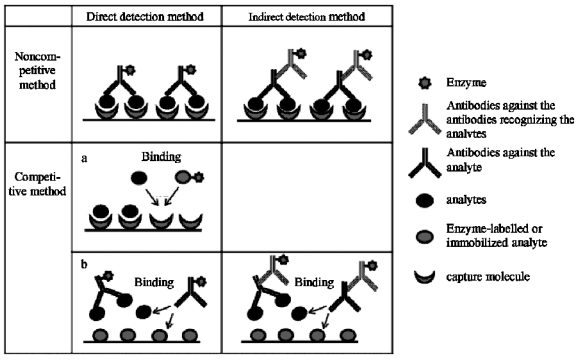


Fig. 1 Classification of ELISA by analytical method

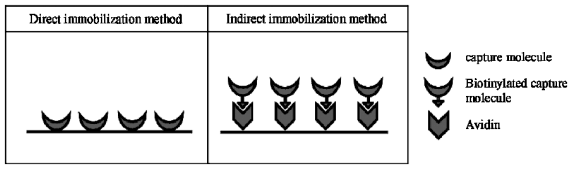


Fig. 2 Examples of direct immobilization method and indirect immobilization method

2.1.2. Competitive method

1) Competitive method (a): Add a solution containing capture molecules onto a plate, then incubate so that the capture molecules bind to a solid phase. Wash off the unbound capture molecules.
Competitive method (b): Add an analyte prepared for immobilizing onto a plate, and incubate so that the analyte bind to the solid phase. Wash off the unbound analyte.
2) Add a blocking reagent to bind on the solid phase surface that is not occupied by the operation of 1). Wash off the unbound blocking reagent.
3) Competitive method (a): Add a solution containing a reference material and an enzyme-labeled analyte, or a test sample and an enzyme-labeled analyte onto each well of the plate. Then have the analyte and the enzyme-labeled analyte bind on the solid phase. Wash off the unbound molecules.
Competitive method (b): In the direct detection method, add a solution containing a reference material and an enzyme-labeled antibody, or a test sample and an enzyme-labeled antibody onto each well of the plate, and then have the enzyme-labeled antibody bind to the solid phase. Wash off the unbound molecules. In the indirect detection method, add a solution containing a reference material and an antibody against the analyte, or a test sample and an antibody against the analyte onto each well of the plate. After washing, add the enzyme-labeled antibody which binds to the antibody against the analyte. Wash off the unbound enzyme-labeled antibody.
4) Add the substrate of the enzyme, incubate and then add a stopping solution if required. Measure the amount of the substrate converted by the enzyme reaction by measuring absorbance, luminescent intensity or fluorescent intensity.
5) Calculate the binding activity or the concentration of the analyte from the dose-response curve (calibration curve) of the reference material.

2.2. Data analysis

2.2.1. Quantitation

When ELISA is applied to determine the concentration of an analyte, use an appropriately diluted test sample and calculate the concentration of the analyte in the test sample from the calibration curve obtained from the reference material. Usually, the calibration curve is prepared by using an equation of such as 4-parameter logistic regression, setting the log concentrations of the target molecule on the x-axis and responses obtained on the y-axis.

4-parameter logistic model

$$y = D + \frac{A - D}{1 + \left(\frac{x}{C}\right)^{B}}$$

A: Lower asymptote
B: Slope parameter at EC_{50} (IC_{50})
C: EC_{50} (IC_{50})
D: Upper asymptote
x: Concentration of test sample
y: Response

When the calibration curve is not bilateral symmetric as a sigmoid curve, applying 5-parameter logistic regression may improve the analytical result. As for the noncompetitive method, a calibration curve may be obtained by linear regression by limiting the concentration at the lower range.

2.2.2. Biological activity

For evaluating biological activity, the methods such as 1) to 3) described below are used.

1) Use the test sample diluted with an appropriate dilution ratio. Determine the relative activity against the reference material by calculating the relative concentration based on the dose-response curve (the calibration curve) of the reference material.

2) Obtain the dose-response curves of the reference material and the test sample, respectively. Calculate the relative activity of the test sample against the reference material from the ratio of the concentration corresponding to 50% of the maximum response (EC_{50} for the noncompetitive method, and IC_{50} for the competitive method).

3) Use the range that can be approximated linear regression in the dose-response curve. Calculate the relative activity of the test sample against the reference material based on the ratio of the dose that arise the same response.

1) uses the same method as 2.2.1. and calculate the relative concentration against the reference material. 2) uses the same method as 2.2.1. to lead the regression equation on the reference material and the test sample. Better regression can be obtained by weighting to equalize the contribution of each concentration response in leading the regression equation. The methods of weighting are to use $1/y^2$, $1/y$ and $1/x$. Upon the establishment of the test method, choose a regression method to obtain a better result based on the accuracy and precision. 3) uses the concentration region near EC_{50} or IC_{50} that can be approximated as a straight line for analysis.

2.3. Reagents, test Solutions

2.3.1. Capture reagents

Use molecules (antigen, antibody, etc.) which can specifically bind to the analyte. Physical adsorption is frequently used for immobilizing a capture reagent on a plate, and covalent binding is also possible to use for its binding to the plate which is covered by materials having the binding activity with an amino or sulfhydryl functional group. Note that there is a case that binding activity with an analyte may be changed due to the conformational change by the binding onto the plate.

The capture reagent is a critical reagent that affects assay performance, and therefore, its quality should be controlled by setting necessary specifications. Establish the procedure for lot renewal as well.

2.3.2. Blocking reagents

A buffer solution containing protein such as albumin, gelatin, or casein, which is supplemented with surfactants such as polysorbate 20 as required, is used as a blocking reagent.

2.3.3. Detection reagents

As enzymes for detection, peroxidase, alkaline phosphatase, and β-galactosidase are typically used. As a labeling method for an enzyme, covalent binding with a target protein is used; A *N*-hydroxysuccinimide ester group introduced into an enzyme binds to the amino group of the labeled protein, and a maleimide group introduced into the enzyme binds to the sulfhydryl group of the labeled protein. As a method used for enzyme labeling of antibodies, covalent binding with which the maleimide group introduced into the enzyme is bound to the sulfhydryl group of the antibody is often used.

Detection Reagents are the critical reagents affecting assay performance, and therefore, their quality should be controlled by setting necessary specifications. Establish the procedures for lot renewal as well. As for the indirect detection method, unlabeled antibodies against an analyte are also used as a detection reagent, and therefore, it is necessary to control by setting necessary specifications.

2.3.4. Substrates

Use substrates which are appropriate for each enzyme. There are chromogenic, chemiluminescent and fluorescent substrates. Chemiluminescent substrates or fluorescent substrates are suitable when high sensitivity is required.

2.4. Points to consider

Since types of plates, amount of immobilized capture molecule, and incubation time as well as incubation temperature may affect test results, determine these procedures including materials and reagents of use. Also determine the test conditions and the sample placement in plates to prevent that the sample placement on the plates (the position of the well where the test is performed) affects the test results.

3. Application on specifications

3.1. Identification

In monographs of biotechnological/biological products, ELISA is used as an identification test which uses specific antibodies against the target product to evaluate the binding with the antibodies. As to therapeutic antibodies ELISA is also used as an identification test which evaluates the binding of the antibodies with antigen. Usually it is used as a qualitative test. In the meantime, acceptance criteria can also be set regarding the binding activity compared with a reference material when used as an identification test which evaluates the binding of therapeutic antibodies with antigen.

3.2. Purity test

ELISA is used mainly as a purity test for process-related impurities such as host cell proteins, impurities derived from culture media and ligands eluted from affinity column resin. When ELISA is used as a test to determine the amount of impurities, calculate the concentrations in a test sample by using calibration curves. When it is used as a limit test, confirm the test sample response is not higher than that of the control containing the impurities equal to the upper limits of the acceptance criteria.

In general, samples include much more amount of a target product than impurities, and therefore, the target product may disturb the detection of the impurities. Especially when ligands of affinity column are analytes, pay attention to the disturbance by the target product as the target product binds to the ligands. Consider a recovery rate when sample pretreatment is performed.

Table 1 Examples of substrates

Enzyme	Chromogenic substrate	Chemiluminescent substrates	Fluorescent substrates
peroxidase	TMB OPD ABTS	Luminol	
alkaline phosphatase	pNPP		
β-galactosidase			MG NG

TMB: 3,3′,5,5′-Tetramethylbenzidine
OPD: *o*-Phenylenediamine
ABTS: 2,2′-Azino-bis[3-ethylbenzothiazoline-6-sulfonate]
pNPP: *p*-Nitrophenyl phosphate
MG: 4-Methylumbelliferyl galactoside
NG: Nitrophenyl galactoside

3.3. Biological assay

ELISA is used as a test to determine the binding activity of a therapeutic antibody as the target product with its target molecule, and used to quantitate bioactive proteins secreted from the cells treated with test samples containing the target product in cell-based assay.

Determine relative activity by the method indicated in 2.2.2. 1) to 3).

3.4. Assay

ELISA is used for measuring the amount of target products. Obtain the calibration curve of reference materials and calculate the concentration of the target products.

4. Validity of test

In general, the validity of the test can be set as follows; use those in combination as necessary.

4.1. Identification

Confirm the results of reference materials and a negative control pass the acceptance criteria specified in the monograph.

4.2. Purity test

As for a quantitative test, confirm the reliability of calibration curves. Accuracy and/or precision of each concentration of material solutions for the calibration curve and the coefficient of determination (R^2 Value) calculated from a regression equation are used to confirm the reliability. Precision of test samples or accuracy of control samples prepared from the known concentration of a reference material (Quality Control Sample: QC sample) could be set as the test suitability. As for a limit test, confirm the response of the control sample containing the analyte at a concentration equal to the upper limit of the acceptance criteria satisfies the criterion specified in the monograph.

4.3. Biological assay

When determining biological activity by using the method of 1) of 2.2.2., confirm the reliability of the dose-response curve (the calibration curve) of the reference material . To confirm the reliability of the dose-response curve, accuracy and/or precision of each concentration of the reference material and R^2 value calculated from the regression equation or each parameter value of the regression equation obtained from the dose-response curve of the reference material can be used. Magnitude of the response of test samples, precision of the relative activity calculated from the response or accuracy of the concentration of QC samples can also be used to confirm the validity of the test.

When determining biological activity by using the method of 2) of 2.2.2., confirm the parallelism of the two regression curves obtained from a reference material and a test sample. As for the parallelism confirmation, following methods are the examples. Obtain the ratio of the difference between the upper asymptote and the lower asymptote ($D - A$ of the 4-parameter regression equation in 2.2.1.) of the test sample to that of the reference material or the ratio of the slope parameter (B of the 4-parameter regression equation in 2.2.1.), then confirm that those ratios are within the predetermined range. R^2 value of the dose-response curves of the reference material and the test sample, and accuracy of the QC samples are also used to confirm the validity of the test.

When determining biological activity by using the method of 3) of 2.2.2., confirm the linearity of the dose-response lines of a reference material and a test sample as well as the parallelism of these lines.

As for 2) and 3) of 2.2.2., there is a method to confirm the parallelism by comparing the residual variances of two regression curves, using the constrained model for control and sample data and using unconstrained models for the control and sample data, and determining the parallelism of the two regression curves by the method of analysis of variance. However, it should be noted that if the precision of the data is low, then the determination can be unrigorous.

4.4. Assay

Confirm the reliability of calibration curves obtained from the dose-response curves of reference materials. To confirm the reliability of the calibration curve, accuracy and/or precision of each concentration of the reference material calculated from the regression equation, each parameter value of the regression equation and R^2 value can be used. Precision of the measured results of test samples or accuracy of QC samples is also used to confirm the validity of the test.

Total Protein Assay <*G3-12-172*>

The following procedures are provided as illustrations of the determination of total protein content in pharmacopoeial preparations. Other techniques, such as HPLC, are also acceptable if total protein recovery is demonstrated. Many of the total protein assay methods described below can be performed successfully using kits from commercial sources.

Note: Where water is required, use distilled water.

Method 1 (UV method)

Protein in solution absorbs UV light at a wavelength of 280 nm, due to the presence of aromatic amino acids, mainly tyrosine and tryptophan. This property is the basis of this method. Protein determination at 280 nm is mainly a function of the tyrosine and tryptophan content of the protein. If the buffer used to dissolve the protein has a high absorbance relative to that of water, there is an interfering substance in the buffer. This interference can be compensated for when the spectrophotometer is adjusted to zero buffer absorbance. If the interference results in a large absorbance that challenges the limit of sensitivity of the spectrophotometer, the results may be compromised. Furthermore, at low concentrations protein can be absorbed onto the cuvette, thereby reducing the content in solution. This can be prevented by preparing samples at higher concentrations or by using a nonionic detergent in the preparation.

Note: Keep the Test Solution, the Standard Solution, and the buffer at the same temperature during testing.

Standard Solution Unless otherwise specified in the individual monograph, prepare a solution of the reference standard or reference material for the protein under test in the same buffer and at the same concentration as the Test Solution.

Test Solution Dissolve a suitable quantity of the protein under test in the appropriate buffer to obtain a solution having a concentration of 0.2 to 2 mg per mL.

Procedure Concomitantly determine the absorbances of the Standard Solution and the Test Solution in quartz cells at a wavelength of 280 nm, with a suitable spectrophotometer, using the buffer as the blank. To obtain accurate results, the response should be linear in the range of protein concentrations to be assayed.

Light-Scattering The accuracy of the UV spectroscopic determination of protein can be decreased by the scattering of light by the test specimen. If the proteins in solution exist as particles comparable in size to the wavelength of the measuring light (250 to 300 nm), scattering of the light beam results in an apparent increase in absorbance of the test specimen. To calculate the absorbance at 280 nm due to light-scattering, determine the absorbances of the Test Solution at wave-

lengths of 320, 325, 330, 335, 340, 345, and 350 nm. Using the linear regression method, plot the log of the observed absorbance versus the log of the wavelength, and determine the standard curve best fitting the plotted points. From the graph so obtained, extrapolate the absorbance value due to light-scattering at 280 nm. Subtract the absorbance from light-scattering from the total absorbance at 280 nm to obtain the absorbance value of the protein in solution. Filtration with a filter having a 0.2-μm porosity or clarification by centrifugation may be performed to reduce the effect of light-scattering, especially if the solution is noticeably turbid.

Calculations Calculate the concentration, C_U, of protein in the test specimen by the formula:

$$C_U = C_S (A_U/A_S),$$

in which C_S is the concentration of the Standard Solution; and A_U and A_S are the corrected absorbances of the Test Solution and the Standard Solution, respectively.

Method 2 (Lowry method)

This method, commonly referred to as the Lowry assay, is based on the reduction by protein of the phosphomolybdictungstic mixed acid chromogen in the Folin-Ciocalteu's phenol reagent, resulting in an absorbance maximum at 750 nm. The Folin-Ciocalteu's phenol reagent (Folin's TS) reacts primarily with tyrosine residues in the protein, which can lead to variation in the response of the assay to different proteins. Because the method is sensitive to interfering substances, a procedure for precipitation of the protein from the test specimen may be used. Where separation of interfering substances from the protein in the test specimen is necessary, proceed as directed below for Interfering Substances prior to preparation of the Test Solution. The effect of interfering substances can be minimized by dilution provided the concentration of the protein under test remains sufficient for accurate measurement. Variations of the Lowry test that are indicated in national regulatory documents[1)] can be substituted for the method described below.

Standard Solutions Unless otherwise specified in the individual monograph, dissolve the reference standard or reference material for the protein under test in the buffer used to prepare the Test Solution. Dilute portions of this solution with the same buffer to obtain not fewer than five Standard Solutions having concentrations between 5 μg and 100 μg of protein per mL, the concentrations being evenly spaced.

Test Solution Dissolve a suitable quantity of the protein under test in the appropriate buffer to obtain a solution having a concentration within the range of the concentrations of the Standard Solutions. An appropriate buffer will produce a pH in the range of 10 to 10.5.

Blank Use the buffer used for the Test Solution and the Standard Solutions.

Reagents and Solutions—

Copper Sulfate Reagent Dissolve 100 mg of copper (II) sulfate pentahydrate and 200 mg of sodium tartrate dihydrate in water, dilute with water to 50 mL, and mix. Dissolve 10 g of anhydrous sodium carbonate in water to a final volume of 50 mL, and mix. Slowly pour the sodium carbonate solution into the copper sulfate solution with mixing. Prepare this solution fresh daily.

5% SDS TS Dissolve 5 g of sodium dodecyl sulfate in water, and dilute with water to 100 mL.

Alkaline Copper Reagent Prepare a mixture of 5% SDS TS, Copper Sulfate Reagent, and Sodium Hydroxide Solution (4 in 125) (2:1:1). This reagent may be stored at room temperature for up to 2 weeks.

Diluted Folin's TS Mix 10 mL of Folin's TS with 50 mL of water. Store in an amber bottle, at room temperature.

Procedure To 1 mL of each Standard Solution, the Test Solution, and the Blank, add 1 mL of Alkaline Copper Reagent, and mix. Allow to stand at room temperature for 10 minutes. Add 0.5 mL of the Diluted Folin's TS to each solution, and mix each tube immediately after the addition, and allow to stand at room temperature for 30 minutes. Determine the absorbances of the solutions from the Standard Solutions and the Test Solution at the wavelength of maximum absorbance at 750 nm, with a suitable spectrophotometer, using the solution from the Blank to set the instrument to zero.

Calculations [Note: The relationship of absorbance to protein concentration is nonlinear; however, if the standard curve concentration range is sufficiently small, it will approach linearity.] Using the linear regression method, plot the absorbances of the solutions from the Standard Solutions versus the protein concentrations, and determine the standard curve best fitting the plotted points. From the standard curve so obtained and the absorbance of the Test Solution, determine the concentration of protein in the Test Solution.

Interfering Substances In the following procedure, deoxycholate-trichloroacetic acid is added to a test specimen to remove interfering substances by precipitation of proteins before testing. This technique also can be used to concentrate proteins from a dilute solution.

Sodium Deoxycholate Reagent Prepare a solution of sodium deoxycholate in water having a concentration of 150 mg in 100 mL.

Trichloroacetic Acid Reagent Prepare a solution of trichloroacetic acid in water having a concentration of 72 g in 100 mL.

Procedure Add 0.1 mL of Sodium Deoxycholate Reagent to 1 mL of a solution of the protein under test. Mix on a vortex mixer, and allow to stand at room temperature for 10 minutes. Add 0.1 mL of Trichloroacetic Acid Reagent, and mix on a vortex mixer. Centrifuge at 3000 × g for 30 minutes, decant the liquid, and remove any residual liquid with a pipet. Redissolve the protein pellet in 1 mL of Alkaline Copper Reagent. Proceed as directed for the Test Solution. [Note: Color development reaches a maximum in 20 to 30 minutes during incubation at room temperature, after which there is a gradual loss of color. Most interfering substances cause a lower color yield; however, some detergents cause a slight increase in color. A high salt concentration may cause a precipitate to form. Because different protein species may give different color response intensities, the standard protein and test protein should be the same.]

Method 3 (Bradford method)

This method, commonly referred to as the Bradford assay, is based on the absorption shift from 470 nm to 595 nm observed when Coomassie brilliant blue G-250 binds to protein. Coomassie brilliant blue G-250 binds most readily to arginyl and lysyl residues in the protein, which can lead to variation in the response of the assay to different proteins.

Standard Solutions Unless otherwise specified in the individual monograph, dissolve the reference standard or the reference material for the protein under test in the buffer used to prepare the Test Solution. Dilute portions of this solution with the same buffer to obtain not fewer than five Standard Solutions having concentrations between 100 μg and 1 mg of protein per mL, the concentrations being evenly spaced.

Test Solution Dissolve a suitable quantity of the protein

under test in the appropriate buffer to obtain a solution having a concentration within the range of the concentrations of the Standard Solutions.

Blank Use the buffer used to prepare the Test Solution and the Standard Solutions.

Coomassie Reagent Dissolve 100 mg of Coomassie brilliant blue G-250[2)] in 50 mL of ethanol (95). [Note: Not all dyes have the same brilliant blue G content, and different products may give different results.] Add 100 mL of phosphoric acid, dilute with water to 1000 mL, and mix. Filter the solution through filter paper (Whatman No.1 or equivalent), and store the filtered reagent in an amber bottle at room temperature. [Note: Slow precipitation of the dye will occur during storage of the reagent. Filter the reagent before use.]

Procedure Add 5 mL of the Coomassie Reagent to 100 μL of each Standard Solution, the Test Solution, and the Blank, and mix by inversion. Avoid foaming, which will lead to poor reproducibility. Determine the absorbances of the solutions from the Standard Solutions and the Test Solution at 595 nm, with a suitable spectrophotometer, using the Blank to set the instrument to zero.

[Note: Do not use quartz (silica) spectrophotometer cells: the dye binds to this material. Because different protein species may give different color response intensities, the standard protein and test protein should be the same.] There are relatively few interfering substances, but detergents and ampholytes in the test specimen should be avoided. Highly alkaline specimens may interfere with the acidic reagent.

Calculations [Note: The relationship of absorbance to protein concentration is nonlinear; however, if the standard curve concentration range is sufficiently small, it will approach linearity.] Using the linear regression method, plot the absorbances of the solutions from the Standard Solutions versus the protein concentrations, and determine the standard curve best fitting the plotted points. From the standard curve so obtained and the absorbance of the Test Solution, determine the concentration of protein in the Test Solution.

Method 4 (Bicinchoninic acid method)

This method, commonly referred to as the bicinchoninic acid or BCA assay, is based on reduction of the cupric (Cu^{2+}) ion to cuprous (Cu^{+}) ion by protein. The bicinchoninic acid reagent is used to detect the cuprous ion. The method has few interfering substances. When interfering substances are present, their effect may be minimized by dilution, provided that the concentration of the protein under test remains sufficient for accurate measurement.

Standard Solutions Unless otherwise specified in the individual monograph, dissolve the reference standard or the reference material for the protein under test in the buffer used to prepare the Test Solution. Dilute portions of this solution with the same buffer to obtain not fewer than five Standard Solutions having concentrations between 10 μg and 1200 μg of protein per mL, the concentrations being evenly spaced.

Test Solution Dissolve a suitable quantity of the protein under test in the appropriate buffer to obtain a solution having a concentration within the range of the concentrations of the Standard Solutions.

Blank Use the buffer used to prepare the Test Solution and the Standard Solutions.

Reagents and Solutions—

BCA Reagent Dissolve about 10 g of bicinchoninic acid, 20 g of sodium carbonate monohydrate, 1.6 g of sodium tartrate dihydrate, 4 g of sodium hydroxide, and 9.5 g of sodium hydrogen carbonate in water. Adjust, if necessary, with sodium hydroxide or sodium hydrogen carbonate to a pH of 11.25. Dilute with water to 1000 mL, and mix.

Copper Sulfate Reagent Dissolve about 2 g of copper (II) sulfate pentahydrate in water to a final volume of 50 mL.

Copper-BCA Reagent Mix 1 mL of Copper Sulfate Reagent and 50 mL of BCA Reagent.

Procedure Mix 0.1 mL of each Standard Solution, the Test Solution, and the Blank with 2 mL of the Copper-BCA Reagent. Incubate the solutions at 37°C for 30 minutes, note the time, and allow to come to room temperature. Within 60 minutes following the incubation time, determine the absorbances of the solutions from the Standard Solutions and the Test Solution in quartz cells at 562 nm, with a suitable spectrophotometer, using the Blank to set the instrument to zero. After the solutions are cooled to room temperature, the color intensity continues to increase gradually. If substances that will cause interference in the test are present, proceed as directed for Interfering Substances under Method 2. Because different protein species may give different color response intensities, the standard protein and test protein should be the same.

Calculations [Note: The relationship of absorbance to protein concentration is nonlinear; however, if the standard curve concentration range is sufficiently small, it will approach linearity.] Using the linear regression method, plot the absorbances of the solutions from the Standard Solutions versus the protein concentrations, and determine the standard curve best fitting the plotted points. From the standard curve so obtained and the absorbance of the Test Solution, determine the concentration of protein in the Test Solution.

Method 5 (Biuret method)

This method, commonly referred to as the Biuret assay, is based on the interaction of cupric (Cu^{2+}) ion with protein in an alkaline solution and the resultant development of absorbance at 545 nm.

Standard Solutions Unless otherwise specified in the individual monograph, prepare a solution of Albumin Human for which the protein content has been previously determined by nitrogen analysis (using the nitrogen-to-protein conversion factor of 6.25) or of the reference standard or reference material for the protein under test in sodium chloride solution (9 in 1000). Dilute portions of this solution with sodium chloride solution (9 in 1000) to obtain not fewer than three Standard Solutions having concentrations between 0.5 mg and 10 mg per mL, the concentrations being evenly spaced. [Note: Low responses may be observed if the sample under test has significantly different level of proline than that of Albumin Human. A different standard protein may be employed in such cases.]

Test Solution Prepare a solution of the test protein in sodium chloride solution (9 in 1000) having a concentration within the range of the concentrations of the Standard Solutions.

Blank Use sodium chloride solution (9 in 1000).

Biuret Reagent Dissolve about 3.46 g of copper (II) sulfate pentahydrate in 10 mL of water, with heating if necessary, and allow to cool (Solution A). Dissolve about 34.6 g of sodium citrate dihydrate and 20.0 g of anhydrous sodium carbonate in 80 mL of water, with heating if necessary, and allow to cool (Solution B). Mix Solutions A and B, and dilute with water to 200 mL. This Biuret Reagent is stable at room temperature for 6 months. Do not use the reagent if it develops turbidity or contains any precipitate.

Procedure To one volume of the Standard Solutions and a solution of the Test Solution add an equal volume of sodium

hydroxide solution (6 in 100), and mix. Immediately add a volume of Biuret Reagent equivalent to 0.4 volume of the Test Solution, and mix. Allow to stand at a temperature between 15°C and 25°C for not less than 15 minutes. Within 90 minutes after the addition of the Biuret Reagent, determine the absorbances of the Standard Solutions and the solution from the Test Solution at the wavelength of maximum absorbance at 545 nm, with a suitable spectrophotometer, using the Blank to set the instrument to zero. [Note: Any solution that develops turbidity or a precipitate is not acceptable for calculation of protein concentration.]

Calculations Using the least-squares linear regression method, plot the absorbances of the Standard Solutions versus the protein concentrations, and determine the standard curve best fitting the plotted points, and calculate the correlation coefficient for the line. [Note: Within the given range of the standards, the relationship of absorbance to protein concentration is approximately linear.] A suitable system is one that yields a line having a correlation coefficient of not less than 0.99. From the standard curve and the absorbance of the Test Solution, determine the concentration of protein in the test specimen, making any necessary correction.

Interfering Substances To minimize the effect of interfering substances, the protein can be precipitated from the initial test specimen as follows. Add 0.1 volume of 50% trichloroacetic acid to 1 volume of a solution of the test specimen, withdraw the supernatant layer, and dissolve the precipitate in a small volume of 0.5 mol/L sodium hydroxide TS. Use the solution so obtained to prepare the Test Solution.

Comments This test shows minimal difference between equivalent IgG and albumin samples. Addition of the sodium hydroxide and the Biuret Reagent as a combined reagent, insufficient mixing after the addition of the sodium hydroxide, or an extended time between the addition of the sodium hydroxide solution and the addition of the Biuret Reagent will give IgG samples a higher response than albumin samples. The trichloroacetic acid method used to minimize the effects of interfering substances also can be used to determine the protein content in test specimens at concentrations below 500 μg per mL.

Method 6 (Fluorometric method)

This fluorometric method is based on the derivatization of the protein with *o*-phthalaldehyde (OPA), which reacts with the primary amines of the protein (i.e., NH_2-terminal amino acid and the ε-amino group of the lysine residues). The sensitivity of the test can be increased by hydrolyzing the protein before testing. Hydrolysis makes the α-amino group of the constituent amino acids of the protein available for reaction with the OPA reagent. The method requires very small quantities of the protein.

Primary amines, such as tris(hydroxymethyl)aminomethane and amino acid buffers, react with OPA and must be avoided or removed. Ammonia at high concentrations will react with OPA as well. The fluorescence obtained when amine reacts with OPA can be unstable. The use of automated procedures to standardize this procedure may improve the accuracy and precision of the test.

Standard Solutions Unless otherwise specified in the individual monograph, dissolve the reference standard or the reference material for the protein under test in the buffer used to prepare the Test Solution. Dilute portions of this solution with the same buffer to obtain not fewer than five Standard Solutions having concentrations between 10 and 200 μg of protein per mL, the concentrations being evenly spaced.

Test Solution Dissolve a suitable quantity of the protein under test in the appropriate buffer to obtain a solution having a concentration within the range of the concentrations of the Standard Solutions.

Blank Use the buffer used to prepare the Test Solution and the Standard Solutions.

Reagents and Solutions—

Borate Buffer Dissolve about 61.83 g of boric acid in water, and adjust with potassium hydroxide to a pH of 10.4. Dilute with water to 1000 mL, and mix.

Stock OPA Reagent Dissolve about 120 mg of *o*-phthalaldehyde in 1.5 mL of methanol, add 100 mL of Borate Buffer, and mix. Add 0.6 mL of polyoxyethylene (23) lauryl ether, and mix. This solution is stable at room temperature for at least 3 weeks.

OPA Reagent To 5 mL of Stock OPA Reagent add 15 μL of 2-mercaptoethanol. Prepare at least 30 minutes prior to use. This reagent is stable for one day.

Procedure Adjust each of the Standard Solutions and the Test Solution to a pH between 8.0 and 10.5. Mix 10 μL of the Test Solution and each of the Standard Solutions with 100 μL of OPA Reagent, and allow to stand at room temperature for 15 minutes. Add 3 mL of 0.5 mol/L sodium hydroxide TS, and mix. Using a suitable fluorometer, determine the fluorescent intensities of solutions from the Standard Solutions and the Test Solution at an excitation wavelength of 340 nm and an emission wavelength between 440 nm and 455 nm. [Note: The fluorescence of an individual specimen is read only once because irradiation decreases the fluorescent intensity.]

Calculations The relationship of fluorescence to protein concentration is linear. Using the linear regression method, plot the fluorescent intensities of the solutions from the Standard Solutions versus the protein concentrations, and determine the standard curve best fitting the plotted points. From the standard curve so obtained and the fluorescent intensity of the Test Solution, determine the concentration of protein in the test specimen.

Method 7 (Nitrogen method)

This method is based on nitrogen analysis as a means of protein determination. Interference caused by the presence of other nitrogen-containing substances in the test protein can affect the determination of protein by this method. Nitrogen analysis techniques destroy the protein under test but are not limited to protein presentation in an aqueous environment.

Procedure A Determine the nitrogen content of the protein under test as directed elsewhere in the Pharmacopoeia. Commercial instrumentation is available for the Kjeldahl nitrogen assay.

Procedure B Commercial instrumentation is available for nitrogen analysis. Most nitrogen analysis instruments use pyrolysis (i.e., combustion of the sample in oxygen at temperatures approaching 1000°C), which produces nitric oxide (NO) and other oxides of nitrogen (NO_x) from the nitrogen present in the test protein. Some instruments convert the nitric oxides to nitrogen gas, which is quantified with a thermal conductivity detector. Other instruments mix nitric oxide (NO) with ozone (O_3) to produce excited nitrogen dioxide (NO_2) which emits light when it decays and can be quantified with a chemiluminescence detector. A protein reference standard or reference material that is relatively pure and is similar in composition to the test proteins is used to optimize the injection and pyrolysis parameters and to evaluate consistency in the analysis.

Calculations The protein concentration is calculated by dividing the nitrogen content of the sample by the known

nitrogen content of the protein. The known nitrogen content of the protein can be determined from the chemical composition of the protein or by comparison with the nitrogen content of the appropriate reference standard or reference material.

◆1) Example: Minimum Requirements for Biological Products and individual monograph in JP.◆
2) Purity of the reagent is important.

Basic Requirements for Viral Safety of Biotechnological/Biological Products listed in Japanese Pharmacopoeia <*G3-13-141*>

Introduction

The primary role of specification of biotechnological/biological products listed in the Japanese Pharmacopoeia (JP) is not only for securing quality control or consistency of the quality but also for assuring their efficacy and safety. In the meantime, the requirements to assure quality and safety of drugs have come to be quite strict recently, and a rigid attitude addressing safety assurance is expected for biotechnological/biological products. The key points for quality and safety assurance of biotechnological/biological products are selection and appropriate evaluation of source material, appropriate evaluation of manufacturing process and maintenance of manufacturing consistency, and control of specific physical properties of the products. Now, how to assure quality and safety of such drugs within a scope of the JP has come to be questioned. This General Information describes what sorts of approaches are available to overcome these issues.

It is desired that quality and safety assurance of JP listed products are achieved by state-of-the-art methods and concepts which reflect progress of science and accumulation of experiences. This General Information challenges to show the highest level of current scientific speculation. It is expected that this information will contribute to promotion of scientific understanding of quality and safety assurance of not only JP listed products but also the other biotechnological/biological products and to promotion of active discussion of each Official Monograph in JP.

1. Fundamental measures to ensure viral safety of JP listed biotechnological/biological products

JP listed biotechnological/biological products includes the products derived from living tissue and body fluid (urine, blood, etc.) of mammals, etc. Protein drugs derived from cell lines of human or animal origin (e.g., recombinant DNA drug, cell culture drug) are also included. The fundamental measures required for comprehensive viral safety of JP listed biotechnological/biological products are as follows: 1) acquaintance of possible virus contamination (source of contamination); 2) careful examination of eligibility of raw materials and their sources, e.g. human/animal, and thorough analysis and screening of the sample chosen as a substrate for drug production (e.g., pooled body fluid, cell bank, etc.) to determine any virus contamination and determination of type and nature of the virus, if contaminated; 3) evaluation to determine virus titer and virus-like particles hazardous to human, if exists; 4) selection of production related material (e.g., reagent, immune antibody column) free from infectious or pathogenic virus; 5) performance of virus free test at an appropriate stage of manufacturing including the final product, if necessary; 6) adoption of effective viral clearance method in the manufacturing process to remove/inactivate virus. A combined method sometimes achieves higher level of clearance; 7) development of a deliberate viral clearance scheme; 8) performance of the test to evaluate viral removal and inactivation. It is considered that the stepwise and supplemental adoption of the said measures will contribute to ensure viral safety and its improvement.

2. Safety assurance measures described in the Official Monograph and this General Information

As mentioned in above 1, this General Information describes, in package, points to be concerned with and concrete information on the measures taken for viral safety of JP listed products. Except where any specific caution is provided in Official Monograph of a product in question, Official Monograph provides in general that ''Any raw material, substrate for drug production and production related materials used for production of drug should be derived from healthy animals and should be shown to be free of latent virus which is infectious or pathogenic to human'', ''Cell line and culture method well evaluated in aspects of appropriateness and rationality on viral safety are used for production, and the presence of infectious or pathogenic latent virus to human in process related materials derived from living organisms should be denied'', and ''biotechnological/biological drug should be produced through a manufacturing process which is capable of removing infectious or pathogenic virus'', etc., to raise awareness on viral safety and on necessity to conduct test and process evaluation for viral safety.

3. Items and contents described in this General Information

As for viral safety of protein drug derived from cell line of human or animal origin, there is a Notice in Japan entitled ''Viral safety evaluation of biotechnology products derived from cell lines of human or animal origin'' (Iyakushin No. 329 issued on February 22, 2000 by Director, Evaluation and Licensing Division, Pharmaceutical and Medical Safety Bureau, Ministry of Health and Welfare) to reflect the internationally harmonized ICH Guideline, and as for blood plasma protein fraction preparations, there is a document entitled ''Guideline for ensuring viral safety of blood plasma protein fraction preparations''. This General Information for ensuring viral safety of JP listed biotechnological/biological products has been written, referencing the contents of those guidelines, to cover general points and their details to be concerned for ensuring viral safety of not only JP listed biotechnological/biological products but also all products which would be listed in the JP in future, i.e., biological products derived from living tissue and body fluid, such as urine, and protein drugs derived from cell lines of human or animal origin (Table 1).

3.1. Purpose

The purpose of this document is to propose the comprehensive concepts of the measures to be taken for ensuring viral safety of biotechnological/biological products derived from living tissue or body fluid of mammals, etc. and of protein drugs derived from cell lines of human or animal origin. That is to say, this document describes the measures and the points of concern on the items, such as ① consideration of the source of virus contamination; ② appropriate evaluation on eligibility at selecting the raw material and on qualification of its source, e.g. human or animal; ③ virus test, and its analysis and evaluation at a stage of cell substrate for drug production; ④ appropriate evaluation to choose product related materials derived from living organisms (e.g. reagent, immune antibody column, etc.); ⑤ con-

duct of necessary virus test on the product at an appropriate stage of manufacturing; ⑥ development of viral clearance test scheme; ⑦ performance and evaluation of viral clearance test. This document is also purposed to comprehensively describe in details that supplemental and combining adoption of the said measures will contribute to secure viral safety and its improvement.

3.2. Background

One of the most important issues to be cautioned for safety of a biological product, which is directly derived from human or animal, or of a protein drug, which is derived from cell line of human or animal origin (recombinant DNA derived product, cell culture derived product, etc.), is a risk of virus contamination. Virus contamination may cause serious situation at clinical use once it occurs. Virus contamination may be from a raw material or from a cell substrate for drug production, or may be from an adventitious factor introduced to the manufacturing process.

JP listed biological drugs or protein drugs derived from cell line have achieved drastic contribution to the medical society, and to date, there has not been any evidence of any safety problem on them caused by virus. But, social requirement of health hazard prevention is strong, and it is now very important to prevent accidental incidence, taking security measures carefully supported by scientific rationality. It is always great concern among the persons involved that under what sort of viewpoint and to what extent we have to pursue for ensuring viral safety of a biotechnological/biological product.

Before discussing these issues, two fundamental points have to be reconfirmed. One is that; we have to consider scientific, medical, and social profiles a drug has. In other words, ''Medicine is a social asset which is utilized in medical practice paying attention to the risk and benefit from the standpoints of science and society''. It is the destination and the mission of the medical/pharmaceutical society to realize prompt and stable supply of such a social asset, drug, among the medical work front to bring gospel to the patients.

The other is that; issue of viral safety is independent from safety of the components of a drug per se (narrow sense of safety). It is important to consider that this is the matter of general safety of a drug (broad sense of safety). In case of a drug which has been used for a long time in the medical front, such as a JP listed product, its broad sense of safety is considered to have been established epidemiologically, and its usage past records have a great meaning. However, different from safety of drug per se (its components), taking into account any possibility of virus contamination, we have to say that only the results accumulated can not always assure viral safety of a drug used in future. Accordingly, the basis for securing broad sense of viral safety of JP listed biotechnological/biological products is to pay every attention to the measures to take for prevention, while evaluating the accumulated results.

Adopting strict regulations and conducting tests at maximum level to the extent theoretically considered may be the ways off assuring safety, but applying such way generally, without sufficient scientific review of the ways and evaluation of usage results, causes the excessive requirement of regulations and tests not having scientific rationality. As the results, effective and prompt supply of an important drug, already having enough accumulation of experiences, to the medical work front will be hampered, and the drug, a social asset, may not to be utilized effectively. Medicine is a sword used in medical field having double-edge named effectiveness and safety. Effectiveness and safety factors have to be derived as the fruits of leading edge of science, and relatively evaluated on a balance sheet of usefulness. Usefulness evaluation should not be unbalanced in a way that too much emphasis is placed on safety concern without back-up of appropriate scientific rationality. A drug can play an important role as a social asset only when well balanced appropriate scientific usefulness evaluation in addition to social concern of the age are given. In other words, a drug is a common asset utilized by society for medication as a fruit of science of the age, and the key point of its utilization lies on a balance of risk and benefit produced from scientific and social evalu-

Table 1 Items described in General Information for viral safety assurance of JP listed biotechnological/biological products

I. Introduction
1. Fundamental measures to ensure viral safety of JP listed biotechnological/biological products
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1. Purpose
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5. Possible viral contamination to a JP listed biotechnological/biological product (source of virus contamination)
6. Basis for ensuring viral safety
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III. Raw material/substrate for drug production
1. Issues relating to animal species and its region as a source of raw material substrate for drug production and countermeasures to be taken thereto
2. Qualification evaluation test on human or animal as a source of raw material/substrate for drug production

IV. Points of concern with respect to manufacturing and virus testing
1. Virus test conducted in advance of purification process
2. Virus test as an acceptance test of an intermediate material, etc.
3. Virus test on a final product

V. Process evaluation on viral clearance
1. Rationale, objective and general items to be concerned with respect to viral clearance process evaluation
2. Selection of virus
3. Design of viral clearance studies
4. Interpretation of viral clearance studies
   1) Evaluation of viral clearance factor
   2) Calculation of viral clearance index
   3) Interpretation of results and items to be concerned at evaluation

VI. Statistics
1. Statistical considerations for assessing virus assays
2. Reproducibility and confidence limit of viral clearance studies

VII. Re-evaluation of viral clearance

VIII. Measurement for viral clearance studies
1. Measurement of virus infective titer
2. Testing by nucleic-acid amplification test (NAT)

IX. Reporting and preservation

X. Others

ation. So, those factors have to be taken into account when target and pursuance levels for ensuring viral safety of a JP listed biotechnological/biological product are reviewed.

And, in general, the risk and benefit of drugs should be considered with the relative comparison to alternative drugs or medical treatment. The usefulness of a certain drug should be reviewed finally after the competitive assessment on the risk and benefit on the alternative drugs, relevant drugs and/or alternative medical treatment.

Under such background, the purpose of this article is to describe the scientific and rational measures to be taken for ensuring viral safety of JP listed biotechnological/biological products. Giving scientific and rational measures mean that; appropriate and effective measures, elaborated from the current scientific level, are given to the issues assumable under the current scientific knowledge. In other words, a possible contaminant virus is assumed to have the natures of genus, morph, particle size, physical/chemical properties, etc. which are within the range of knowledge of existing virology, and is those assumed to exist in human and animal, tissue and body fluid, which are the source of biotechnological/biological products, reagent, material, additives, etc. Accordingly, viral clearance studies using a detection method which target those viruses have to be designed.

3.3. Unknown risk on the measures taken for ensuring viral safety

There are known and unknown risks.

It is easy to determine a test method and an evaluation standard on the known risk, which exists in the drug per se (pharmaceutical component) or inevitably exists due to a quality threshold, and quantification of such risk is possible. In other words, it is easy to evaluate the known risk on a balance sheet in relation to the benefit, and we can say that the valuation of JP listed products even in this respect has been established to some extent.

On the other hand, as for the unknown risk which is inevitable for ensuring viral safety, the subject of the risk can not be defined and quantitative concept is hard to introduce, and, therefore, taking a counter measure and evaluating its effect are not so easy. Therefore, this is the subject to be challenged calling upon wisdom of the related parties among the society of drugs.

Talking about the unknown risk, there are view points that say ''It is risky because it is unknown.'' and ''What are the unknowns, and how do we cope with them in ensuring safety?''.

The view of ''It is risky because it is unknown.'' is already nothing but a sort of evaluation result, and directly connects to a final decision if it can be used as a drug. Such evaluation/decision has to be made based upon a rational, scientific or social judgment.

For example, in the case that ''In a manufacturing process of a drug, virus, virus-like particle or retrovirus was detected, but its identification could not be confirmed, and, therefore, its risk can not be denied.'', the evaluation of ''It is risky because it is unknown.'' is scientifically rational and reasonable. On the other hand, however, if we reach a decision of ''It is risky because it is unknown.'' due to the reason that ''In a manufacturing process of a drug, virus, virus-like particle or retrovirus was not detected, but there is a 'concern' that something unknown may exist.'', it can not be said that such evaluation is based upon a rational, scientific or social judgment. It goes without saying that the utmost care has to be taken for viral safety, but the substance of 'concern' has to be at least clearly explainable. Otherwise, the 'concern' may result in causing contradiction in the meaningful mission to utilize a social asset, drug, in medical practice.

From a scientific view point, we should not be narrow minded by saying ''it is risky'' because ''there is a 'concern' that something unknown may exists'', but challenge to clarify the subject of ''What is unknown, and how to cope with it for ensuring safety'' using wisdom. What is important at the time is to define ''what is unknown'' based upon current scientific knowledge. Only through this way, is it possible for us to elaborate the measures for ensuring safety.

Once we chase up the substance of unknown risk for viral safety without premises of ''what is unknown'', ''unknown'' will be an endless question because it theoretically remains unresolved forever. If this kind of approach is taken, the issue and the measure can not be scientifically connected to each other, which will result in the excessive requirement of regulations and of tests to be conducted. Yet, it is unlikely that the measure which has no relation with science will be effective to the subject of ''What is unknown is unknown.''

For example, ''what is unknown'' at the ''evaluation of a purification process which can completely clear up every virus that contaminated in a manufacturing process'' should be the subject of ''what sort of existing virus that contaminated is unknown'', not on the subject of ''what sort of virus that exist in the world is unknown. In the former subject, the premise of the study is based on all the knowledge on viruses including DNA/RNA-virus, virus with/without envelope, particle size, physical/chemical properties, etc. The premise is that the virus contaminated should be within range of existing wisdom and knowledge of virus such as species, type, nature, etc., even though the virus that contaminated is unknown. Under such premise, when evaluation is made on a purification process to decide its capability of clearing a derived virus, which is within the range of existing wisdom and learning, specific viral clearance studies designed to combine a few model viruses with different natures, such as type of nucleic acid, with/without envelope, particle size, physical/chemical properties, etc., would be enough to simulate every sort of the virus already known, and will be a good measure for ''ensuring safety''.

The issue of ''the sort of viruses that exist in the world is unknown'' may be a future study item, but it is not an appropriate subject for the viral clearance test. Further, even if the subject of ''unknown viruses, which have a particle size smaller than that of currently known viruses, may exists'' or ''unknown viruses, which have special physical/chemical properties that can not be matched to any of the currently known viruses, may exists'' is set up as an armchair theory, any experimental work can not be pursued under the current scientific level, since such virus model is not available. Further, any viral clearance test performed by using the currently available methods and technologies will be meaningless ''for ensuring safety'', since particle size or natures of such speculated virus are unknown. Likewise, any counter measures can not be taken on the subject of ''unknown virus, which can not be detected by currently available screening method, may exist'', and conducting any virus detection test at any stage will be useless ''for ensuring safety''.

The requirement of regulations or tests excessively over scientific rationality will raise human, economical and temporal burden to the pharmaceutical companies, and will adversely affect prompt, effective and economical supply of a drug to the medical front. As a drug is a sort of social asset, which has to be scientifically evaluated, how to assure maximization of its safety by means of scientifically rational approach at minimum human, economical and time resources is important.

It is also important to reconfirm that achievement of those

issues is on the premise that appropriate measures are taken on the supply source of drugs. For example, in a case of ''In a manufacturing process of drug, virus, virus-like particle or retrovirus was not detected, but there is a 'concern' that something unknown may exist.'', appropriateness of the test, which resulted in the judgment that ''virus, virus-like particle or retrovirus was not detected in a process of drug production'', should be a prerequisite premise when judged by the science standard at the time. If there is any question on the premise, it is quite natural that the question of ''there is a 'concern' that unknown something may exist.'' will be effective.

3.4. Applicable range

This General Information is on JP listed biological products, derived from living tissue or body fluid, and protein drugs, derived from human or animal cell line, that in Japan. In the case of protein drugs derived from human or animal cell line, the products developed and approved after enforcement of the Notice Iyakushin No. 329 entitled ''Viral safety evaluation of biotechnology products derived from cell lines of human or animal origin'' should have been treated under the Notice, and it is inevitable that some products approved before the Notice might not have been sufficiently treated. It is expected that such biodrug will be sufficiently examined to meet such General Information before being listed in the JP. On the other hand, blood preparations listed in the biological products standard and covered by ''Guideline for securing safety of blood plasma protein fraction preparations against virus'', are out of the scope of this General Information. Further, in case of a relatively lower molecular biogenous substance, such as amino acid, saccharide and glycerin, and of gelatin, which is even classified as infectious or pathogenic polymer, there are cases that viral contamination can not be considered due to its manufacturing or purification process, and that potent viral inactivation /removal procedure that can not be applied to protein, can be used, and, therefore, it is considered reasonable to omit such substances from the subject for application. However, some part of this General Information may be used as reference. Further, a comprehensive assurance measure for viral safety is recommendable on a biotechnological/biological product not listed in the JP using this document as a reference so long as it is similar to JP listed biotechnological/biological products.

3.5. Possible viral contamination to a JP listed biotechnological/biological product (source of virus contamination)

Promoting awareness of virus contamination to a JP listed biotechnological/biological product (source of virus contamination) and citing countermeasures are important for eradicating any possible virus contamination and raising probability of safety assurance. Many biotechnological/biological products are produced from a ''substrate for drug production'' which is derived from human or animal tissue, body fluid, etc. as an origin/raw material, and in purification or pharmaceutical processing of such products column materials or additives, which are living organism origin, are occasionally used. Accordingly, enough safety measures should be taken against diffusion of the contaminant virus. Further, as mentioned in Notice Iyakushin No. 329, any protein drug derived from cell lines of human or animal origin should be carefully examined with respect to the risk of virus contamination through the cell line, the cell substrate for drug production, and through the manufacturing process applied thereafter.

''Substrate for drug production'' is defined as a starting material which is at a stage where it is deemed to be in a position to ensure quality/safety of a drug substance. The ''substrate for drug production'' is sometimes tissue, body fluid, etc. of human or animal per se and pooled material such as urine, and sometimes a material after some treatment. In many cases, it is considered rational that the starting point of a full-scale test, evaluation and control should be at the stage of ''substrate for drug production''. The more strict levels of test, evaluation and control achieved at the stage of ''substrate for drug production'' can more rationalize evaluation and control of the raw material or individual level of an upper stream. On the contrary, strict evaluation and control of the raw material or individual level at an upper stream stage can rationalize tests, evaluation or quality control at the stage of ''substrate for drug production''.

The measures taken for ensuring viral safety on a biotechnological/biological product currently listed in the JP can be assumed from the provisions of the manufacturing method, specification and test methods of each preparation. However, unitary principles or information with respect to the measures to be taken for ensuring viral safety, totally reviewing the entire process up to the final product rationally and comprehensively, including source/raw material/substrate, purification process, etc. have not been clarified. The most important thing for ensuring viral safety is to take thorough measures to eliminate the risk of virus contamination at any stage of source animal, raw material and substrate. Although not the cases of a biotechnological/biological product, known examples of a virus contamination from a raw material/substrate for drug production in old times are Hepatitis A Virus (HAV) or Hepatitis C Virus (HCV) contamination in blood protein fraction preparations. It is also well known that Human Immunodeficiency Virus (HIV) infection caused by blood plasma protein fraction preparations occurred in 1980s. The aim of this General Information is to show concrete guidelines for comprehensive viral safety assurance of JP listed biotechnological/biological products. The pathogenic infectious viruses, currently known to contaminate to raw materials, etc. of drug and have to be cautioned, are HIV, HAV, Hepatitis B Virus (HBV), HCV, Human T-Lymphotropic Virus (HTLV-I/II), Human Parvovirus B19, Cytomegalovirus (CMV), etc. Biotechnological/biological products produced from raw material/substrate for drug production derived from tissue or body fluid of human or animal origin always have a risk of contamination of pathogenic or other latent virus. Therefore, safety measures should be thoroughly taken. There is also the case that a material, other than the biological component such as raw material/substrate for drug production, causes virus contamination. Using an enzymatic or monoclonal antibody column or using albumin etc. as a stabilizer, is the example of the case, in which caution has to be taken on risk of virus contamination from the source animal or cell. Further, there is a possibility of contamination from environment or personnel in charge of production or at handling of the product. So, caution has to be taken on these respects as well.

In case of protein drugs derived from cell line of human or animal origin, there may be cases where latent or persistent infectious viruses (e.g., herpesvirus) or endogenous retroviruses exist in the cell. Further, adventitious viruses may be introduced through the routes such as: 1) derivation of a cell line from an infected animal; 2) use of a virus to drive a cell line; 3) use of a contaminated biological reagent (e.g., animal serum components); 4) contamination during cell handling. In the manufacturing process of drugs, an adventitious virus may contaminate the final product through the routes, such as 1) contamination through a reagent of living being origin, such as serum component, which is used for

culturing, etc.; 2) use of a virus for introduction of a specific gene expression to code an objective protein; 3) contamination through a reagent used for purification such as monoclonal antibody affinity column; 4) contamination through an additive used for formulation production; 5) contamination at handling of cells and culture media, etc. It is reported that monitoring of cell culture parameters may be helpful for early detection of an adventitious viral contamination.

3.6. Basis for ensuring viral safety

Viral safety of a biotechnological/biological product produced from a raw material/substrate, which derived from tissue, body fluid, cell line, etc. of human or animal origin, can be achieved by supplemental and appropriate adoption of the following plural methods.

(1) Acquaintance of possible virus contamination (source of contamination).
(2) Careful examination of eligibility of the raw material and its source, i.e., human or animal, thorough analysis and screening of the sample chosen as the substrate for drug production to determine virus contamination and through examination of the type of virus and its nature, if contaminated.
(3) Evaluation to determine hazardous properties of the virus or virus-like particle to human, if exists.
(4) Choosing a product related material of living organism origin (e.g., reagent, immune anti-body column, etc.) which is free from an infectious or pathogenic virus.
(5) Conduct virus free test at an appropriate stage of manufacturing including the final product, if necessary.
(6) Adoption of an effective method to remove/inactivate the virus in the manufacturing process for viral clearance. Combined processes sometimes achieve higher level of viral clearance.
(7) Develop a deliberate viral clearance scheme.
(8) Conduct the test and evaluation to confirm removal/inactivation of the virus.

Manufacturers are responsible for explaining rationality of the way of approach adopted among the comprehensive strategy for viral safety on each product and its manufacturing process. At the time, the approach described in this General Information shall be applicable as far as possible.

3.7. Limit of virus test

A virus test has to be conducted to define existence of virus, but it should be noted that the virus test alone can not reach a conclusion of inexistence of virus nor sufficient to secure safety of the product. Examples of a virus not being detected are as follows: 1) Due to statistical reason, there is an inherent quantitative limit, such as detection sensitivity at lower concentration depending upon the sample size. 2) Generally, every virus test has a detection limit, and any negative result of a virus test can not completely deny existence of a virus. 3) A virus test applied is not always appropriate in terms of specificity or sensitivity for detection of a virus which exists in the tissue or body fluid of human or animal origin.

A virus testing method is improved as science and technology progress, and it is important to apply scientifically the most advanced technology at the time of testing so that it can be possible to raise the assurance level of virus detection. It should be noted, however, that the limit as mentioned above can not always be completely overcome. Further, risk of virus contamination in a manufacturing process can not be completely denied, and, therefore, it is necessary to elaborate the countermeasure taken these effects into account.

Reliable assurance of a viral free final product can not be obtained only by negative test results on the raw material/substrate for drug production or on the product in general, it is also necessary to demonstrate inactivation/removal capability of the purification process.

3.8. Roles of viral clearance studies

Under the premises as mentioned in the preceding clause that there is a limit of a virus test, that there is a possibility of existence of latent virus in a raw material/substrate for drug production of human or animal origin and that there is a risk of entry of a non-endogenous virus in a manufacturing process, one of the important measures for viral safety is how to remove or inactivate the virus, which exists in a raw material, etc. and can not be detected, or the virus, which is contingently contaminated in a manufacturing process. The purpose of a viral clearance study is to experimentally evaluate the viral removal/inactivation capability of a step that mounted in a manufacturing process. So, it is necessary to conduct an experimental scale spike test using an appropriate virus that is selected by taking account the properties, such as particle size, shape, with or without envelope, type of nucleic acid (DNA type, RNA type), heat and chemical treatment tolerance, etc., with an aim to determine removal/inactivation capability of the virus that can not be detected in a raw material or contingently contaminated.

As mentioned above, the role of the viral clearance study is to speculate removal/inactivation capability of a process through a model test, and it contributes to give scientific basis to assure that a biotechnological/biological product of human or animal origin has reached an acceptable level in aspect of viral safety.

At a viral clearance study, it is necessary to adopt an appropriate approach method which is definitive and rational and can assure viral safety of a final product, taking into consideration the source and the properties of the raw material/substrate for drug production as well as the manufacturing process.

4. Raw material/substrate for drug production

4.1. Issues relating to animal species and its region as a source of raw material/substrate for drug production and countermeasures to be taken thereto

For manufacturing JP listed biotechnological/biological products, which require measures for viral safety, a raw material/substrate for drug production derived mainly from human, bovine, swine or equine is used, and it is obvious that such human and animal has to be healthy nature. A wild animal should be avoided, and it is recommended to use animals derived from a colony controlled by an appropriate SPF (Specific Pathogen-Free) condition and bred under a well designed hygienic control, including appropriate control for prevention of microbial contamination and contamination monitoring system. If a meat standard for food is available, an animal meeting this standard has to be used. The type of virus to be concerned about depend on animal species, but it may be possible to narrow down the virus for investigation by means of examining the hygiene control, applicability of a meat standard for food, etc. On the other hand, even with the animals of the same species, a different approach may be necessary depending upon the region where the specimen for a raw material/substrate for drug production is taken. For example, in case of obtaining raw material /substrate for drug production from blood or other specific region, it is necessary to be aware of the risk level, virus multiplication risk, etc. which may specifically exists depending upon its region. Such approach may be different from those applied to body waste such as urine, milk, etc. as a source of raw material/substrate for drug production. Further, cau-

tion has to be taken on transmissible spongiform encephalopathy (TSE) when pituitary gland, etc. is used as a raw material. This report does not include detailed explanation on TSE, but recommendations are to use raw material derived from 1) animals originated in the countries (area) where incidence of TSE has not been reported; 2) animals not infected by TSE; or 3) species of animal which has not been reported on TSE. It is recommended to discuss the matters concerned with TSE with the regulatory authority if there is any unclear point.

Followings are the raw material/substrate used for manufacturing biotechnological/biological products in Japan.

(1) Biological products derived from human

Blood plasma, placenta, urine, etc. derived from human are used as the sources of raw material of biotechnological/biological products. As for these raw materials, there are two cases: 1) Appropriateness can be confirmed by interview or by examination of the individual who supplies each raw material, and 2) Such sufficient interview or examination of the individual can not be made due to the type of raw material. In case that sufficient examination of individual level is not possible, it is necessary to perform the test to deny virus contamination at an appropriate manufacturing stage, for example, the stage to decide it as a substrate for drug production.

(2) Biological products derived from animal besides human

Heparin, gonadotropin, etc. are manufactured from blood plasma or from various organs of bovine, swine and equine.

(3) Protein drug derived from cell line of human or animal origin

In the case of protein drugs derived from cell line of human or animal origin, a cell line of human or animal is the raw material per se, and the substrate for drug production is a cell bank prepared from a cloned cell line (master cell bank or working cell bank). Examination at cell bank level is considered enough for viral safety qualification, but it goes without saying that the more appropriate and rational qualification evaluation test of the master cell bank can be realized when more information is available on the virus of the source animal or on the prehistory of driving the cell line, the base of the cell bank.

4.2. Qualification evaluation test on human or animal as a source of raw material/substrate for drug production

(1) Biological products derived from human

Body fluid etc. obtained from healthy human must be used for biological products production. Further, in case that interview or examination of the individual, who supplies the raw material, can be possible and is necessary, interview under an appropriate protocol and a serologic test well evaluated in aspects of specificity, sensitivity and accuracy have to be performed, so that only the raw material, which is denied latent HBV, HCV and HIV, will be used. In addition to the above, it is necessary to test for the gene of HBV, HCV and HIV by a nucleic amplification test (NAT) well evaluated in aspects of specificity, sensitivity and accuracy.

In case of the raw material (e.g., urine), which can not be tested over the general medical examination of the individual who supplies the material, or of the raw material which is irrational to conduct individual test, the pooled raw material, as the substrate for drug production, has to be conducted at least to deny existence of HBV, HCV and HIV, using a method well evaluated in aspects of specificity, sensitivity and accuracy, such as the antigen test or NAT.

(2) Biological products derived from animal besides human

The animal used for manufacturing biological products has to be under appropriate health control, and has to be confirmed of its health by various tests. Further, it is necessary that the population, to which the animal belongs, has been under an appropriate breeding condition, and that no abnormal individual has been observed in the population. Further, it is necessary to demonstrate information or scientific basis which can deny known causes infection or disease to human, or to deny such animal inherent latent virus by a serologic test or by a nucleic amplification test (NAT). The infectious virus that is known to be common between human and animal, and known to cause infection in each animal are tentatively listed in Table 2. It is necessary that the table is completed under careful examination, and denial of all of them, by means of tests on individual animal, tissue, body fluid, etc. as a raw material, or on pooled raw material (as a direct substrate for drug production), is not always necessary. Table 2 can be used as reference information, in addition to the other information, such as; source of animal, health condition, health and breeding control, conformity to the meat standard for food, etc., to elaborate to which virus what kind of test has to be performed, and for which virus it is not always necessary to test for, etc. It is important to clarify and record the basis of choosing the virus and the test conducted thereof.

(3) Protein drug derived from cell line of human or animal origin

It is important to conduct thorough investigation on latent endogenous and non-endogenous virus contamination in a master cell bank (MCB), which is the cell substrate for drug production, in accordance with the Notice Iyakushin No. 329 entitled ''Viral safety evaluation of biotechnology products derived from cell lines of human or animal origin''. Further, it is necessary to conduct an appropriate adventitious virus test (e.g., *in vitro* and *in vivo* test) and a latent endogenous virus test on the cell at the limit of *in vitro* cell age (CAL) for drug production. Each working cell bank (WCB) as a starting cell substrate for drug production should be tested for adventitious virus either by direct testing or by analysis of cells at the CAL, initiated from the WCB. When appropriate non-endogenous virus tests have been performed on the MCB and cells cultured up to or beyond the CAL have been derived from the WCB and used for testing for the presence of adventitious viruses, similar tests need not be performed on the initial WCB.

5. Points of concern with respect to manufacturing and virus testing

To ensure viral safety of a biological product derived from tissue, body fluid etc. of human or animal origin, it is necessary to exclude any possibility of virus contamination from a raw material, such as tissue and body fluid, or a substrate, paying attention to the source of virus contamination as mentioned in above 3.5, and to adopt appropriate manufacturing conditions and technologies in addition to enhancement of manufacturing environment, so that virus contamination in the course of process and handling and from operators, facilities and environment can be minimized.

In addition to the above, effective virus tests and viral inactivation/removal technology, which are reflected by rapid progress of science, have to be introduced. Adoption of two or more steps with different principles is recommended for virus inactivation/removal process. Further, it is important to minimize any possible virus derivation by using a reagent, of which quality is equivalent to that of a drug. Examples of virus inactivation/removal processes are ① heating (It is reported that almost viruses are inactivated by heating at 55 – 60°C for 30 minutes with exceptions of hepatitis virus, etc. and that liquid heating at 60°C for 10 – 24 hours or dry

heating is effective in case of the products of blood or urine origin.), ② treatment with organic solvent/surfactant (S/D treatment), ③ membrane filtration (15 – 50 nm), ④ acid treatment, ⑤ irradiation (γ-irradiation, etc.), ⑥ treatment with column chromatograph (e.g. affinity chromatography, ion-exchange chromatography), ⑦ fractionation (e.g. organic solvent or ammonium sulfate fractionation), ⑧ extraction.

5.1. Virus test conducted in advance of purification process

(1) Biological products derived from human

In many cases, samples for virus test before purification process are body fluid or tissue of individual collected as a raw material, or its pooled material or extraction as a substrate for drug production. As mentioned in 4.2. (1), it is necessary to deny latent HBV, HCV and HIV by the test evaluated enough in aspects of specificity, sensitivity and accuracy. Even in a case that a non-purified bulk before purification process is produced from a substrate for drug production, it is not always necessary to conduct a virus test again at the stage before purification, so long as the presence of any latent virus can be denied at the stage of substrate by an appropriate virus test, with cases where the non-purified bulk is made from the substrate by adding any reagent etc. of living organisms origin are an exception.

(2) Biological products derived from animal besides human

Similar to 5.1. (1), samples for virus tests before purification process are, in many cases, body fluid or tissue of individual collected as a raw material, or its pooled material or extraction as a substrate for drug production. In these cases, it is necessary to have a data, which can deny latent virus of probable cause of human infection or disease as mentioned in the above 4.2. (2), or to have a result of a serologic test or a nucleic amplification test (NAT) evaluated enough in aspects of specificity, sensitivity and accuracy. The concept, which is applied to a case that non-purified bulk before purification process is produced from a substrate for drug production, is the same as those provided in the above 4.2. (1).

(3) Protein drug derived from cell line of human or animal origin

Generally, a substrate for drug production in this case is a cell bank, and the sample for testing before purification process is a harvested cell after cell culturing or unprocessed bulk which consists of single or pooled complex culture broth. The unprocessed bulk may be sometimes culture broth without cells. Denial of latent virus, which is determined by a virus test at a MCB or WCB level, does not always deny latent virus in unprocessed bulk after culturing. Further, it is noted that the viral test at the CAL is meaningful as a validation but can not guarantee definite assurance of latent virus denial, since the test is generally performed only once. In case of using a serum or a component of blood origin in a culture medium, definite denial of latent virus at the level of unprocessed bulk can not be assured so long as the viral test has not been conducted on each lot at the CAL, since lot renewal can be a variable factor on viral contamination.

A representative sample of the unprocessed bulk, removed from the production reactor prior to further processing, represents one of the most suitable levels at which the possibility of adventitious virus contamination can be determined with a high probability of detection. Appropriate testing for viruses should be performed at the unprocessed bulk level unless virus testing is made more sensitive by initial partial processing (e.g., unprocessed bulk may be toxic in test cell cultures, whereas partially processed bulk may not be toxic). In certain instances it may be more appropriate to test a mixture consisting of both intact and disrupted cells and their cell culture supernatants removed from the production reactor prior to further processing.

In case of unprocessed bulk, it is required to conduct virus tests on at least 3 lots obtained from pilot scale or commercial scale production. It is recommended that manufacturers develop programs for the ongoing assessment of adventitious viruses in production batches. The scope, extent and frequency of virus testing on the unprocessed bulk should be determined by taking several points into consideration including the nature of the cell lines used to produce the desired products, the results and extent of virus tests performed during the qualification of the cell lines, the cultivation method, raw material sources and results of viral clearance studies. Screening *in vitro* tests, using one or several cell lines, are generally employed to test unprocessed bulk. If appropriate, a NAT test or other suitable methods may be used.

Generally, harvest material in which adventitious virus has been detected should not be used to manufacture the product. If any adventitious viruses are detected at this level, the process should be carefully checked to determine the cause of the contamination, and appropriate actions taken.

5.2. Virus test as an acceptance test of an intermediate material, etc.

When a biological product is manufactured from tissue, body fluid etc. of human or animal origin, there are cases that an intermediate material, partially processed as a raw material or substrate for drug production by outside manufacturer, is purchased and used for manufacturing. In such case, if any test to meet this General Information has been conducted by such outside manufacturer, it is necessary for the manufacturer of the biological product, who purchased the intermediate material, to examine what sort of virus test has to be conducted as acceptance tests, and to clarify the basis of rationality including the details of the test conducted.

On the other hand, if no test to meet this General Information has been conducted by such outside manufacturer of the raw material, all necessary virus free test has to be conducted to meet this General Information on the intermediate material regarding it as the direct substrate for drug production.

5.3. Virus test on a final product

Virus tests to be conducted on a final product (or on a product to reach the final product) has to be defined under comprehensive consideration of the type of raw material or substrate for drug production, the result of virus tests conducted on raw material/substrate for drug production, the result of evaluation on viral removal/inactivation processes, any possibility of virus contamination in the manufacturing process, etc. Comprehensive viral safety assurance can only be achieved by appropriate selection of the raw material/substrate for drug production, an appropriate virus test conducted on the raw material/substrate for drug production/intermediate material, the virus test conducted at an appropriate stage of manufacturing, an appropriate viral clearance test, etc. However, there are cases of having specific backgrounds, such as 1) use of the raw material derived from unspecified individual human, 2) possible existence of virus at a window period, 3) specific detection limit of virus test, etc. and in these cases, virus contamination to the final product may occur if there is any deficiency on the manufacturing process (e.g., damage of membrane filter) or any mix-up of the raw materials, etc. To avoid such accidental virus contamination, it may be recommended to conduct a nucleic

Table 2 Infectious viruses known to be common between human and animal and known to cause infection to each animal

	bovine	swine	sheep	goat	equine
Cowpox virus	◎				
Paravaccinia virus	◎	◎	◎	◎	
Murray valley encephalitis virus	◎	◎			
Louping ill virus	◎	◎	◎	◎	
Wesselsbron virus			◎		
Foot-and-mouth disease virus	◎	◎			
Japanese encephalitis virus		◎			
Vesicular stomatitis virus		◎			
Bovine papular stomatitis virus	◎				
Orf virus			◎		
Borna disease virus			◎		◎
Rabies virus	◎	◎	◎	◎	◎
Influenza virus		◎			
Hepatitis E virus		◎			
Encephalomyocarditis virus	◎	◎			
Rotavirus	◎				
Eastern equine encephalitis virus					◎
Western equine encephalitis virus					◎
Venezuelan equine encephalitis virus					◎
Morbillivirus					◎
Hendra virus					◎
Nipah virus		◎			
Transmissible gastroenteritis virus		◎			
Porcine respiratory coronavirus		◎			
Porcine epidemic diarrhea virus		◎			
Hemagglutinating encephalomyelitis virus		◎			
Porcine respiratory and reproductive syndrome virus		◎			
Hog cholera virus		◎			
Parainfluenza virus Type 3		◎			
Talfan/Teschen disease virus		◎			
Reovirus		◎			
Endogenous retrovirus		◎			
Porcine adenovirus		◎			
Porcine circovirus		◎			
Porcine parvovirus		◎			
Porcine poxvirus		◎			
Porcine cytomegalovirus		◎			
Pseudorabies virus		◎			
Russian spring-summer encephalitis virus			◎	◎	
Rift Valley fever virus			◎	◎	
Crimean-Congo hemorrhagic fever virus (Nairovirus)	◎		◎	◎	
Torovirus	◎				

amplification test (NAT) on the final product focusing on the most risky virus among those that may possibly to exist in the raw material.

6. Process evaluation on viral clearance

6.1. Rationale, objective and general items to be concerned with respect to viral clearance process evaluation

Evaluation of a viral inactivation/removal process is important for ensuring safety of a biological product derived from tissue or body fluid of human or animal origin. Conducting evaluation on viral clearance is to assure, even to some extent, elimination of the virus, which may exist in a raw material, etc. or may be derived to the process due to an unexpected situation. Viral clearance studies should be made by a carefully designed appropriate method, and has to be rationally evaluated.

The objective of viral clearance studies is to assess process step(s) that can be considered to be effective in inactivating/removing viruses and to estimate quantitatively the overall level of virus reduction obtained by the process. This should be achieved by the deliberate addition (''spiking'') of significant amounts of a virus at different manufacturing/purification steps and demonstrating its removal or inactivation during the subsequent steps. It is not necessary to evaluate or characterize every step of a manufacturing process if adequate clearance is demonstrated by the use of fewer steps. It should be borne in mind that other steps in the process may have an indirect effect on the viral inactivation/removal achieved. Manufacturers should explain and justify the approach used in studies for evaluating viral clearance.

The reduction of virus infectivity may be achieved by removal of virus particles or by inactivation of viral infectivity. For each production step assessed, the possible mechanism of loss of viral infectivity should be described with regard to whether it is due to inactivation or removal. For inactivation steps, the study should be planned in such a way that samples are taken at different times and an inactivation curve constructed.

6.2. Selection of virus

To obtain a broad range of information of viral inactivation/removal, it is desirable that a model virus used for viral clearance studies should be chosen from the viruses with a broad range of characteristics in aspects of DNA/RNA viruses, with or without envelope, particle size, significant resistance to physical/chemical treatment, etc. and it is necessary to combine about 3 model viruses to cover these characteristics.

At choice of a model virus, there are also the ways to choose a virus closely related to or having the same characteristics of the virus known to exist in the raw material. In such case, it is in principle recommendable to choose a virus which demonstrates a higher resistance to inactivation/removal treatment if two or more candidate viruses are available for choice. Further, a virus which can grow at a high titer is desirable for choice, although this may not always be possible. In addition to the above, choosing a virus, which will provide effective and reliable assay result at each step, is necessary, since sample condition to be tested at each step of a production process may influence the detection sensitivity. Consideration should also be given to health hazard which may pose to the personnel performing the clearance studies.

For the other items taken for consideration at choice of virus, the Notice, Iyakushin No. 329 can be used as a reference. Examples of the virus which have been used for viral clearance studies are shown in Table 3 which was derived from Iyakushin No. 329. However, the Notice, Iyakushin No. 329, is on viral safety of a product derived from cell lines of human or animal origin, and a more appropriate model virus has to be chosen taking into account the origin/raw material of biological products.

6.3. Design of viral clearance studies

The purpose of viral clearance studies is to quantitatively evaluate removal or inactivation capability of a process, in which a virus is intentionally spiked to a specific step of a manufacturing process.

Following are the precautions to be taken at planning viral clearance studies.

(1) Care should be taken in preparing the high-titer virus to avoid aggregation which may enhance physical removal and decrease inactivation thus distorting the correlation with actual production.

(2) Virus detection methods give great influence to the viral clearance factor. Accordingly, it is advisable to gain detection sensitivity of the methods available in advance, and use a method with a detection sensitivity as high as possible. Quantitative infectivity assays should have adequate sensitivity and reproducibility in each manufacturing process, and should be performed with sufficient replicates to ensure adequate statistical validity of the result. Quantitative assays not associated with infectivity may be used if justified. Appropriate virus controls should be included in all infectivity assays to ensure the sensitivity of the method. Also, the statistics of sampling virus when at low concentrations (for example, number of virus is 1-1000/L) should be considered.

(3) Viral clearance studies are performed in a miniature size system that simulates the actual production process of the biotechnological/biological product used by the manufacturer. It is inappropriate to introduce any virus not used for manufacturing into a production facility because of GMP constraints. Therefore, viral clearance studies should be conducted in a separate laboratory equipped for virological work and performed by a staff with virological expertise in conjunction with production personnel involved in designing and preparing a scaled-down version of the purification process. The viral clearance studies should be performed under the basic concept of GLP.

(4) Each factor on a viral clearance study of a process, which is performed in miniature size, should reflect that of actual manufacturing as far as possible, and its rationality should be clarified. In case of chromatograph process, length of column bed, linear velocity, ratio of bed volume per velocity (in other words, contact time), buffer, type of column packing, pH, temperature, protein concentration, salt concentration and concentration of the objective product are all correspondent to those of the actual production. Further, similarity of the elution profile should be achieved. For the other process, similar concept should be applied. If there is any factor which can not reflect the actual production, its effect to the result should be examined.

(5) It is desirable that two or more inactivation/removal processes of different principles are selected and examined.

(6) As for the process which is expected to inactivate/remove virus, each step should be evaluated in aspect of clearance capability, and carefully determined if it is the stage of inactivation, removal or their combination for designing the test. Generally, in viral clearance tests, a virus is spiked in each step which is the object of the test, and after passing through the process in question, the reduction level of infectivity is evaluated. But, in some case, it is accepted that a high potential virus is spiked at a step of the process, and virus concentration of each succeeding step is carefully monitored. When removal of virus is made by separation or fractionation, it is desirable to investigate how the virus is separated or fractionated (mass balance).

(7) For assessment of viral inactivation, unprocessed crude material or intermediate material should be spiked with infectious virus and the reduction factor calculated. It should be recognized that virus inactivation is not a simple, first order reaction and is usually more complex, with a fast ''phase 1'' and a slow ''phase 2''. The study should, therefore, be planned in such a way that samples are taken at different times and an inactivation curve constructed. It is recommended that studies for inactivation include at least one time point less than the minimum exposure time and greater than zero, in addition to the minimum exposure time. The reproducible clearance should be demonstrated in at least two independent studies. When there is a possibility that the virus is a human pathogen, it is very important that the effective inactivation process is designed and more detailed data (more points) for the virus (or the same or closely related viruses) are obtained. The initial virus load should be determined from the virus which can be detected in the spiked starting material. If this is not possible, the initial virus load may be calculated from the titer of the spiking virus preparation. Where inactivation is too rapid to plot an inactivation curve using process conditions, appropriate controls should be performed to demonstrate that infectivity is indeed lost by inactivation.

(8) If antibody against virus exists in an unprocessed material, caution should be taken at clearance studies, since it may affect the behavior of virus at viral removal or inactivation process.

(9) The amount of virus spiked in unprocessed material should be sufficient enough to evaluate viral removal or inactivation capability of the process. However, the virus ''spike'' to be added to the unprocessed material should be as small as possible in comparison with the sample volume of the unprocessed material so as not to cause characteristic change of the material by addition of the virus nor to cause behavioral change of the protein in the material by dilution.

(10) It is desirable that the virus in the sample is subject for quantitative determination without applying ultracen-

Table 3 Example of viruses which have been used for viral clearance studies

Virus	Family	Genus	Natural host	Genome	Env	Size (nm)	Shape	Resistance
Vesicular Stomatitis Virus	Rhabdo	Vesiculovirus	Equine Bovine	RNA	yes	70 × 150	Bullet	Low
Parainfluenza Virus	Paramyxo	Type 1,3 Respirovirus Type 2,4 Rubulavirus	Various	RNA	yes	100 – 200 +	Pleo-Spher	Low
MuLV	Retro	Type C oncovirus	Mouse	RNA	yes	80 – 110	Spherical	Low
Sindbis Virus	Toga	Alphavirus	Human	RNA	yes	60 – 70	Spherical	Low
BVDV	Flavi	Pestivirus	Bovine	RNA	yes	50 – 70	Pleo-Spher	Low
Pseudorabies Virus	Herpes	Varicellovirus	Swine	DNA	yes	120 – 200	Spherical	Med
Poliovirus Sabin Type 1	Picorna	Enterovirus	Human	RNA	no	25 – 30	Icosahedral	Med
Encephalomyocarditis Virus	Picorna	Cardiovirus	Mouse	RNA	no	25 – 30	Icosahedral	Med
Reovirus Type 3	Reo	Orthoreovirus	Various kind	RNA	no	60 – 80	Spherical	Med
SV 40	Papova	Polyomavirus	Monkey	DNA	no	40 – 50	Icosahedral	Very high
Parvovirus: canine, porcine	Parvo	Parvovirus	Canine Porcine	DNA	no	18 – 24	Icosahedral	Very high

trifuge, dialysis, storage, etc. as far as possible. However, there may be a case that any handling before a quantitative test, such as remove procedure of an inhibitor or a toxic substance, storage for a period to realize test at a time, etc., is inevitable. If any manipulation, such as dilution, concentration, filtration, dialysis, storage, etc., is applied for preparation of the sample for testing, a parallel control test, which passes through a similar manipulation, should be conducted to assess infectivity variance at the manipulation.

(11) Buffers and products (desired protein or other component contained therein) should be evaluated independently for toxicity or interference in assays used to determine the virus titer, and measures should be taken so as not to interfere with the assays. If the solutions are toxic to the indicator cells, dilution, adjustment of the pH, or dialysis of the buffer containing spiked virus might be necessary. If the product itself has anti-viral activity, the clearance study may need to be performed without the product in a mock run, although omitting the product or substituting a similar protein that does not have anti-viral activity could affect the behaviour of the virus in some production steps.

(12) Many purification schemes use the same or similar buffers or columns, repetitively. The effects of this approach should be taken into account when analyzing the data. The effectiveness of virus elimination by a particular process may vary with the stage in manufacture at which it is used.

(13) Overall reduction factors may be underestimated where production conditions or buffers are too cytotoxic or virucidal and should be discussed on a case-by-case basis. Overall reduction factors may also be overestimated due to inherent limitations or inadequate design of viral clearance studies.

(14) It has to be noted that clearance capability of viral removal/inactivation process may vary depending upon the type of virus. The viral removal/inactivation process, which displays viral clearance by a specific principle or mechanism, may be quite effective to the virus, which meets such mechanism of action, but not effective to the other type of viruses. For example, S/D (Solvent/Detergent) treatment is generally effective to the virus with lipid membrane, but not effective to the non-enveloped virus. Further, some virus is resistant to the general heating process (55 – 60°C, 30 minutes). When clearance is expected for such virus, introduction of a further severe condition or process, which has a different principle or mechanism, is necessary. Virus removal by membrane filtration, which is different from S/D or heat treatment in aspect of principle, is effective to a broad range of virus that can not pass through the membrane. Affinity chromatography process, which specifically absorbs the objective protein, can thoroughly wash out the materials other than the objective protein including virus etc. and is generally effective for viral removal. Separation/fractionation of a virus from an objective protein is sometimes very difficult, but there are not so rare that ion exchange chromatography, ethanol fractionation, etc. is effective for clearance of a virus which can not be sufficiently inactivated or removed by the other process.

(15) Effective clearance may be achieved by any of the following: multiple inactivation steps, multiple complementary separation steps, or combinations of inactivation and separation steps. Separation methods may be dependent on the extremely specific physico/chemical properties of a virus which influence its interaction with gel matrices and precipitation properties, and the isolation may differ for each virus. However, despite these potential variables, effective removal can be obtained by a combination of complementary separation steps or combinations of inactivation and separation steps. Well designed separation steps that have been thoroughly examined on the items that affect the separation of a target virus and a mode virus, such as chromatographic procedures, filtration steps and extractions, can be also effective virus removal steps provided that they are performed under appropriately controlled conditions.

(16) An effective virus removal step should give reproducible reduction of virus load shown by at least two independent studies.

(17) Over time and after repeated use, the ability of chromatography columns and other devices used in the purification scheme to clear virus may vary. Some estimate of the stability of the viral clearance after several uses may

provide support for repeated use of such columns.

(18) The Notice, Iyakushin No. 329, would be used as a reference when viral clearance studies on biological products are designed.

6.4. Interpretation of viral clearance studies

6.4.1. Evaluation on viral clearance factor

The viral clearance factor is a logarithm of reduction ratio of viral amount (infectious titer) between each step applied for viral clearance of a manufacturing process. The total viral clearance factor throughout the process is sum of the viral clearance factor of each step appropriately evaluated.

Whether each and total viral clearance factor obtained are acceptable or should not be evaluated in aspects of every virus that can be realistically anticipated to derive into the raw material or the manufacturing process, and its rationality should be shown.

In case that existence of any viral particle is recognized in a substrate for drug production, e.g., a substrate of rodent origin for biodrug production, it is important not only to demonstrate removal or inactivation of such virus, but also to demonstrate that the purification process has enough capability over the required level to assure safety of the final product at an appropriate level. The virus amount removed or inactivated in a manufacturing process should be compared with the virus amount assumed to exist in the substrate etc. used for manufacturing drug, and for this purpose, it is necessary to obtain the virus amount in the raw materials/substrate for drug production, etc. Such figure can be obtained by measuring infectious titer or by the other method such as transmission electron microscope (TEM). For evaluation of overall process, a virus amount, far larger than that assumed to exist in the amount of the raw materials/substrate for drug production which is equivalent to single administration of the final product, has to be removed. It is quite rare that existence of virus can be assumed in a substrate for drug production, with the exception of the substrate of rodent origin, and such suspicious raw material/substrate for drug production should not be used for manufacturing drug with a special exceptional case that the drug in question is not available from the other process and is clinically indispensable, and that the information including infectious properties of the virus particle assumed to exist has been clarified.

Any virus contaminations in the substrates for drug production of biotechnological/biological products are usually denied by some tests or examinations. In such case, a specific virus that can possibly contaminate may be used as a model. However, in general, it would be necessary to perform a viral clearance test by choosing a combination of appropriate model viruses that can show the capability for clearance of a wide variety of viruses in the process, as indicated in 6.2. In this case, a common numerical goal cannot be established on the viral clearance. Therefore, the validity of viral clearance factor of the process should be taken into account, considering various information on factual possibility of virus contaminations of the substrates and others, detection sensitivity of the virus free test, and other cases in publications.

6.4.2. Calculation of viral clearance factor

The viral clearance factor, "R", for viral removal/inactivation process can be calculated by the following formula.

$$R = \log[(V_1 \times T_1)/(V_2 \times T_2)]$$

In which

R: Logarithm of reduction ratio

V_1: Sample volume of the unprocessed material

T_1: Virus amount (titer) of the unprocessed material

V_2: Sample volume of the processed material

T_2: Virus amount (titer) of the processed material

At the calculation of the viral clearance factor, it is recommendable to use the virus titer detected in the sample preparation of the unprocessed material after addition of virus, not the viral titer added to the sample preparation wherever possible. If this is not possible, loaded virus amount is calculated from virus titer of the solution used for spike.

6.4.3. Interpretation of results and items to be concerned at evaluation

At the interpretation and the evaluation of the data on effectiveness of viral inactivation/removal process, there are various factors to be comprehensively taken into account, such as ① appropriateness of the virus used for the test, ② design of the viral clearance studies, ③ virus reduction ratio shown in logarithm, ④ time dependence of inactivation, ⑤ factors/items which give influence to the inactivation/removal process, ⑥ sensitivity limit of virus assay method, ⑦ possible effect of the inactivation/removal process which is specific to certain class of viruses.

Additional items to be concerned at appropriate interpretation and evaluation of the viral clearance data are as follows:

(1) Behavior of virus used to the test

At interpretation of the vial clearance results, it is necessary to recognize that clearance mechanism may differ depending upon the virus used for the test. Virus used for a test is generally produced in tissue culture, but behavior of the virus prepared in the tissue culture may be different from that of the native virus. Examples are possible differences of purity and degree of aggregation between the native and the cultured viruses. Further, change of surface properties of a virus, e.g., addition of a sucrose chain which is ascribed to specific nature of a separation process, may give effect to the separation. These matters should be also considered at interpretation of the results.

(2) Design of test

Viral clearance tests should have been designed taking into account variation factors of the manufacturing process and scaling down, but there still remain some variance from actual production scale. It is necessary to consider such variance at the interpretation of the data and limitation of the test.

(3) Acceptability of viral reduction data

The total viral clearance factor is expressed as a sum of logarithm of reduction ratio obtained at each step. The summation of the reduction factors of multiple steps, particularly of steps with little reduction (e.g., below 1 $\log_{10}$), may overestimate viral removal/inactivation capability of the overall process. Therefore, virus titer of the order of 1 $\log_{10}$ or less has to be ignored unless justified. Further, a viral clearance factor achieved by repeated use of the same or similar method should be ignored for calculation unless justified.

(4) Time dependence of inactivation

Inactivation of virus infectivity frequently shows biphasic curve, which consists of a rapid initial phase and subsequent slow phase. It is possible that a virus not inactivated in a step may be more resistant to the subsequent step. For example, if an inactivated virus forms coagulation, it may be resistant to any chemical treatment and heating.

(5) Evaluation of viral reduction ratio shown in logarithm

The viral clearance factor shown in logarithm of reduction ratio of virus titer can demonstrate drastic reduction of residual infectious virus, but there is a limit that infectious titer can never be reduced to zero. For example, reduction in

infectivity of a preparation containing 8 $\log_{10}$ infectious unit per mL by a factor of 8 $\log_{10}$ leaves zero $\log_{10}$ per mL or one infectious unit per mL, taking into account the detection limit of the assay.

(6) Variable factor of manufacturing process

Minor variance of a variation factor of a manufacturing process, e.g., contact time of a spiked sample to a buffer or a column, will sometimes give influence to viral removal or inactivation effect. In such case, it may be necessary to investigate to what extent such variance of the factor has given influence to the process concerned in aspect of viral inactivation.

(7) Existence of anti-viral antibody

Anti-viral antibody that exists in the sample preparation used for a test may affect sensitivity of distribution or inactivation of a virus, which may result in not only defusing the virus titer but complicating interpretation of the test result. So, existence of anti-viral antibody is one of the important variable factors.

(8) Introduction of a new process for removal/inactivation

Viral clearance is an important factor for securing safety of a drug. In case that an achievement level of infective clearance of a process is considered insufficient, a process which is characterized by an inactivation/removal mechanism to meet the purpose or an inactivation/removal process which can mutually complement to the existence process has to be introduced.

(9) Limit of viral clearance studies

Viral clearance studies are useful for contributing to the assurance that an acceptable level of safety in the final product is achieved but do not by themselves establish safety. However, a number of factors in the design and execution of viral clearance studies, and the interpretation of the results may lead to an incorrect estimate of the ability of the process to remove virus infectivity, as described above.

7. Statistics

The viral clearance studies should include the use of statistical analysis of the data to evaluate the results. The study results should be statistically valid to support the conclusions reached.

7.1. Statistical considerations for assessing virus assays

Virus titrations suffer the problems of variation common to all biological assay systems. Assessment of the accuracy of the virus titrations and the reduction factors derived from them and the validity of the assays should be performed to define the reliability of a study. The objective of statistical evaluation is to establish that the study has been carried out to an acceptable level of virological competence.

1. Assay methods may be either semiquantitative or quantitative. Both semiquantitative and quantitative assays are amenable to statistical evaluation.

2. Variation can arise within an assay as a result of dilution errors, statistical effects and differences within the assay system which are either unknown or difficult to control. These effects are likely to be greater when different assay runs are compared (between-assay variation) than when results within a single assay run are compared (within-assay variation).

3. The 95% confidence limits for results of within-assay variation normally should be on the order of ± 0.5 $\log_{10}$ of the mean. Within-assay variation can be assessed by standard textbook methods. Between-assay variation can be monitored by the inclusion of a reference preparation, the estimate of whose potency should be within approximately 0.5 $\log_{10}$ of the mean estimate established in the laboratory for the assay to be acceptable. Assays with lower precision may be acceptable with appropriate justification.

7.2. Reproducibility and confidence limit of viral clearance studies

An effective virus inactivation/removal step should give reproducible reduction of virus load shown by at least two independent studies.

The 95% confidence limits for the reduction factor observed should be calculated wherever possible in studies of viral clearance. If the 95% confidence limits for the viral assays of the starting material are $\pm s$, and for the viral assays of the material after the step are $\pm a$, the 95% confidence limits for the reduction factor are $\pm \sqrt{s^2 + a^2}$.

8. Re-evaluation of viral clearance

Whenever significant changes in the production or purification process are made, the effect of that change, both direct and indirect, on viral clearance should be re-evaluated as needed. Changes in process steps may also change the extent of viral clearance.

9. Measurement for viral clearance studies

9.1. Measurement of virus infective titer

Assay methods may be either semiquantitative or quantitative. Semiquantitative methods include infectivity assays in animals or in cultured cell infections dose (CCID) assays, in which the animal or cell culture is scored as either infected or not. Infectivity titers are then measured by the proportion of animals or culture infected. In quantitative methods, the infectivity measured varies continuously with the virus input. Quantitative methods include plaque assays where each plaque counted corresponds to a single infectious unit. The methods should be sufficiently sensitive and reproducible, and controls should be used to obtain statistically analyzable results. Both quantal and quantitative assays are amenable to statistical evaluation.

9.2. Testing by nucleic-acid-amplification test (NAT)

NAT can detect virus genomes in individual samples, pooled raw material/cell substrate for drug production or products at a high sensitivity even in a stage that a serum test on each virus is negative. Further, it can detect HBV or HCV gene, which can not be measured in culture system. Window periods can be drastically shortened at the test on HBV, HCV and HIV, and the method is expected to contribute as an effective measure for ensuring viral safety. However, depending upon a choice of primer, there may be a case that not all the subtype of objective virus can be detected by this method, and, therefore, it is recommendable to evaluate, in advance, if subtypes of a broad range can be detected.

NAT will be an effective evaluation method for virus removal capability for viral clearance. However, in case of viral inactivation process, viral inactivation obtained by this method may be underrated, since there is a case that inactivated virus still shows positive on nucleic acid. Further, at introduction of NAT, cautions should be taken on rationality of detection sensitivity, choice of a standard which is used as run-control, quality assurance and maintenance of a reagent used for primer, interpretation of positive and negative results, etc.

10. Reporting and preservation

All the items relating to virus test and viral clearance studies should be reported and preserved.

11. Others

The Notice, Iyakushin No. 329, should be used as a reference at virus tests and viral clearance studies.

Conclusion

As mentioned at the Introduction, assurance of quality/

safety etc. of JP listed drugs should be achieved by state-of-the-art methods and concepts reflecting the progress of science and accumulation of experiences.

The basis for ensuring viral safety of JP listed biotechnological/biological products is detailed in this General Information. What is discussed here is that an almost equal level of measures are required for both development of new drugs and for existing products as well, which means that the similar level of concerns should be paid on both existing and new products in aspect of viral safety. This document is intended to introduce a basic concept that quality and safety assurance of JP listed product should be based upon the most advanced methods and concepts. This document has been written to cover all conceivable cases, which can be applied to all biotechnological/biological products. Therefore, there may be cases that it is not so rational to pursue virus tests and viral clearance studies in accordance with this document on each product, which has been used for a long time without any safety issue. So, it will be necessary to elaborate the most rational ways under a case-by-case principle taking into due consideration source, origin, type, manufacturing process, characteristics, usages at clinical stage, accumulation of the past usage record, etc. relating to such biotechnological/biological products.

Mycoplasma Testing for Cell Substrates used for the Production of Biotechnological/Biological Products <*G3-14-170*>

This document describes the currently available methods of mycoplasma testing that should be performed for cell substrates that are used in the manufacture of biotechnological/biological products.

Methods suggested for detection of mycoplasma are, A. culture method, B. indicator cell culture method, and C. nucleic acid amplification test (NAT) method.

Mycoplasma testing should be performed on the master cell bank (MCB) and the working cell bank (WCB), as well as on the cell cultures used during the manufacturing process of the product. For the assessment of these cells, mycoplasma testing should be performed using both methods A and B. Note that method C may be used as an alternative to methods A and/or B after suitable validation.

Prior to mycoplasma testing by methods A or B, the sample should be tested to detect the presence of any factors inhibiting the growth of mycoplasma. If such growth-inhibiting factors are detected, they should be neutralized or eliminated by an appropriate method, such as centrifugation or cell passage.

If the test will be performed within 24 hours of obtaining the sample, the sample should be stored at 2 – 8°C. If more than 24 hours will elapse before the test is performed, the sample should be stored at −60°C or lower.

If mycoplasma is detected, additional testing to identify the species may be helpful in determining the source of contamination.

A. Culture Method

1. Culture Medium

Both agar plates and broth are used. Each batch of agar and broth medium should be free of antibiotics except for penicillin. Refer to the Minimum Requirements for Biological Products regarding selection of the culture media. Other culture media may be used if they fulfill the requirements described in the following section 2.

2. Suitability of Culture Medium

Each batch of medium should be examined for mycoplasma growth-promoting properties. To demonstrate the capacity of the media to detect known mycoplasma, each test should include control cultures of at least two known species or strains of mycoplasma, one of which should be a dextrose fermenter (i.e., *Mycoplasma pneumoniae* ATCC 15531, NBRC 14401 or equivalent species or strains) and one of which should be an arginine hydrolyser (i.e., *Mycoplasma orale* ATCC 23714, NBRC 14477 or equivalent species or strains). The mycoplasma strains used for the positive control tests should be those with a low number of passages obtained from an official or suitably accredited agency, and handled appropriately. Inoculate the culture medium with 100 colony-forming units (CFU) or 100 color-changing units (CCU) or less.

3. Culture and Observation

1) Inoculate no less than 0.2 mL of the test sample (cell suspension) in evenly distributed amounts over the surface of each of two or more agar plates. After the surfaces of the inoculated plates are dried, the plates should be incubated in an atmosphere of nitrogen containing 5 – 10% carbon dioxide and adequate humidity at 35 – 37°C for no less than 14 days.

2) Inoculate no less than 10 mL of the test sample (cell suspension) into each of one or more vessels containing 100 mL of broth medium, and incubate at 35 – 37°C.

If the culture medium for the sample cells contains any growth-inhibiting factors, such as antibiotics, these factors should be removed. Refer to the Validation tests for growth-inhibiting factors described in the Minimum Requirements for Biological Products for the detection of growth-inhibiting factors.

3) Subculture 0.2 mL of broth culture from each vessel on the 3rd, 7th, and 14th days of incubation onto two or more agar plates. Observe the broth media every 2 or 3 days and if a color change occurs, subculture. The plates should be incubated in nitrogen containing 5 – 10% carbon dioxide and adequate humidity at 35 – 37°C for no less than 14 days.

4) Examination of all plates for mycoplasma colonies should be done microscopically on the 7th and 14th day at 100 times magnification or greater.

B. Indicator Cell Culture Method

Using Vero cell culture substrate, pretest the suitability of the method using an inoculum of 100 CFU or 100 CCU or less of *Mycoplasma hyorhinis* (ATCC 29052, ATCC 17981, NBRC 14858 or equivalent species or strains) and *M. orale* (ATCC 23714, NBRC 14477 or equivalent species or strains).

Indicator cell substrate equivalent to Vero cells and suitable mycoplasma strains may be acceptable if data demonstrate at least equal sensitivity for the detection of known mycoplasma contaminants. The mycoplasma strains should be those with a low number of passages obtained from an official or suitably accredited agency, and handled appropriately, and the unit of inoculation should be determined before use. The cell substrate used should be obtained from a qualified cell bank and certified to be mycoplasma free. The acquired cells should be carefully cultured and propagated, and sufficient volumes of seed stock should be prepared with the proper precautions to avoid mycoplasma contamination. The stock should be tested for mycoplasma contamination using at least one of the methods described in this document, then frozen for storage. For each test this stock should be thawed and used within 6 passages.

Indicator cell cultures should be grown on cover slips submerged in culture dishes or equivalent containers for one day. Inoculate no less than 1 mL of the test sample (cell culture supernatant) into two or more of the culture dishes.

The test should include a negative (non-infected) control and two positive mycoplasma controls, such as *M. hyorhinis* (ATCC 29052, ATCC 17981, NBRC 14858 or equivalent species or strains) and *M. orale* (ATCC 23714, NBRC 14477 or equivalent species or strains). Use an inoculum of 100 CFU or 100 CCU or less for the positive controls.

Incubate the cell cultures at 35 – 38°C for 3 – 6 days in an atmosphere of air containing 5% carbon dioxide.

Examine the cell cultures after fixation for the presence of mycoplasma by epifluorescence microscopy (400 to 600 times magnification or greater) using a DNA-binding fluorochrome, such as bisbenzimide or an equivalent stain. Compare the microscopical appearance of the test cultures with that of the negative and positive controls.

Procedure

1) Aseptically place a sterilized glass cover slip into each cell culture dish (35 mm diameter).

2) Prepare Vero cell suspension in Eagle's minimum essential medium containing 10% fetal calf serum at a concentration of 1×10^4 cells per mL. The fetal calf serum should be tested and confirmed to be free from mycoplasma prior to use.

3) Inoculate aliquots of 2 mL of the Vero cell suspension into each culture dish. Ensure that the cover slips are completely submerged, and not floating on the surface of the culture medium. Incubate the cultures at 35 – 38°C in an atmosphere of air containing 5% carbon dioxide for one day, so that the cells are attached to the glass cover slip.

4) Replace 2 mL of the culture medium with fresh medium, then add 0.5 mL of the test sample (cell culture supernatant) to each of two or more culture dishes. Perform the same procedure for the positive (2 types of mycoplasmas, such as *M. hyorhinis* (ATCC 29052, ATCC 17981, NBRC 14858 or equivalent species or strains) and *M. orale* (ATCC 23714, NBRC 14477 or equivalent species or strains) and negative controls.

5) Incubate the cultures at 35 – 38°C for 3 – 6 days in an atmosphere of air containing 5% carbon dioxide.

6) Remove the culture medium from the culture dishes, and add 2 mL of a mixture of acetic acid (100) and methanol (1:3) (fixative) to each dish; then, allow them to stand for 5 minutes.

7) Remove the fixative from each dish, then add the same amount of fixative again, and leave the dishes to stand for 10 minutes.

8) Remove the fixative and then completely air-dry all the dishes.

9) Add 2 mL of bisbenzimide fluorochrome staining solution to each culture dish. Cover the dishes and let them stand at room temperature for 30 minutes.

10) Aspirate the staining solution and rinse each dish with 2 mL of distilled water 3 times. Take out the glass cover slips and dry them.

11) Mount each cover slip with a drop of a mounting fluid. Blot off surplus mounting fluid from the edges of the cover slips.

12) Examine by epifluorescence microscopy at 400 to 600 times magnification or greater.

13) Compare the microscopic appearance of the test sample with those of the negative and positive controls.

14) The test result is judged to be positive if there are more than 5 cells per 1000 (0.5%) that have minute fluorescent spots that appear to surround, but are outside, the cell nucleus.

C. Nucleic Acid Amplification Test (NAT)

Nucleic acid amplification test (NAT) is a detection method of genes or mRNA transcribed from genes of target cells or viruses by enzymatic amplification with specific primers for target nucleic acid sequences, and the amplified products are detected by several ways. When NAT is used for detection of mycoplasma, high sensitivity detection is expected for the presence or absence of the target sequence derived from mycoplasma by amplification of nucleic acid extracted from a test sample (cell suspension or cell culture supernatant) with specific primers/probes. NAT indicates the presence of a target sequence and not necessarily the presence of viable mycoplasmas.

A number of different NAT methods are available. This general information does not prescribe a particular method. NAT method applied should be validated for sufficient sensitivity, specificity, and robustness of results that remain unaffected by small variations in extraction method parameters or in composition of the reaction mix. Any NAT method is available if the specificity and the sensitivity is properly validated as described in this section. Where a commercial kit is used, certain elements of the validation may be carried out by the manufacturer and information provided to the user. However, it should be remembered that the different results might be obtained by user depending on the instrument used and the target cells tested. The user should confirm the manufacturer's validation results by own facilities. Especially, when the target cell substrate is different from cells validated by the manufacturer, the detection limit and reproducibility of the kit should be confirmed with the cells of interest. When the user's extraction method or instruments used for detection etc. are different from the method or the instrument specified by the manufacturer, the employed method or the instrument should be validated.

In addition, when the information on the primers/probes or the kit reagents may not be available from the manufacturer, countermeasure is required to obtain the information from the manufacturer about the modification of the kit production when modified. If the composition of the kit reagents is modified, user should confirm that the detection limit and the detection accuracy of the modified kit for target mycoplasma is comparable to the previous one, as needed. On the other hand, appropriate alternative method should be considered, since the production of the kit may be discontinued.

Basically, cell suspension but not cell culture supernatant will be used as a test sample, since mycoplasma contaminated in cell culture mainly growth in a cell-dependent manner. When cell culture supernatant is used as test samples, validation is required that the method employed is able to fully detect the mycoplasma contamination in cell cultures.

NAT may be used instead of methods A and/or B, after suitable validation described below, and the validation revealed sufficient sensitivity for all of the listed mycoplasma species.

In order to increase the detection sensitivity of nucleic acid derived from infectious mycoplasma, it is possible to perform NAT after enrichment of mycoplasma that may be present in test samples by culturing with Vero cells. In this case, again, validation is required to show sufficient sensitivity for all of the listed mycoplasma species.

C-1. Mycoplasma testing by NAT

The tests should include both a positive control (run control) (such as *M. hyorhinis* (ATCC 17981, NBRC 14858 or equivalent species or strains) of 100 CFU or 100 CCU or

less) and a negative control. The mycoplasma strains used for the positive control tests should be those within a low number of passages obtained from an official or suitably accredited agency, and handled appropriately. The unit of inoculation should be determined before use. When cell suspension is used as a test sample, a preliminary test is required for the effect of cellular nucleic acid to NAT with the cells confirmed to be mycoplasma-free as a negative control, and confirm that no positive signals are obtained from the negative control. The test result is judged to pass the test if no mycoplasma sequences are amplified from the test sample.

C-2. Precautions for the test

Because NAT enables the detection of trace amounts of nucleic acid, false positive results may be obtained by contamination of the facilities, instruments and reagents etc. with amplified products. To prevent the risk of contamination, wherever possible, each step of the storage and preparation of reagents, the extraction of nucleic acid, the amplification of nucleic acid, and the detection of amplified products should be performed in separate facilities or equipments with special precautions for handling. To exclude false-positive results by contamination of carry-over amplified products, Uracil-N-glycosylase (UNG) procedure may be available. To exclude the false-negative results by low efficiency of extraction or interfering substances for NAT in test samples, simultaneously detection of house-keeping genes of the test cells as internal control is recommended.

On the other hand, if an automatic closed system from extraction to amplification is used to prevent cross-contamination, segregation of the area is not always required. However, measures to prevent contamination are required when disposing the amplified products from the automatic system.

C-3. Validation of NAT for the detection of mycoplasmas

NAT methods for the detection of target sequences are either qualitative or quantitative tests. To detect mycoplasma contamination of cell substrates, qualitative tests are adequate and may be considered to be limit tests. This section describes methods to validate qualitative NAT analytical procedures for assessing mycoplasma contamination. These validation methods may also be applicable for quantitative NAT with an optimal cut-off point.

The most important parameters for validation of the analytical procedure by NAT are the specificity and the detection limit. In addition, the robustness of the analytical procedure should be evaluated. Note that for the purpose of this document, validation of NAT method is defined as the complete procedure from extraction of nucleic acid to detection of the amplified products.

Where commercial kits are used for a part or all of the analytical procedure, documented full validation data already covered by the kit manufacturer can replace validation data by the user, and a full validation by the user is unnecessary. Nevertheless, the performance of the kit with respect to its intended use and user's test system should be demonstrated by the user (e.g. specificity, detection limit).

NAT may be used as:

- a test for in-process control purposes;
- an alternative method to replace methods A and/or B.

This section will thus separate these 2 objectives by presenting first a guideline for the validation of the NAT themselves, and second, a guideline for a comparability study between NAT and methods A or B.

Mycoplasma reference strains evaluated for concentration either in CFUs or equivalent copies are required at various stages during validation of specificity or detection limit of NAT. During routine application of the test, mycoplasma reference strains or the test sample calibrated for concentration using reference strains are used as positive controls. In the test, mycoplasma or mycoplasma nucleic acid (e.g. plasmid) may be used as a positive control. Mycoplasma is required for validation of the procedure including extraction efficiency.

1) Evaluation parameters

Three parameters should be evaluated: specificity, detection limit, and robustness.

2) Specificity

Specificity of NAT is the ability to unequivocally detect a target nucleic acid in the presence of test samples that may be expected to be present. The specificity of NAT is dependent on the choice of primers/probes and the strictness of the test conditions (both of the amplification and the detection steps).

It is important to use primers/probes by choosing nucleic acid sequences that are specific and well conserved for a wide range of mycoplasmas (the bacterial class *Mollicutes* such as the genus *Mycoplasma* and related genera such as *Ureaplasma*, *Spiroplasma*, *Acholeplasma* etc.). The ability of NAT to detect a large panel of mycoplasma species should be demonstrated by experimental results using reference mycoplasmas described in 3), and evaluation only by the theoretical analysis of primers/probes comparing with databases is not recommended.

3) Detection limit

The detection limit of an individual analytical procedure is the lowest amount of a target nucleic acid in a sample that can be detected but not necessarily quantitated as an exact value. For establishment of the detection limit, a positive cut-off point should be determined for NAT. The positive cut-off point is the target sequence copies per volume of sample that can be detected in 95% of test runs. This positive cut-off point is influenced by the nucleic acid sequences of target mycoplasma in the individual samples being tested and by factors such as enzyme efficiency, and can result in different 95% cut-off values for individual analytical test runs. To determine the positive cut-off point, a dilution series of characterized and calibrated (either in CFUs or nucleic acid copies) mycoplasma reference strains or international standards should be tested on different days to examine variation between test runs.

For validation of the limit of detection, the following species should be used. These species represent an optimal selection in terms of the frequency of occurrence as contaminants of mammalian culture cells used for production of biotechnological/biological products, phylogenetic relationships, and animal-derived components used during culture and production processes. Note that the list is only for validation of NAT and not for used as positive run control in routine tests.

- *Acholeplasma laidlawii* (ATCC 23206, NBRC 14400 or equivalent strains)
- *Mycoplasma arginini* (ATCC 23838 or equivalent strains)
- *Mycoplasma fermentans* (ATCC 19989, NBRC 14854 or equivalent strains)
- *Mycoplasma hyorhinis* (ATCC 17981, NBRC 14858 or equivalent strains)
- *Mycoplasma orale* (ATCC 23714, NBRC 14477 or equivalent strains)
- *Mycoplasma pneumoniae* (ATCC 15531, NBRC 14401 or equivalent strains)
- *Mycoplasma salivarium* (ATCC 23064, NBRC 14478 or equivalent strains)

Where there is use of insect or plant cells during produc-

tion, mycoplasma strains derived from insect or plant (e.g. *Spiroplasma citri*) should be tested in addition to the above list. Where there is use of avian cells or materials during production, mycoplasma species derived from avian should be tested whether avian mycoplasmas (e.g. *Mycoplasma synoviae*) can be detected.

For establishment of the detection limit, appropriate dilution series (10-fold or $10^{0.5}$-fold dilution) should be prepared from the undiluted mycoplasma evaluated for concentration (CFU etc.), and tests by NAT should be performed for each dilution. Based on the dilution factor that shows the limit of the detection, a positive cut-off point should be determined as the minimum number of CFUs of target sequences in the test sample. In case amplified products are separated by electrophoresis and the positive band is detected by fluorescent staining, confirmation is required whether no positive band is appeared from the test sample of mycoplasma-free cells. Detection using quantitative real-time PCR requires to set an adequate cut-off point of amplification cycles, and the setting of the cut-off point should be validated. Since extraction efficiency of nucleic acid from the test sample affects the detection, the detection sensitivity of mycoplasma in cell suspension should be evaluated.

For each mycoplasma reference strain described above, at least 3 independent 10-fold dilution series should be tested, with a sufficient number of replicates at each dilution to give a total number of 24 test results for each dilution, to enable a statistical analysis of the results. For example, a laboratory may test 3 dilution series on different days with 8 replicates for each dilution, 4 dilution series on different days with 6 replicates for each dilution, or 6 dilution series on different days with 4 replicates for each dilution. In order to keep the number of dilutions at a manageable level, a preliminary test should be performed to obtain a preliminary value for the positive cut-off point (i.e. the highest dilution giving a positive signal). The range of dilutions can then be chosen around the determined preliminary cut-off point. The concentration of mycoplasmas (CFUs, etc.) that can be detected in 95% of test runs can then be calculated using an appropriate statistical evaluation. These results may also serve to evaluate the variability of the analytical procedure.

4) Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters, and provides an indication of its reliability during normal usage. The evaluation of robustness should be considered during the development phase. It should show the reliability of the analytical procedure with respect to deliberate variations in method parameters. For NAT, small variations in the method parameters can be crucial. However, the robustness of the method can be demonstrated during its development when small variations in the concentrations of reagents (e.g. $MgCl_2$, primers, and deoxyribonucleotides) are tested. Modifications of extraction kits or extraction procedures as well as different thermal cycler types may also be evaluated.

5) Use of NAT instead of methods A and/or B

NAT may be used instead of methods A (culture method) and/or B (indicator cell culture method). In this case, a comparability study should be carried out. This comparability study should include mainly a comparison of the respective detection limits of the NAT and methods A and/or B. However, specificity (mycoplasma panel detected, putative false positive results) should also be considered.

For the detection limit, acceptability criteria are defined as follows:

• if the alternative method is proposed to replace method A (the culture method), the NAT system should be shown to detect 10 CFU/mL for each mycoplasma test species described in 3).

• if the alternative method is proposed to replace method B (the indicator cell culture method), the NAT system should be shown to detect 100 CFU/mL for each mycoplasma test species described in 3).

For both cases, suitable references calibrated for the number of CFUs may be used for establishing that these acceptability criteria are reached.

One of the following 2 strategies can be used to perform this comparability study:

• perform the NAT alternative method in parallel with the methods A or B to evaluate simultaneously the detection limit of both methods using the same samples of calibrated strains with CFUs.

• compare the performance of the NAT alternative method using previously obtained data from methods A or B. In this case, calibration of CFUs of reference strains used for both validations as well as their stabilities should be described carefully.

Alternatively, comparability of detection limit may be demonstrated by the number of nucleic acid copies, etc. of mycoplasma in test samples. In this case, the relation between CFUs and the number of nucleic acid copies for the reference preparations should be previously established.

6) Controls

• Internal controls: For validation, internal controls are useful to confirm appropriate nucleic acid amplification without effect of inhibitory substances derived from test samples. Internal controls are also necessary for routine verification of extraction and absence of inhibition to NAT reaction. The internal control may contain the primer binding-site, or some other suitable sequence may be used. It is preferably added to the test material before isolating the nucleic acid and therefore acts as an overall control for extraction, reverse transcription, amplification, and detection. Cellular genes derived from test samples may also be used as the internal control.

• External controls: The external positive control contains a defined number of target-sequence copies or CFUs from one or more suitable species of mycoplasma chosen from those used during validation of the test conditions. One of the positive controls is set close to the positive cut-off point to demonstrate that the expected sensitivity is achieved. The external negative control contains no target sequence but does not necessarily represent the same matrix as the test article.

7) Interpretation of results

The primers/probes used may also amplify non-mycoplasma nucleic acid, leading to false-positive results. Procedures are established at the time of validation for dealing with confirmation of positive results, where necessary.

C-4 Method of cultivating mycoplasma with Vero cells

1) Use at least two cell culture dishes for each of the test sample, positive control and negative control.

2) Into each cell culture dish (35 mm diameter), inoculate 2 mL of the Vero cell suspension (1×10^4 cells per mL) in Eagle's minimum essential medium containing 10% fetal calf serum (tested in advance using the NAT method to verify that it does not contain any detectable mycoplasma DNA). Incubate the cultures at 35 – 38°C in an atmosphere of air containing 5% carbon dioxide for one day.

3) Replace the culture media with fresh media, and add 0.5 mL of the test sample (cell culture supernatant) to each of two or more Vero cell culture dishes. Perform the same procedure for the positive (such as 100 CFU or 100 CCU or less of *M. hyorhinis* (ATCC 17981, NBRC 14858 or equiva-

lent species or strains)) and negative controls.

4) Incubate the Vero cell culture dishes for the test sample, positive and negative controls for 3 – 6 days at 35 – 38°C in an atmosphere of air containing 5% carbon dioxide.

Qualification of Animals as Origin of Animal-derived Medicinal Products provided in the General Notices of Japanese Pharmacopoeia and Other Standards <*G3-15-141*>

Introduction

The Official Gazette issued on March 29, 2002 announced that General Notices of the Japanese Pharmacopoeia and other standards were amended to add a provision that ''When a drug product or a drug substance which is used to manufacture a drug product, is manufactured from a raw material of animal origin, the animal in question should be in principle a healthy subject, if not otherwise provided.''.

The Notice Iyaku-hatsu No. 0329001, which was issued on the same date, provided that ''Healthy subject herein provided is the animal which does not cause any disease or any infection to human being at an appropriate use of the drug product, and as for the oral or external drug for example, the animal, as its raw material of animal origin, should be confirmed at this stage to meet the Food Standard. It has to be noted that this standard of healthy subject has to be revised timely taking into account the up-to-date information with respect to the amphixenosis infections common between human beings and animals.''.

This General Information describes safety assurance against infection associated with the use of drugs, which are manufactured from raw materials of animal origin, to follow up the Notice as mentioned above.

1. Basic concept

When drugs derived from raw materials of animal origin including human are used, it is important to take into account any possibility that communicable disease agents such as virus may cause infectious disease or any possible hazards to patients. In such case, it goes without saying that the primary subject that has to be considered is the absence of any infectious agents such as virus in the raw materials of animal origin including human as the source of the drug. More important point is whether there is any possibility of transmission of infectious agents when the drugs containing such infectious agents are administered to patient. The eligibility of animals including human, as the source of raw materials of drugs, in other words ''the subject which is free from any disease or transmission of infectious agents that is infectious to human being at an appropriate use of the drug product'' is that ''The drug should be entirely free from any risk of infections by means of whole procedures which include evaluation of appropriateness of the animals including human as the source of their raw materials, establishment of appropriate production processes and their appropriate control, and strict adherence to the clinical indications of the final product.''

2. Animals including human as the source of raw materials of drugs

The most clear and appropriate preventive measures against infection to human being due to administration of drugs which are derived from animals including human are to assure the absence of any infectious agents such as virus in its raw materials or an appropriate critical raw material by either of the following: (1) the use of raw materials of healthy animal origin, which are proved to be free from communicable disease agents to human, or (2) the use of appropriate critical raw materials for drug production, which are proved to be free from communicable disease agents after certain appropriate processing on raw materials of animal origin.

As for raw materials of drugs of human origin, cell/tissue, blood, placenta, urine, etc. are used. Whenever it is possible for each donor of such raw materials to be asked or inspected about his (her) health condition, the appropriateness as a donor should be confirmed at this stage from the standpoint of safety concerning communicable disease agents such as virus.

For example, ''Basic concept on handling and use of a drug product, etc. which is derived from cell/tissue'' (Attachment 1 of the Notice Iyaku-Hatsu No. 1314 dated December 26, 2000) issued by the Director-General of the Medicinal Safety Bureau, Ministry of Health and Welfare, states that since the cell/tissue supplied by a human donor comes to be applied to patients without processing through any sufficient inactivation or removal of communicable disease agents, the selection and qualification criteria on such donor has to be established. These criteria are to be composed with the respect to the check items on the case history and the physical conditions as well as the test items on the various transmission of infectious agents through cell/tissue, and that the appropriateness of these criteria has to be clarified. Hepatitis Type-B (HBV), Hepatitis Type-C (HCV), Human Immune Deficiency Viral infections (HIV), Adult T-Cell Leukemia and Parvovirus B19 Infections should be denied through the interview to the donor and the tests (serologic test, nucleic acid amplification test, etc.). Further, if necessary, Cytomegalovirus infection and EB Virus infection should be denied by tests. ''Infections caused by bacteria such as Treponema pallidum, Chlamydia, Gonococci, Tubercule bacillus, etc.'', ''septicemia and its suspicious case'', ''vicious tumor'', ''serious metabolic or endocrine-related disorders'', ''collagenosis and haematological disorder'', ''hepatic disease'' and ''dementia (transmissible spongiform encephalopathies and its suspicious case)'' should be checked on the case history or by the interview, etc. and the experience of being transfused or/and transplanted should be checked to confirm eligibility as a donor. The most appropriate check items and test methods then available are to be used, which need to be reconsidered at appropriate timing taking into account the updated knowledge and the progress of the science and the technologies. At screening of a donor, reexaminations have to be made at appropriate timing using the eligible check items and the test methods taking into account the window period (Initial period after infection, in which antibody against bacteria, fungi or virus is not detected).

In the case of plasma derivatives produced from the donated blood in Japan, the donor should be checked by means of self-assessed report about health conditions, and a serologic check and a nucleic acid amplification test (NAT) on mini pooled plasma targeted for HBV, HCV and HIV should be performed at the stage of donated blood. Further, the plasma material for fractionation should be stored 4 months in minimum so that the arrangement could be taken based on the information available after collection of the blood and the blood infusion to exclude the possibility of using any critical raw material which might cause infection to patients.

On the other hand, as for the materials such as urine which are taken from the unspecified number of the donors and come to be critical raw materials for drug production after some treatments, it is unrealistic and not practical to conduct the tests of virus infection, etc. on the individual donor. Consequently, appropriate tests such as virus test has to be performed on such pooled raw materials for drug production.

In the case of the animals besides human, the wild ones should be excluded. Only the animals, which are raised under well sanitarily controlled conditions taken to prevent bacterial contamination or under the effective bacterial pollution monitoring systems, have to be used, and it is recommended that the animals from a colony appropriately controlled under specific pathogen-free (SPF) environment are to be used as far as possible. Further, for the animals regulated under the Food Standard, only the animals that met this standard should be used. It should be confirmed by appropriate tests that the animals were free from pathogen, if necessary.

The concrete measures to avoid transmittance or spread of infectivity of prion, which is considered to be the pathogen of transmissible spongiform encephalopathies (TSEs), as far as possible are the followings: ① avoidance of use of animals, which are raised in the areas where high incidence or high risk of TSEs (Scrapie in sheep and goat, bovine spongiform encephalopathies (BSE) in cattle, chronic wasting disease (CWD) in deer, new type of Creutzfeldt-Jacob-Disease (CJD) in human, etc.) is reported, and humans, who have stayed long time (more than 6 months) in such areas, as raw materials or related substances of drugs; ② avoidance of use of any substances that are derived from the individual infected with scrapie, BSE, CJD, etc.; ③ avoidance of using a material derived from organ, tissue and cell, etc. of high risk of TSEs; and ④ taking appropriate measures basing on the information collected, which includes incidence of TSEs, the results of epidemiological investigation and the experimental research on prion, and incidence of tardive infection on donors after collecting raw materials, etc.

3. Human or animal cells which are used as critical raw materials for drug production

Cell substrates derived from humans or animals are used for drug production. In such case, it is desirable that the humans or the animals, which are the origins of the cell substrates, are healthy subjects. However, it is considered practical that viral safety of the drugs derived from the cell substrates are evaluated on the cells, which are so called critical raw materials for production of such drugs. In such case, the safety should be confirmed through the test and analysis on established cell bank thoroughly with respect to virus etc., as far as possible. The items and the methods of the tests that have been followed in this case are described in detail in the Notice of Japanese version on the internationally accepted ICH Guideline entitled ''Viral safety evaluation of biotechnology products derived from cell lines of human or animal origin'' (Iyakushin No. 329 issued on February 22, 2000 by Director, Evaluation and Licensing Division, Pharmaceutical and Medical Safety Bureau, Ministry of Health and Welfare). In the meantime, it is important how to handle the cell in case that any virus has been detected under the cell level tests. This Notice describes how to cope with this situation as follows: ''It is recognised that some cell lines used for the manufacture of product will contain endogenous retroviruses, other viruses or viral sequences. In such circumstances, the action plan recommended for manufacturer is described in Section V (Rationale and action plan for viral clearance studies and virus tests on purified bulk) of the Notice. The acceptability of cell lines containing viruses other than endogenous retroviruses will be considered on an individual basis by the regulatory authorities, by taking into account a risk/benefit analysis based on the benefit of the product and its intended clinical use, the nature of the contaminating viruses, their potential for infecting humans or for causing disease in humans, the purification process for the product (e.g., viral clearance evaluation data), and the extent of the virus tests conducted on the purified bulk.'' For example, it is well known that Type A-, R- and C-particles like endogenous retrovirus are observed in the cells of the rodents used most often for drug production. It is also known that they are not infectious to human and is not dangerous, and CHO cells are generally used for drug production. The established cell lines (e.g., NAMALWA Cell, BALL-1 Cell, etc.) derived from cancer patients are sometimes used, but through the thorough virus tests, etc., their safety is confirmed. The established cell lines are assumed to be safer than the primary cultured cells which are hard to conduct the thorough virus test.

4. Establishment and control of appropriate production process and adherence to the clinical indication of final product for safety assurance

Safety assurance against potential infections at only the level of animals that are source of raw materials of drugs is limited. Further, ''health of animal'' can not be defined univocally, and the various factors have to be taken into account. The final goal of this subject is to protect human from any infectious disease caused by drugs. Achieving this goal, the establishment and control of appropriate production processes of each drug and the adherence to the clinical indications of the final product are important.

As mentioned above, the rodent cells used most often for the production of the drugs are known to have endogenous retrovirus-like particles sometimes. The reason why such cells can be used for the production of the drugs is that multiple measures are applied for safety in the purification stages which include appropriate inactivation or removal processes. There are cases in which the production procedure involves intentional use of a virus or a microorganism. In this case, relevant measures capable of removing or inactivating of such virus or microorganism are appropriately incorporated in the purification process, so that the risk of infection to human can be fully denied and its safety can be assured when it is used as a drug. Further, even in the case that it is difficult to clarify the risk of contamination of the infectious agents or that the raw material is contaminated by viruses etc., the raw material in question may be used for the production of drugs so long as appropriate inactivation or removal processes are introduced, their effectiveness can be confirmed and the safety can be assured by appropriate control of the manufacturing processes under GMP, etc.

5. Conclusion

The qualification of animals including human, as the source of raw materials of drugs, in other words ''the subject which does not cause any infectious diseases to human being at an appropriate use of the drug product'' is that ''the drug has to be entirely free from any risk of infections by means of whole procedures which include evaluation of appropriateness of the animal including human as the source of their raw materials, establishment of appropriate production processes and their appropriate control, and strict adherence to the clinical indication of the final product.''

To cope with this subject, the advanced scientific measures, which actually reflect the updated knowledge and

progress of the science and the technology about infectious diseases in human and infection of animal origin, have to be taken into account timely.

G4 Microorganisms

Microbial Attributes of Non-sterile Pharmaceutical Products
<*G4-1-170*>

This chapter is harmonized with the European Pharmacopoeia and the U.S. Pharmacopeia. The parts of the text that are not harmonized are marked with symbols (◆ ◆).

Information on the harmonization with the European Pharmacopoeia and the U.S. Pharmacopeia is available on the website of the Pharmaceuticals and Medical Devices Agency.

The presence of certain micro-organisms in non-sterile preparations may have the potential to reduce or even inactivate the therapeutic activity of the product and has a potential to adversely affect the health of the patient. Manufacturers have therefore to ensure a low bioburden of finished dosage forms by implementing current guidelines on Good Manufacturing Practice during the manufacture, storage and distribution of pharmaceutical preparations. ◆This chapter provides guidelines for acceptable limits of viable microorganisms (bacteria and fungi) existing in raw materials and non-sterile pharmaceutical products.◆ Microbial examination of non-sterile products is performed according to the methods given in the Microbiological Examination of Non-sterile Products <*4.05*> on Microbiological Examination of Non-sterile Products: I. Microbial Enumeration Tests and II. Tests for Specified Micro-organisms. ◆When these tests are carried out, a microbial control program must be established as an important part of the quality management system of the product. Personnel responsible for conducting the tests should have specialized training in microbiology, biosafety measures and in the interpretation of the testing results.◆

◆1. Definitions

(i) Non-sterile pharmaceutical products: Non-sterile drugs shown in monographs of the JP and non-sterile finished dosage forms.

(ii) Raw materials: All materials, including raw ingredients and excipients, used for the preparation of drugs, except for water and gases.

(iii) Bioburden: Number and type of viable micro-organisms existing in non-sterile pharmaceutical products.

(iv) Action levels: Established bioburden levels that require immediate follow-up and corrective action if they are exceeded.

(v) Alert levels: Established bioburden levels that give early warning of a potential drift from normal bioburden level, but which are not necessary grounds for definitive corrective action, though they may require follow-up investigation.

(vi) Quality management system: The procedures, operation methods and organizational structure of a manufacturer (including responsibilities, authorities and relationships between these) needed to implement quality management.

2. Scope

In general, Microbial Enumeration Tests is not applied to drugs containing viable micro-organisms as an active ingredient.

3. Sampling plan and frequency of testing

3.1. Sampling methods

Microbial contaminants are usually not uniformly distributed throughout the batches of non-sterile pharmaceutical products or raw materials. A biased sampling plan, therefore, cannot be used to estimate the real bioburden in the product. A sampling plan which can properly reflect the status of the product batch should be established on the basis of the bioburden data obtained by retrospective validation and/or concurrent validation. In general, a mixture of samples randomly taken from at least different three portions, almost the same amount for each portion, is used for the tests of the product.

When the sampling is difficult in a clean area, special care is required during sampling to avoid introducing microbial contamination into the product or affecting the nature of the product bioburden. If it is confirmed that the product bioburden is stable for a certain period, as in the case of non-aqueous or dried products, it is not necessary to do the tests, immediately after the sampling.

3.2. Testing frequency

The frequency of the tests should be established on the basis of a variety of factors unless otherwise specified. These factors include:

(i) Dosage forms of non-sterile pharmaceutical products (usage);

(ii) Manufacturing processes;

(iii) Manufacturing frequency;

(iv) Characteristics of raw materials (natural raw material, synthetic compound, etc.);

(v) Batch sizes;

(vi) Variations in bioburden estimates (changes in batches, seasonal variations, etc.);

(vii) Changes affecting the product bioburden (changes in manufacturing process, supplier of raw materials, batch number of raw materials, etc.);

(viii) Others.

In general, the tests may be performed at a high frequency during the initial production of a drug to get information on the microbiological attributes of the product or raw materials used for the production. However, this frequency may be reduced as bioburden data are accumulated through retrospective validation and/or concurrent validation. For example, the tests may be performed at a frequency based on time (e.g., weekly, monthly or seasonally), or on alternate batches.

4. Microbial control program

When the ''Microbiological Examination of Non-sterile Products <*4.05*>'' is applied to a non-sterile pharmaceutical product, the methods for the recovery, cultivation and estimation of the bioburden from the product must be validated and a ''Microbial control program'' covering the items listed below must be prepared.

(i) Subject pharmaceutical name (product name);

(ii) Frequency of sampling and testing;

(iii) Sampling methods (including responsible person, quantity, environment, etc. for sampling);

(iv) Transfer methods of the samples to the testing area (including storage condition until the tests);

(v) Treatment of the samples (recovery methods of microbial contaminants);

(vi) Enumeration of viable micro-organisms (including

testing quantity, culture media, growth-supporting test of the media, culturing methods, etc.);

(vii) Detection of specified micro-organisms (including testing quantity, culture media, growth-supporting test of the media, culturing methods, etc.);

(viii) Estimation of the number of and characterization of microbial contaminants;

(ix) Establishment of ''Microbial acceptance criteria'' (including alert level and action level);

(x) Actions to be taken when the levels exceed ''Microbial acceptance criteria'';

(xi) Persons responsible for the testing and evaluation, etc.;

(xii) Other necessary items.◆

5. Microbial acceptance criteria for non-sterile pharmaceutical products

By establishing ''Microbial acceptance criteria'' for non-sterile pharmaceutical products based upon the total aerobic microbial count (TAMC) and the total combined yeasts/moulds count (TYMC), ◆it is possible to evaluate at the initial processing stage of the product whether the microbiological quality of the raw materials is adequate or not. Furthermore, it is then possible to implement appropriate corrective action as needed to maintain or improve the microbiological quality of the product.◆

The target limits of microbial levels for raw materials (synthetic compounds and minerals) are shown in Table 1. ◆In general, synthetic compounds have low bioburden levels due to the high temperatures, organic solvents, etc., used in their manufacturing processes. Raw materials originated from plants and animals in general have higher bioburdens than synthetic compounds.

The microbial quality of the water used in the processing of active ingredients or non-sterile pharmaceuticals may have a direct effect on the quality of the finished dosage form. This means it is necessary to keep the level of microbial contaminants in the water as low as possible.◆

Acceptance criteria for microbiological quality for non-sterile finished dosage forms are shown in Table 2. ◆These microbial limits are based primarily on the type of dosage form, water activity, and so on. For oral liquids and pharmaceutical products having a high water activity, in general, low microbial acceptance criteria are given.◆

Table 2 includes a list of specified micro-organisms for which acceptance criteria are set. The list is not necessarily exhaustive and for a given preparation it may be necessary to test for other micro-organisms depending on the nature of the starting materials and the manufacturing process.

If it has been shown that none of the prescribed tests will allow valid enumeration of micro-organisms at the level prescribed, a validated method with a limit of detection as close as possible to the indicated acceptance criterion is used.

In addition to the micro-organisms listed in Table 2, the significance of other micro-organisms recovered should be evaluated in terms of:

(i) the use of the product: hazard varies according to the route of administration (eye, nose, respiratory tract);

(ii) the nature of the product: does the product support growth, does it have adequate antimicrobial preservation?

(iii) the method of application;

(iv) the intended recipient: risk may differ for neonates, infants, the debilitated;

(v) use of immunosuppressive agents, corticosteroids;

(vi) presence of disease, wounds, organ damage.

Where warranted, a risk-based assessment of the relevant factors is conducted by personnel with specialized training in microbiology and the interpretation of microbiological data.

For raw materials, the assessment takes account of processing to which the product is subjected, the current technology of testing and the availability of materials of the desired quality. Acceptance criteria are based on individual results or on the average of replicate counts when replicate counts are performed (e.g. direct plating methods).

When an acceptance criterion for microbiological quality is prescribed it is interpreted as follows:

—10^1 CFU: maximum acceptable count = 20,

—10^2 CFU: maximum acceptable count = 200,

—10^3 CFU: maximum acceptable count = 2000, and so forth.

◆6. Acceptance criteria for crude drugs and crude drug-containing preparations

Target limits of microbial contamination for crude drugs and crude drug-containing preparations are shown in Table 3. Category 1 includes crude drugs and crude drug preparations which are used for extraction by boiling water or to which boiling water is added before use. Category 2 includes crude drugs which are taken directly without extraction process and directly consumed crude drug preparations containing powdered crude drugs. In this guideline, bile-tolerant gram-negative bacteria, *Escherichia coli* and *Salmonella* are mentioned as specified micro-organisms, but other micro-organisms (such as certain species of *Bacillus cereus*, *Clostridia*, *Pseudomonas*, *Burkholderia*, *Staphylococcus aureus*, *Asperigillus* and *Enterobacter* species) are also necessary to be tested depending on the origin of raw materials for crude drugs or the preparation method of crude drug-containing preparations. The target limit of microbial contamination for the raw materials is to be set based on the risk assessment being taken into account the provided process of those materials or the desired quality specification for them.◆

Table 1 Acceptance criteria for microbiological quality of non-sterile substances for pharmaceutical use

	Total Aerobic Microbial Count (CFU/g or CFU/mL)	Total Combined Yeasts/Moulds Count (CFU/g or CFU/mL)
Substances for pharmaceutical use	10^3	10^2

Table 2 Acceptance criteria for microbiological quality of non-sterile dosage forms

Route of administration	Total Aerobic Microbial Count (CFU/g or CFU/mL)	Total Combined Yeasts/Moulds Count (CFU/g or CFU/mL)	Specified Micro-organism
Non-aqueous preparations for oral use	10^3	10^2	Absence of *Escherichia coli* (1 g or 1 mL)
Aqueous preparations for oral use	10^2	10^1	Absence of *Escherichia coli* (1 g or 1 mL)
Rectal use	10^3	10^2	—
Oromucosal use Gingival use Cutaneous use Nasal use Auricular use	10^2	10^1	Absence of *Staphylococcus aureus* (1 g or 1 mL) Absence of *Pseudomonas aeruginosa* (1 g or 1 mL)
Vaginal use	10^2	10^1	Absence of *Pseudomonas aeruginosa* (1 g or 1 mL) Absence of *Staphylococcus aureus* (1 g or 1 mL) Absence of *Candida albicans* (1 g or 1 mL)
Transdermal patches (limits for one patch including adhesive layer and backing)	10^2	10^1	Absence of *Staphylococcus aureus* (1 patch) Absence of *Pseudomonas aeruginosa* (1 patch)
Inhalation use (more rigorous requirements apply to liquid preparations for nebulization)	10^2	10^1	Absence of *Staphylococcus aureus* (1 g or 1 mL) Absence of *Pseudomonas aeruginosa* (1 g or 1 mL) Absence of bile-tolerant gram-negative bacteria (1g or 1 mL)

◆**Table 3** Acceptance criteria for crude drugs and crude drug-containing preparations

	Total Aerobic Microbial Count (CFU/g or CFU/mL)	Total Combined Yeasts/Moulds Count (CFU/g or CFU/mL)	Specified Micro-organism
Category 1	Acceptance criterion: 10^7 Maximum limit: 50,000,000	Acceptance criterion: 10^5 Maximum limit: 500,000	Acceptance criterion for *Escherichia coli*: 10^3 (1 g or 1 mL) Absence of *Salmonella* (10 g or 10 mL)
Category 2	Acceptance criterion: 10^5 Maximum limit: 500,000	Acceptance criterion: 10^4 Maximum limit: 50,000	Acceptance criterion for bile-tolerant gram-negative bacteria: 10^4 (1 g or 1 mL) Absence of *Escherichia coli* (1 g or 1 mL) Absence of *Salmonella* (10 g or 10 mL)

◆

Control of Culture Media and Strains of Microorganisms Used for Microbial Tests <*G4-2-180*>

This General Information describes points to consider in the control of culture media and strains of microorganisms used for microbial tests in a laboratory.

Use apparatuses which are appropriately maintained, controlled, and calibrated.

1. Media preparation and quality control

1.1. Media preparation

Select culture media or medium components suitable for microbial tests to be conducted when preparing culture media. Dehydrated media are accompanied by component compositions and instructions for preparation. Because each media may have different preparation requirements (e.g., heating, additives, and pH adjustment), it is important to follow their instructions to prepare media with appropriate quality. Records of the date of preparation, name/lot number/mass of dehydrated media or medium components, the volume of

water used, sterilization conditions, pH after sterilization, and equipment/instrument used, etc. are useful to investigate cause when a problem occurs.

Dehydrated media or medium components should be weighed appropriately. In addition, clean containers and tools should be used to prevent contamination with foreign matters during preparation. Water suitable for performing the relevant test should be used to prepare culture media, and purified water is most often used.

Dehydrated media should be dissolved in water before sterilization, or shaken thoroughly to disperse sufficiently. When dispensing before sterilization, media should be thoroughly dissolved in water. If heating is necessary to dissolve media, care should be taken not to overheat media. Browning of media by the Maillard reaction etc. is one of the indication of overheating. Appropriate equipment and tools should be used for heating, stirring and mixing in the preparation of media. When adding components that cannot be heated, they should be aseptically added to media cooled to an appropriate temperature after sterilization, and mixed thoroughly.

If poorly cleaned tools are used to prepare media, substances that inhibit the growth of microorganisms may contaminate the media. Inhibitory substances are derived from detergent residues after cleaning tools, etc., substances used before cleaning tools, or residues during manufacturing even when unused tools are used. In the cleaning process, residues and foreign matters should be removed certainly, and finally detergents, etc. should be washed out completely using purified water, etc.

Sterilization of media should be performed within parameters (temperature, pressure, exposure time, etc.) provided by a supplier or parameters validated by users. Sterilization in an autoclave is preferred, unless media contain medium components that are unstable to heat. Sterilization by filtration may be appropriate for some medium compositions.

When an autoclave is used, the sterilization should be performed in the sterilization cycles in which the temperature of all objects to be sterilized (temperature of media) meets specified temperature and exposure time, depending on the loading format of loads (loading pattern). Loading pattern includes the shape, size, number and arrangement of containers, and the type and liquid volume of an object to be sterilized, etc. Sterilization cycle includes the process where the temperature of objects to be sterilized rises to specified temperature and the process from the completion of sterilization until the falling to the temperature to be able to take out the objects. The sterilization cycles in which temperature rises slowly may result in the overheating of media. In general, the more the liquid volume of an object to be sterilized, the longer the sterilization cycle. However, since the cycle is affected by the size and number of dispensing containers even if the total volume of liquid is the same, so appropriate conditions should be selected. After the completion of sterilization cycle, the media should be taken out immediately, and cooled, if necessary. The effects of the sterilization cycles should be verified by the growth promotion test (refer to Microbiological Examination of Non-sterile Products <*4.05*>, Sterility Test <*4.06*>, etc.) together with confirmation of the sterility of media (no microbial contamination).

Take into consideration that improper preparation may result in the deterioration of growth promoting properties and inhibitory properties, and in the deviation of the properties, such as color, clarity, gel strength and pH, from the acceptable range.

The pH of a medium should be confirmed after it has cooled to room temperature or another specified temperature by aseptically withdrawing a test sample every each sterile batch (unit to be sterilized at one time, hereinafter called as ''batch''). If it cannot be measured at the specified temperature, the pH should be corrected for the specified temperature. A flat pH probe is recommended for agar surfaces, and an immersion probe is recommended for liquids. The pH of media should be within a specified range. However, unless when it is confirmed that a wider range is acceptable by the growth promotion test or suitability test (refer to Microbiological Examination of Non-sterile Products <*4.05*>, Sterility Test <*4.06*>, etc.).

Prepared media (agar plate or media dispensed to test tubes etc.) are identified by name, batch number, preparation date, etc. Also, pay attention to the following terms.

(i) Container fracture
(ii) Unequal dispensing volume between containers
(iii) Dirt of containers due to adhesion of medium components, etc.
(iv) Browning or discoloration
(v) Air bubbles
(vi) Status of redox indicator (if applicable)
(vii) Hemolysis (if applicable)
(viii) Formation of crystals, etc.
(ix) Drying that causes cracks and dimples
(x) Microbial contamination

1.2. Media storage

When storing media and medium components, the following points should be noted, including transport conditions until acquisition, in order to prevent the deterioration of quality.

(i) Drying, evaporation, moisture absorption
(ii) Temperature
(iii) Microbial contamination
(iv) Contamination of foreign matters
(v) Fracture

In addition, media or medium components should be labelled with names, batch or lot numbers, storage conditions, expiration dates, etc. and identified.

The storage conditions and the expiration dates of the media after preparation is set after the stability is confirmed by verifying that the performance of the media meet the acceptance criteria up to the end of the expiration date by the growth promotion test and other necessary quality tests when media are stored under the set conditions.

For long-term storage, packaging materials, packaging types, containers and stoppers that can prevent evaporation of water should be selected. Also, protect from light, if necessary. The agar media should be stored avoiding freezing because freezing damage the gel structure of agar.

In addition, agar media that have been remelted after storage can be used within the confirmed expiration date, if performance tests are performed to confirm the suitability. Also, it is desirable to remelt agar media only once to avoid the possibility of deterioration and contamination due to overheating. It is recommended that remelting is performed in a heated water bath or in free-flowing steam. When using a microwave oven or a heating plate for melting media, care should be taken because whole media may not be uniformly heated, and deterioration of the media and breakage of the container due to overheating may occur.

Agar media immediately after sterilization, or remelted media should be held at 45 to 50°C or another specified temperature, but holding for a long-time should be avoided in consideration of the risk of deterioration and contamination. In addition, if media are held in a water bath, be careful of contamination derived from water in a bath when

pouring them into petri dishes.

When discarding used or expired media, sterilize as needed and take care to prevent contamination.

1.3. Quality control testing

Perform the following quality control tests for each batch or lot for all prepared media. The prepared media include ready-prepared media and ready-to use swabs, strips, etc.

(i) Growth promotion (growth promoting properties, and, as needed, inhibitory properties or indicative properties)

(ii) pH (as needed, for ready-prepared media)

Media purchased or stored under a refrigeration condition should be returned to room temperature or a temperature being specified separately, and confirmed.

(iii) Sterility (no microbial contamination)

Media used for environmental monitoring of Grade A and B in processing areas for sterile pharmaceutical products should be multiple-wrapped or carried into the areas according to a specified procedure after disinfecting or decontaminating the exterior. If sterilization after packaging is not performed, all media (100%) should be subjected to incubation prior to use and confirmed to be free of microbial contamination in order to prevent extraneous contamination from being carried into controlled environments and prevent false-positive results.

A certificate of analysis describing storage conditions and an expiration date accompanies ready-prepared media, as well as the standard microbial strain used in growth promotion testing. When the data of testing are obtained from a supplier at the time of acceptance, if the data is reliable as the result of writing or site investigation etc. and the validity of the growth promotion testing result and the expiration date can be confirmed, the quality control test may be performed regularly instead of every batch or lot.

Similar to media, those requiring quality control tests include reagents for microbial identification tests such as Gram staining reagents and oxidase reagents. These should be subjected to quality control tests at the time of acceptance or use using appropriate standard microbial strains selected.

2. Maintenance and control of microbial strains

The appropriate treatment of stock microbial strains is very important to maintain the accuracy and repeatability of results of microbial tests. The storage and handling of microbial strains in a laboratory should be done in such a way that will minimize changes in the growth characteristics of the microorganism, paying attention to contamination. Microbial strains used in the compendial methods are available in frozen, lyophilized, slant cultured or ready-to-use forms from an organization for culture collections or an appropriate supplier. Obtained microbial strains should be confirmed to be contaminated with no other strains before or at use for quality control testing by the observation of emerged colonies being single, etc. when spread on plate media having no selectivity. In addition, if necessary, confirm the distributed microbial species.

Stock microbial strains are resuscitated according to the method specified by an organization for culture collections, etc. The application of seed lot culture maintenance techniques (seed-lot systems) is recommended to control the preservation of microbial strains. The seed-lot system is a system to control the number of passage of stock microbial strains in order to avoid property changes due to passages. One passage is defined as the transfer of organisms from a viable culture to a fresh medium with growth of the microorganisms. Any form of subculturing is considered to be a passage. At least the following terms should be noted in the control.

(i) Count a culture obtained by resuscitating (the number of passage is first) a microbial strain distributed from an organization for culture collections, etc. as the first generation.

(ii) Control the number of passages.

(iii) Microbial strains must not be used more than 5 passages for growth promotion tests and suitability tests.

There are methods by freezing, by drying, by serial subculture, etc. for the preservation of microbial strains, and an example of the method by freezing is shown. A standard microbial strain distributed from an organization for culture collections etc. is resuscitated and grown in appropriate medium. Aliquot of this culture (the first generation) is suspended in a solution containing a protective agent that prevents freezing damage, transferred to a vial or the like, and cryopreserved at an appropriate temperature according to the microbial species. Many microbial strains can be stored for a long time by maintaining at the temperature not exceeding −70°C. If the second and subsequent generations are prepared in large quantities, the frequency of acquiring and preparing standard microbial strains can be reduced.

Microbial strains in once opened containers should be discarded without refreezing to avoid the risk of reduced viability and contamination of stored microbial strains.

3. References

1) WHO, WHO Good Practices for Pharmaceutical Microbiology Laboratories (WHO Technical Report Series, No. 961, Annex 2, 2011).

2) US Pharmacopeia 43 (2020), <1117> Microbiological Best Laboratory Practices.

Preservatives-Effectiveness Tests
<G4-3-170>

The purpose of the Preservatives-Effectiveness Tests is to assess microbiologically the preservative efficacy, either due to the action of product components themselves or any added preservative(s), for multi-dose containers[1,2]. The efficacy of the preservatives is assessed by direct inoculation and mixing of the test strains in the product, and determination of survival of the test strains with time.

Water activity in products plays an important role in the growth of contaminating microorganisms. In the case of articles packaged in a multiple-dose container, the degeneration change in quality could be occurred during use due to the microbial secondary contamination, and if such contaminated product is used it could cause not only a decreasing in medical effect but also a hazard to the patient from infection. From these reasons, to the products packaged in multiple-dose containers addition of appropriate preservatives is permitted by General Rules for Preparations in the JP.

Preservatives must not be used solely to comply with GMP for drugs or to reduce viable aerobic (bacteria and yeasts/moulds) counts. In addition, preservatives show the toxicity depending on quantity. Therefore, preservatives must not be added to products in amounts which might jeopardize the safety of human beings, and consideration must be given to minimizing the amounts of preservative used. These tests are commonly used to verify that products maintain their preservative effectiveness at the design phase of formulation or in the case of periodic monitoring. Although these tests are not performed for lot release testing, antimicrobial action of the product itself or the efficacy of the preservative added to the product should be verified

over the shelf life. Testing for antimicrobial preservative content should normally be performed at release. Under certain circumstances, in-process testing may suffice in lieu of release testing.

1. Products and their Categories

The products have been divided into two categories for these tests (Table 1). Category I products are those made with aqueous bases or vehicles, and having a water activity of not less than 0.6. Category II products are those made with nonaqueous bases or vehicles. Oil-in-water emulsions are considered Category I products, and water-in-oil emulsions Category II products.

2. Test Microorganisms, Growth Promotion Test and Suitability of the Counting Method

2.1. Preparation of test strains

Seed lot culture maintenance techniques (seed-lot systems) are used so that the viable micro-organisms used for inoculation are not more than 5 passages removed from the original master seed-lot. Grow each of the bacterial and fungal test strains separately as described in Table 2. The strains shown in Table 2 or those considered to be equivalent are used as the test microorganisms.

In addition to these strains designated as test microorganisms, it is desirable to use strains that might contaminate the product and grow on or in it, depending on its characteristics. It is desirable, for example, that *Zygosaccharomyces rouxii* (NCYC 381; IP 2021.92; NBRC 1960) is used for the products like as Syrups containing a high concentration of sugar. The test strains can be harvested by growth on solid agar or liquid media.

Cultures on agar media: Inoculate each of the five test strains on the surface of agar plates or agar slants. For growth of bacteria, use Soybean-Casein Digest Agar Medium, and for yeasts and moulds, use Sabouraud Glucose Agar Medium. Incubate bacterial cultures at 30 – 35°C for 18 to 24 hours, the culture of *Candida albicans* at 20 – 25°C for 44 to 52 hours and the culture of *Aspergillus brasiliensis* at 20 – 25°C for 6 to 10 days or until good sporulation is obtained. For the bacteria and *C. albicans*, harvest the cultured cells aseptically. Suspend the collected cells in physiological saline and adjust the viable cell count to about 10^8 CFU/mL. In the case of *A. brasiliensis*, suspend the cultured cells in physiological saline containing 0.05 w/v% of polysorbate 80 and adjust the spore count to about 10^8 CFU/mL. Filter, if needed, the spore suspension through a sterilized gauze or glass wool to remove hyphae. The medium components must be removed from all of the cells so prepared by centrifugation if needed. Use these suspensions as the inocula.

Liquid cultures: After cultivation each of the four strains except for *A. brasiliensis* in Soybean-Casein Digest Medium or in Fluid Sabouraud Glucose Medium, remove the medium by centrifugation. Wash the cells in physiological saline and resuspend them in the same solution with the viable cell count of the inoculum adjusted to about 10^8 CFU/mL.

When strains other than the five listed above are cultured, select a culture medium suitable for growth of the strain concerned. The cell suspension may also be prepared by a method suitable for that strain. If it is not possible to inoculate the microbial suspensions into the test specimens within 2 hours after they have been prepared from the cultivations on agar media or in liquid media, keep them at 2 – 8°C and use within 24 hours. Usually, the spore of *A. brasiliensis* may be stored at 2 – 8°C for up to 7 days. Determine the viable cell count of the inocula immediately before use, and then calculate the theoretical viable cell count per mL or per gram of the product present just after inoculation.

Table 1 Product categories

Category	Products
IA	· Injections · Sterile products made by dissolving or suspending in aqueous vehicles (ophthalmic preparations, ear preparations, nasal preparations, etc.)
IB	· Topically used non-sterile products made by dissolving or suspending in aqueous vehicles or by mixing with aqueous bases (ear preparations, nasal preparations, inhalations, including those applied to mucous membranes, etc.)
IC	· Preparations for oral administration other than antacids made by dissolving or suspending in aqueous vehicles or by mixing with aqueous bases and those applied to the oral cavity.
ID	· Antacids made with aqueous vehicles or bases
II	· All the dosage forms listed under Category I made with non-aqueous bases or vehicles

Table 2 Test microorganisms and culture conditions

Organism	Strain	Medium	Incubation temperature	Inoculum incubation time
Escherichia coli	ATCC 8739 NBRC 3972	Soybean-Casein Digest Medium Soybean-Casein Digest Agar Medium	30 – 35°C	18 – 24 hours
Pseudomonas aeruginosa	ATCC 9027 NBRC 13275	Soybean-Casein Digest Medium Soybean-Casein Digest Agar Medium	30 – 35°C	18 – 24 hours
Staphylococcus aureus	ATCC 6538 NBRC 13276	Soybean-Casein Digest Medium Soybean-Casein Digest Agar Medium	30 – 35°C	18 – 24 hours
Candida albicans	ATCC 10231 NBRC 1594	Fluid Sabouraud Glucose Medium Sabouraud Glucose Agar Medium	20 – 25°C	44 – 52 hours
Aspergillus brasiliensis	ATCC 16404 NBRC 9455	Sabouraud Glucose Agar Medium	20 – 25°C	6 – 10 days

2.2. Growth Promotion of the Media

An appropriate culture medium among Soybean-Casein Digest Ager Medium and Sabouraud Glucose Agar Medium is used for these tests. Other media also may be used if they have similar nutritive ingredients and growth-promoting properties for the microorganisms to be tested. For the media to be used, the growth promotion test should be performed using the strains specified in Table 2 or those considered to be equivalent. The incubation times are not more

than 3 days for Soybean-Casein Digest Agar Medium, and not more than 5 days for Sabouraud Glucose Agar Medium.

By the agar media the colony counts should be obtained at least 50% of the standardized cell counts. For a freshly prepared inoculum, growth of the micro-organisms comparable to that previously obtained with a previously tested and approved batch of medium occurs.

2.3. Suitability of the Counting Method

Dilute 1 mL or 1 g of the product to be examined with 9 times its mass of physiological saline or other appropriate neutral diluting solution (10^{-1} dilution), mix, and prepare more two dilutions of this solution by serial 10-fold dilution (10^{-2} and 10^{-3} dilutions). Add a suitable count of the test strains to each tube of these dilutions, mix, and inoculate them so as to yield less than 250 CFU/plate for bacteria and *C. albicans* (ideally 25 – 250 CFU) or less than 80 CFU/plate for *A. brasiliensis* (ideally 8 – 80 CFU). This plating should be performed minimally in duplicate (or more to minimize variability in the plate count estimate). A positive control for this procedure is to introduce the same inocula into saline and transfer similar volumes of saline to agar plates. A suitable recovery scheme is the one that provides at least 50% of this saline control count (averaged). If the growth of the cells is inhibited an effective inactivator may be added in the buffer solution or liquid medium to be used for dilution of the test specimen, as well as in the agar plate count medium. However, it is necessary to confirm that the inactivator has no effect on the growth of the microorganisms. When the occurrence of the preservative or the product itself affects determination of the viable cell count and there is no suitable inactivator available, calculate the viable cell counts by the Membrane filtration method in Microbiological Examination of Non-sterile Products *<4.05>*. In the case where any change is occurred in the test material or procedure or in the product to be examined which might give any effects to the test result, the validation must be performed for the test once again. In the validation study, if the cell recovery count is not less than 50% of the inoculated cell counts, the inoculated cell counts at 0 day may be used as the theoretical inoculate cell count. See 3.2 for more information to obtain suitable counting method for the Category II products.

3. Test Procedure

3.1. Category I products

Inject each of the cell suspensions aseptically into five containers containing the product and mix uniformly. Single-strain challenges rather than mixed cultures should be used. When it is difficult to inject the cell suspension into the container aseptically or the volume of the product in each container is too small to be tested, transfer aseptically a sufficient volume of the product into each of alternative sterile containers, and mix the inoculum. When the product is not sterile, incubate additional containers containing the uninoculated product as controls and calculate their viable cell counts. The volume of the suspension mixed in the product is 0.5 – 1.0% of the volume of the product. Generally, the cell suspension is inoculated and mixed so that the concentration of viable cells is 1×10^5 to 1×10^6 CPU per mL or per g of the product. For Category ID products (antacids) inoculate so that the final concentration of viable cells is 1×10^3 to 1×10^4 CFU per mL of the product. Incubate these inoculated containers at 20 – 25°C with protection from light, and calculate the viable cell count of the test preparations at 0, 7 (Category IA only), 14 and 28 days. Record any marked changes (e.g., changes in color or the development of a bad odor or fungus) when observed in the test preparations during this time. Such changes should be considered when assessing the preservative efficacy of the product concerned. The sequential changes in the viable counts are expressed as changes in term of log reduction against the inoculated cell counts (CFU/mg or g). Determination of the viable cell counts is based, in principle, on the Plate-count methods (Pour-plate methods; Surface-spread method) or the Membrane filtration method in ''Microbiological Examination of Non-sterile Products *<4.05>*''. Alternative microbiological procedures, including an automated method, may be used for the products of Categories I and II, provided that they give a result equal to or better than that of the Pharmacopoeial methods[3].

3.2. Category II products

The procedures are the same as those described for Category I products, but special procedures and considerations are required for both uniform dispersion of the test microorganism in the product and determination of viable cell counts in the test preparations.

For semisolid ointment bases, heat the test preparation to 45°C to 50°C until it becomes oily, add the cell suspension and disperse the inoculum uniformly with a sterile glass rod or spatula. Surfactants may also be added to achieve uniform dispersion, but it is necessary to confirm that the surfactant added has no effect on survival or growth of the test microorganisms and that it does not potentiate the preservative efficacy of the product. For determination of the viable cell count, a surfactant or emulsifier may be added to disperse the test preparations uniformly in the buffer solution or liquid medium. Sorbitan monooleate, polysorbate 80 or lecithin may be added to improve miscibility between the buffer solution or the liquid medium and semisolid ointments or oils in which test microorganisms were inoculated. These agents serve to inactivate or neutralize many of the most commonly used preservatives.

4. Interpretation

Interpret the preservative efficacy of the product according to Table 3. When the results described in Table 3 are obtained, the product examined is considered to be met the requirement of the test. There is a strong possibility of massive microbial contamination having occurred when microorganisms other than the inoculated ones are found in the sterile product to be examined, and caution is required in the test procedures and/or the control of the manufacturing process of the product. When the contamination level in a nonsterile product to be examined exceeds the microbial enumeration limit specified in ''Microbial Attributes of Nonsterile Pharmaceutical Products *<G4-1-170>*'' in General Information, caution is also required in the test procedures and/or the control of the manufacturing process of the product. The statement ''No increase from the initial count'' means not more than 0.5 $\log_{10}$ increase from the initial calculated count.

5. Culture Media

Culture media used for Preservatives Effectiveness Tests are described below. Other media may be used if they have similar nutritive ingredients and selective and growth-promoting properties for the microorganisms to be tested.

(i) Soybean-Casein Digest Medium

Casein peptone	17.0 g
Soybean peptone	3.0 g
Sodium chloride	5.0 g
Dipotassium hydrogen phosphate	2.5 g
Glucose monohydrate	2.5 g
Water	1000 mL

Adjust the pH so that after sterilization it is 7.1 – 7.5 at 25°C. Sterilize in an autoclave using a validated cycle.

Table 3 Interpretation criteria by product category

Category	Microorganisms	Interpretation criteria
IA	Bacteria	At 7 days: Not less than 1.0 log reduction from the initial count. At 14 days: Not less than 3.0 log reduction from the initial count. At 28 days: No increase from the 14 day's count.
	Yeasts/Moulds	At 7, 14 and 28 days: No increase from the initial count.
IB	Bacteria	At 14 days: Not less than 2.0 log reduction from the initial count. At 28 days: No increase from the 14 day's count.
	Yeasts/Moulds	At 14 and 28 days: No increase from the initial count.
IC	Bacteria	At 14 days: Not less than 1.0 log reduction from the initial count. At 28 days: No increase from the 14 day's count.
	Yeasts/Moulds	At 14 and 28 days: No increase from the initial count.
ID	Bacteria	At 14 and 28 days: No increase from the initial count.
	Yeasts/Moulds	At 14 and 28 days: No increase from the initial count.
II	Bacteria	At 14 and 28 days: No increase from the initial count.
	Yeasts/Moulds	At 14 and 28 days: No increase from the initial count.

(ii) Soybean-Casein Digest Agar Medium

Casein peptone	15.0 g
Soybean peptone	5.0 g
Sodium chloride	5.0 g
Agar	15.0 g
Water	1000 mL

Adjust the pH so that after sterilization it is 7.1 – 7.5 at 25°C. Sterilize in an autoclave using a validated cycle.

(iii) Sabouraud Glucose Agar Medium

Glucose	40.0 g
Peptone (animal tissue and casein 1:1)	10.0 g
Agar	15.0 g
Water	1000 mL

Adjust the pH so that after sterilization it is 5.4 – 5.8 at 25°C. Sterilize in an autoclave using a validated cycle.

iv) Fluid Sabouraud Glucose Medium

Glucose	20.0 g
Peptone (animal tissue and casein 1:1)	10.0 g
Water	1000 mL

Adjust the pH so that after sterilization it is 5.4 – 5.8 at 25°C. Sterilize in an autoclave using a validated cycle.

6. References

1) European Pharmacopoeia. 8.0 (2014), 5.1.3. Efficacy of Antimicrobial Preservation.
2) U.S. Pharmacopeia. 38 (2015), <51> Antimicrobial Effectiveness Testing.
3) The Japanese Pharmacopoeia, General Information "Rapid Microbial Methods <*G4-6-170*>"

Bacterial Endotoxins Test and Alternative Methods using Recombinant Protein-reagents for Endotoxin Assay <*G4-4-180*>

Endotoxins, also called lipopolysaccharides, are present in the outer cell membrane of Gram-negative bacteria and exhibit various biological activities. Endotoxins, when entering the blood stream, can cause fever even in a very small quantity, and a large quantity of endotoxins is very toxic and can cause death due to endotoxin shock. In addition, endotoxins may contaminate pharmaceutical preparations during the production process because these are derived from Gram-negative bacteria widely present in the environment and because these are hard to be inactivated due to their heat-resistance. Endotoxins are designated as substances which should be controlled to ensure the safety of pharmaceutical preparations, etc., because these exhibit higher pyrogenicity than other well-known pyrogens which may contaminate them. Bacterial Endotoxins Test <*4.01*> is an *in vitro* test method that can detect endotoxins with high sensitivity using amoebocyte lysate prepared from blood corpuscle extracts of horseshoe crabs, and is applicable to injections, etc. On the other hand, recombinant protein-reagents for endotoxin assay have been developed as alternatives to lysate reagents for the purpose of protecting horseshoe crabs, ensuring a stable supply of reagents, reducing differences between reagent lots, and improving the continuity of the tests.

This General Information describes procedures and consideration in measurement when using recombinant protein-reagents for endotoxin assay as alternative methods, in addition to lysate reagents and test methods in Bacterial Endotoxins Test <*4.01*>.

1. Measurement principle of the Bacterial Endotoxins Test

Bacterial Endotoxins Test <*4.01*> is a test to detect or quantify bacterial endotoxins using amoebocyte lysate prepared from blood corpuscle extracts of horseshoe crab (*Limulus polyphemus* or *Tachypleus tridentatus*). This test utilizes the reaction in which the hemocyte extract of horseshoe crab is coagulated by endotoxins, and the coagulation reaction is based on a chain reaction by multiple serine proteases triggered by endotoxins (Fig. 1). Endotoxins activate factor C contained in the hemocyte extract of horseshoe crab to convert to an active serine protease, which in turn successively activates factor B, and then proclotting enzyme. Finally, coagulogen, which is a coagulant protein, is hydrolyzed to result in coagulin, and insoluble gel is formed and solidified. In addition, the hemocyte extract of horseshoe crab reacts not only to endotoxins but also mainly to (1→3)-β-D-glucans and coagulates by a chain reaction starting from factor G.

2. Measurement methods in the Bacterial Endotoxins Test

Bacterial Endotoxins Test <*4.01*> includes the gel-clot techniques, which are based on the gel formation of the lysate TS, and the photometric quantitative techniques, which are

based on endotoxin-induced optical changes (Fig. 1).

The gel-clot techniques visually confirm the presence or absence of gel formation and require no special device for the determination. The gel-clot techniques include a limit test and a quantitative test. The former is a method for judging whether a sample contains endotoxins exceeding the endotoxin limit specified in each monograph, using the labeled sensitivity of a lysate reagent as an index. The latter is a method for quantifying the amount of endotoxins in a sample by determining an endpoint, which is defined as the highest dilution of a sample solution showing the gel formation.

The photometric quantitative techniques include the turbidimetric technique and the chromogenic technique (Fig. 1). In both techniques, the lysate TS and a sample solution are mixed, and the reaction solution is measured after a given time or over time using a spectrophotometer. The turbidimetric technique measures turbidity changes accompanying gelation of the lysate TS using absorbance or transmittance, and the chromogenic technique measures the amount of chromophore released from a synthetic chromogenic substrate by the reaction of endotoxins with the lysate TS using absorbance or transmittance.

3. Reagents used for the Bacterial Endotoxins Test

There are several lysate reagents used for the Bacterial Endotoxins Test <*4.01*> corresponding to each test method. The reagents are classified into two types based on their reactivity to endotoxins and β-glucans. One is a reagent type which contains both the cascade starting from factor C and the cascade starting from factor G. The other is a reagent type which detects only endotoxins by the cascade starting from factor C due to the fact that the activity of factor G is either removed or suppressed. Appropriate reagents should be selected depending on the sample to be examined and the purpose of the test.

On lysate reagents used for the gel-clot techniques, the lowest concentration of endotoxins that cause coagulation (gel formation) is set as the labeled lysate reagent sensitivity (endotoxin unit (EU)/mL) by the reagent manufacturers. The acceptance of a sample is judged using the labeled sensitivity as an index. In order to obtain accurate test results, confirm that the labeled sensitivity is appropriate according to 4.1.1. Test for confirmation of labeled lysate reagent sensitivity in Bacterial Endotoxins Test <*4.01*> 4.1. Preparatory testing. If the geometric mean endpoint concentration does not fall within the specified range, repeat the test after adjusting test conditions. If the geometric mean endpoint concentration does not fall within the specified range by the retest, the lysate reagent cannot be used.

When using lysate reagents for the photometric quantitative techniques, for both turbidimetric and chromogenic techniques, a standard curve is prepared using the standard solutions of three or more concentrations within the quantifiable concentration range. Confirm that the test procedures of an operator and test conditions are appropriate according to 5.3.1. Test for assurance of criteria for the standard curve in the Bacterial Endotoxins Test <*4.01*> 5.3. Preparatory testing. Although the labeled sensitivity is not shown on the lysate reagents used for the photometric quantitative techniques, the lowest concentration of the standard solution used for the generation of the standard curve corresponds to the labeled sensitivity.

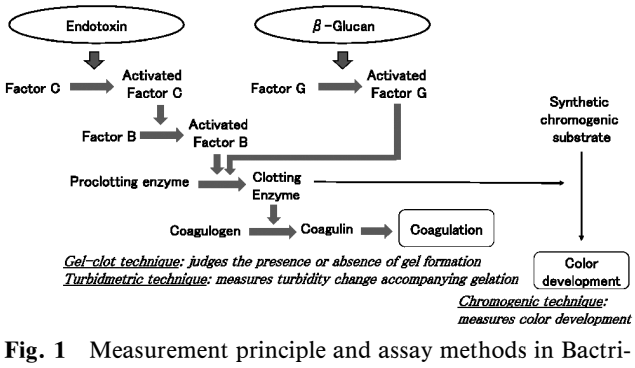


Fig. 1 Measurement principle and assay methods in Bactrial Endotoxins Test <*4.01*>

Most pharmaceuticals are found to interfere with the bacterial endotoxins test performance, although to greater or lesser degrees. In general, the influence of interfering factors present in a sample solution can often be overcome by dilution. In this case, samples should be diluted with water for bacterial endotoxins test within the range that does not exceed a Maximum Valid Dilution for the measurement. The Maximum Valid Dilution is the maximum allowable dilution of a sample solution. As shown in Bacterial Endotoxins Test <*4.01*> 3. Determination of Maximum Valid Dilution, λ is a labeled sensitivity for a lysate reagent in the gel-clot techniques and is the lowest concentration of a standard curve for a lysate reagent in the photometric quantitative techniques; the smaller the λ, the larger the maximum valid dilution. λ for many lysate reagents used for the photometric quantitative techniques is smaller than that for lysate reagents used for the gel-clot techniques. If interfering factors contained in a sample are definite, perform procedures to reduce them. If interfering factors cannot be reduced or interference cannot be avoided because of indefinite interfering factors, consider using other lysate reagents or changing the test method.

4. Measurement by alternative methods using recombinant protein-reagents for endotoxin assay and points to consider in the measurement

Recombinant protein-reagents for endotoxin assay use protein(s) prepared using the gene sequence of fators contained in blood corpuscle extracts of horseshoe crabs. These reagents include reagents that use the recombinant protein prepared using the gene sequence of factor C contained in blood corpuscle extracts of horseshoe crab (e.g. *Carcinoscorpius rotundicauda* or *T. tridentatus*), and reagents that use the recombinant proteins prepared using the gene sequence of factor C, factor B and proclotting enzyme contained in blood corpuscle extracts of horseshoe crab (e.g. *T. tridentatus*). One of the former reagents measures the amount of fluorescence generated by cleavage of a fluorescent synthetic substrate by recombinant factor C which has been activated by endotoxins. In one of the latter reagents composed of the three kinds of recombinant proteins, endotoxins activate recombinant factor C, and it further activates recombinant factor B followed by activation of recombinant proclotting enzyme in the same manner as the chromogenic technique. The change in absorbance at a specific wavelength is measured as a result of chromophore released from the synthetic chromogenic substrate. The both cases, a reagent solution and a sample solution are mixed, the fluorescence intensity or absorbance of the reaction solution is measured optically after a given time or over time.

The recombinant protein-reagents for endotoxin assay do not identical to ''an amoebocyte lysate prepared from blood corpuscle extracts of horseshoe crab'' specified in Bacterial Endotoxins Test <*4.01*>. If these reagents for endotoxin assay are used as an alternative method, confirm that accuracy, precision, sensitivity, specificity, etc. are equal or better

compared to Bacterial Endotoxins Test <4.01> using lysate reagents. Among the recombinant protein-reagents for endotoxin assay, some have been reported to have sensitivity and specificity equal to or better than the method using lysate reagents. When bacterial endotoxins tests <4.01> for pharmaceuticals, etc. are performed using recombinant protein-reagents, it is necessary to conduct 5.3.1.Test for assurance of criteria for the standard curve 5.3. Preparatory testing as with the Photometric quantitative techniques in Bacterial Endotoxins Test <4.01>, and in that case the lowest concentration of the standard curve corresponds to λ (EU/mL). In addition, it is necessary to pay attention to interference, and 5.3.2.Test for interfering factors should be performed. In the case where measurement methods that are not used in Bacterial Endotoxins Test <4.01> such as the methods that measure the amount of fluorescence are used, it is also necessary to be careful of potential interference because even substances that do not disturb the measurement when using lysate reagents may exhibit interference such as the inhibition of the generation of fluorescence. In addition, regarding reagents prepared using the gene sequence of the proteins of different species of horseshoe crab from horseshoe crab (*L. polyphemus* or *T. tridentatus*) specified in Bacterial Endotoxins Test <4.01>, it should be noted that difference of the recombinant proteins may affect the reactivity to endotoxins.

Decision of Limit for Bacterial Endotoxins <G4-5-131>

The endotoxin limit for injections is to be decided as follows:

$$\text{Endotoxin limit} = K/M$$

where K is a threshold pyrogenic dose of endotoxin per kg body mass (EU/kg), and depending on the administration route, values for K are set as in the following table.

Intended route of administration	K (EU/kg)
Intravenous	5.0
Intravenous, for radiopharmaceuticals	2.5
Intraspinal	0.2

M is equal to the maximum bolus dose of product per kg body mass. When the product is to be injected at frequent intervals or infused continuously, M is the maximum total dose administered in a single hour period. M is expressed in mL/kg for products to be administered by volume, in mg/kg or mEq/kg for products to be administered by mass, and in Unit/kg for products to be administered by biological units. Notes:

1) For products to be administered by mass or by units, the endotoxin limit should be decided based on the labeled amount of the principal drug.

2) Sixty kg should be used as the average body mass of an adult when calculating the maximum adult dose per kg.

3) The pediatric dose per kg body mass should be used when this is higher than the adult dose.

4) The K values for the intravenous route are applicable to drugs to be administered by any route other than those shown in the table.

Rapid Microbial Methods <G4-6-170>

Advances in science and technology have provided new methods to perform high-precision measurements of bacterial physiological activities, intracellular components, and so on, and consequently new techniques for bacterial detection, enumeration and quantification have appeared. Since the 1980s it has become clear that the majority of bacteria in the natural environment have low growth ability in conventional culture media, and the detection, enumeration and identification of these bacteria are difficult by means of culture methods alone. The bacterial cell counts obtained vary from method to method, and it should be noted that it is difficult to obtain a reliable value even applied with a new method. Moreover, even if type strains exist for method validation, it is not easy to standardize the physiological activity.

Compared to the conventional methods, these new methods are not necessarily superior in every respect, but they usually offer greater speed and accuracy, and can be applied not only to bacteria, but also to fungi and viruses. Therefore, these new methods are very useful to improve the standards of microbial control in critical areas, and to decrease the risk of hazardous microbial contamination.

The conventional cultivation-based methods use colony formation or turbidity change due to cell growth as an indicator, whereas the new methods vary greatly as regards the detection target and the detection principle. The new methods may be more suitable for obtaining a comprehensive understanding of the microbial community, as well as for identifying specific microorganisms. Among these methods, phylogenic analysis based on gene sequences has become popular, and the dramatic development of sequencing techniques in recent years now allows us to analyze the composition of the microbial community in a short time. In this information chapter, the principles of the new methods and their range of applicability are introduced, and key points in the usage of these methods are described.

1. Detection targets and principles

Name	Target	Principles of measurement	Examples of measurement device
1) Direct Method			
Solid phase cytometry	Microorganism	Directly detect the signals from the bacteria trapped onto a filter. The signals on their physiological activities can be obtained by choosing suitable dyes. Autofluorescence may also be used. To selectively detect specific bacteria, gene probe, antibody or fluorescent-labeled phage may be utilized. Various optical devices including a fluorescent microscope and laser microscope are used as detection/measurement apparatus.	Fluorescence microscope, Laser scanning cytometer, etc.
Flow cytometry	Microorganism	Directly detect the signals given by the bacteria passing through fluid or air. The signals on their physiological activities can be obtained by choosing suitable dyes. Autofluorescence may also be used. To selectively detect specific bacteria, gene probe, antibody or fluorescent-labeled phage may be utilized. Various optical devices are used as detection/measurement apparatus.	Flow cytometer, etc.
2) Indirect Method			
Immunological methods	Antigen	React the antigen of bacteria with the specific antibody, and detect the color or fluorescence visually or by a microplate reader. Immunochromatography is a simple and easy method for the purpose.	Immunochromatography, Micro plate reader
Nucleic acid amplification	Nucleic acid	Amplify a nucleic acid of microorganism by using the primers specific to the target microorganism, and analyze the amplified nucleic acid fragments. Quantitative determination is possible by performing of quantitative PCR.	Electrophoresis apparatus, Quantitative PCR
Bioluminescence/ fluorescence	ATP, etc.	Measure ATP which is released from microorganisms on the basis of luminous or fluorescence phenomena occurred by enzyme reaction.	luminescence detector, fluorescence detector
Micro colony method	Growth (Micro colony)	Detect and count the micro colony that appears in early stage of colonization. The same culture conditions (medium composition, temperature, etc.) as the plate culture method can be used.	Fluorescence microscopy etc.
Impedance method	Growth (Electrical characteristic)	Utilize the change in electrical properties of medium due to the metabolites produced by the growth of microorganisms.	Electrodes
Gas measuring method	Growth (Gas production, etc.)	Utilize the change in amount of gases caused by CO_2 production, O_2 consumption, etc. with the growth of microorganisms.	Gas measuring instrument Color change of medium
Fatty acid profiles	Fatty acid	Utilize the fatty acid profile of cell components that differs depending on the taxonomic groups of microorganism.	Gas chromatography
Infrared spectroscopy	Cell component	Utilize the pattern of infrared spectrum obtained by infrared light irradiation to whole microorganism.	Fourier transformation infrared spectroscope
Mass spectrometry	Cell component	Measure the cell component by means of a mass spectrometer, and identify it by database.	Mass spectrometry
Genetic fingerprinting method	DNA	Utilize the electrophoresis pattern of DNA fragments obtained by cleaving the DNA extracted from sample with a restriction enzyme. It can be identified by database. Analysis of community structure is possible by T-RFLP.	Electrophoresis apparatus
High throughput sequencing	Nucleic acid	Determine the sequence of nucleic acids extracted from bacteria exist in sample, and analyze the community structure phylogenetically.	Sequencer, etc.

Note) PCR: Polymerase Chain Reaction T-RFLP: Terminal Restriction Fragment Length Polymorphism

2. Validation

To qualify introduced equipment, a standard component or strain, which represents the target of each method, should be utilized. That is, in direct measurement, type strains should be used, while in indirect measurement, standard components, etc., of the target bacteria are used.

To validate a protocol/procedure, it is required to demonstrate that the detection target is a suitable index/indicator for bacterial number or quantity. It is also important to state whether any special precautions are necessary in applying the protocol/procedure. When using a type strain, the result of validation should be equivalent to or better than that of the conventional method. However, because the detection principles of new methods are usually different from that of conventional methods, the correlation between them is not always required. For detection of environmental bacteria, it is important that the physiological state of the type strain should be maintained as close as possible to that of environmental bacteria, in order to obtain reliable results.

3. Applications and particular considerations:

New methods are expected to find application in a variety of fields. However, since their detection targets and detection protocols/procedures are different from the conventional methods, the resulting data may not show a good correlation with existing data. Although, it is important in principle that a new method should have an equal or greater capability than the conventional method, a new method may be used after verifying their validity, even in the absence of equivalence to conventional methods.

Because the new methods are rapid, product testing, environmental monitoring, bioburden evaluation, raw materials control, etc. can be performed in real-time, and this is highly advantageous for process control, allowing alert levels, action levels and so on to be set up based on trend analysis of the obtained data.

These new rapid methods may be applied to;

- Quality control of pharmaceutical manufacturing water
- Microbial evaluation of processing areas
- Sterility test
- Microbial limit test
- Antimicrobial and preservatives effectiveness test
- Raw material acceptance test

etc.

Rapid Identification of Microorganisms Based on Molecular Biological Method
<G4-7-160>

This chapter describes the methods for the identification or estimation of microorganisms (bacteria and fungi), found in in-process control tests or lot release tests of pharmaceutical products, at the species or genus level based on their DNA sequence homology. The identification of isolates found in the sterility test or aseptic processing can be helpful for investigating the causes of contamination. Furthermore, information on microorganisms found in raw materials used for pharmaceutical products, processing areas of pharmaceutical products, and so on is useful in designing measures to control the microbiological quality of drugs. For the identification of microorganisms, phenotypic analysis is widely used, based on morphological, physiological, and biochemical features and analysis of components. Commercial kits based on differences in phenotype patterns have been used for the identification of microorganisms, but are not always applicable to microorganisms found in raw materials used for pharmaceutical products and in processing areas of pharmaceutical products. In general, the identification of microorganisms based on phenotypic analysis needs special knowledge and judgment is often subjective. It is considered that the evolutionary history of microorganisms is memorized in their ribosomal RNAs (rRNAs), so that systematic classification and identification of microorganisms in recent years have been based on the analysis of these sequences. This chapter presents a rapid method to identify or estimate microorganisms based on partial sequences of divergent regions of the 16S rRNA gene for bacteria and of the internal transcribed spacer 1 (ITS1) region located between 18S rRNA and 5.8S rRNA for fungi, followed by comparison of the sequences with those in the database. Methods described in this chapter do not take the place of usual other methods for the identification, and can be modified based on the examiner's experience, and on the available equipment or materials. Other gene regions besides those mentioned in this chapter can be used if appropriate.

1. Apparatuses

(i) DNA sequencer

Various types of sequencers using a gel board or capillary can be used.

(ii) DNA amplifier

To amplify target DNA and label amplified (PCR) products with sequencing reagents.

2. Procedures

The following procedures are described as an example.

2.1. Preparation of template DNA

It is important to use a pure cultivated bacterium or fungus for identification. In the case of colony samples, colonies are picked up with a sterilized toothpick (in the case of fungi, a small fragment of colony sample is picked up), and suspended in 0.3 mL of DNA releasing solution in a 1.5 mL centrifuge tube. In the case of culture fluid, a 0.5 mL portion of fluid is put in a 1.5 mL centrifuge tube and centrifuged at 10,000 rpm for 10 minutes. After removal of the supernatant, the pellet is suspended in 0.3 mL of DNA releasing solution, and then heated at 100°C for 10 minutes. In general, PCR can be run for bacteria and yeasts heated in DNA releasing solution. For fungi, DNA extraction from culture fluid is better because some of colony samples can disturb PCR reaction.

2.2. PCR

Add 2 μL of template DNA in PCR reaction solution. Use 10F/800R primers (or 800F/1500R primers in the case to analyze also a latter part of 16S rRNA) for bacteria and ITS1F/ITS1R primers for fungi, and then perform 30 amplification cycles at 94°C for 30 seconds, 55°C for 60 seconds, and 72°C for 60 seconds. DNA fragments are amplified about 800 bp in the case of bacteria and about 150 – 470 bp depending on the strain in the case of fungi. Include a negative control (water instead of the test solution) in the PCR.

2.3. Confirmation of PCR products

Mix 5 μL of PCR product with 1 μL of loading buffer solution, place it in a 1.5 w/v% agarose gel well, and carry out electrophoresis with TAE buffer solution (1-fold concentration). Carry out the electrophoresis together with appropriate DNA size markers. After the electrophoresis, observe PCR products on a trans-illuminator (312 nm) and confirm the presence of a single band of the targeted size. If multiple bands are observed, cut the targeted band out of the gel, and

extract DNA by using appropriate commercial DNA extraction kit.

2.4. Purification of PCR products

Various methods are available for removing unreached substances (dNTP, prime, etc.). Purify according to the protocol of the method adopted.

2.5. Quantification of purified DNA

When purified DNA is measured by spectrophotometer, calculate 1 $OD_{260\,nm}$ as 50 μg/mL.

2.6. Labeling of PCR products with sequencing reagents

Use an appropriate fluorescence-labeled sequencing reagent suitable for the available DNA sequencer or its program and label the PCR products according to the instructions provided with the reagent.

2.7. Purification of sequencing reagent-labeled PCR products

Transfer the product in 75 μL of diluted ethanol (7 in 10) into a 1.5 mL centrifuge tube, keep in an ice bath for 20 minutes, and centrifuge at 15,000 rpm for 20 minutes. After removal of supernatant, add 250 μL of diluted ethanol (7 in 10) to the precipitate and centrifuge at 15,000 rpm for 5 minutes. Remove the supernatant and dry the precipitate.

2.8. DNA homology analysis

Place sequencing reagent-labeled PCR products in the DNA sequencer and read the nucleotide sequences of the PCR products. Compare the partial nucleotide sequence with those in the BLAST database.

3. Judgment

If sequencing data show over 90% identity with a sequence in the database, in general, judgment may be made as follows.

(i) In the case of bacteria, compare the nucleotides in the product obtained with the 10F primer (the 800F primer when 800F/1500R primers are used) with the BLAST database. Higher ranked species are judged as identified species or closely related species.

(ii) In the case of fungi, compare sequencing data for the product obtained with the ITS1F primer with the BLAST database. Higher ranked species are judged as identified species or closely related species.

4. Reagents, Test Solutions

(i) 0.5 mol/L Disodium dihydrogen ethylenediamine tetraacetate TS: Dissolve 18.6 g of disodium dihydrogen ethylenediamine tetraacetate dihydrate in water to make 100 mL.

(ii) 1 mol/L Tris buffer solution (pH 8.0): Dissolve 24.2 g of 2-amino-2-hydroxymethyl-1,3-propanediol in a suitable amount of water, adjust the pH to 8.0 with 0.2 mol/L hydrochloric acid TS, and add water to make 200 mL.

(iii) TE buffer solution: Mix 1.0 mL of 1 mol/L tris buffer solution (pH 8.0) and 0.2 mL of 0.5 mol/L disodium dihydrogen ethylenediamine tetraacetate TS, and add water to make 100 mL.

(iv) DNA releasing solution: Divide TE buffer solution containing 1 vol% of polyoxyethylene (10) octylphenyl ether into small amounts and store frozen until use.

(v) PCR reaction solution

10-fold buffer solution*	5 μL
dNTP mixture**	4 μL
10 μmol/L Sense primer	1 μL
10 μmol/L Anti-sense primer	1 μL
Heat-resistant DNA polymerase (1 U/μL)	1 μL
Water	36 μL

* Being composed of 100 mmol/L 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride (pH 8.4), 500 mmol/L potassium chloride, 20 mmol/L magnesium chloride and 0.1 g/L gelatin.

** A solution containing 2.5 mmol/L each of dGTP (sodium 2′-deoxyguanosine 5′-triphosphate), dATP (sodium 2′-deoxyadenosine 5′-triphosphate), dCTP (sodium 2′-deoxycytidine 5′-triphosphate) and dTTP (sodium 2′-deoxythymidine 5′-triphosphate). Adequate products containing these components as described above may be used.

(vi) Sequencing reagent: There are many kinds of sequencing methods, such as the dye-primer method for labeling of primer, the dye-terminator method for labeling of dNTP terminator and so on. Use an appropriate sequencing reagent kit for the apparatus and program to be used.

(vii) 50-Fold concentrated TAE buffer solution: Dissolve 242 g of 2-amino-2-hydroxymethyl-1,3-propanediol in 57.1 mL of acetic acid (100) and 100 mL of 0.5 mol/L disodium dihydrogen ethylenediamine tetraacetate TS, and add water to make 1000 mL.

(viii) 1-Fold concentrated TAE buffer solution: Diluted 50-fold concentrated TAE buffer solution (1 in 50) prepared before use is referred to as 1-fold concentrated TAE buffer solution.

(ix) Agarose gel: Mix 1.5 g of agarose, 2.0 mL of 50-fold concentrated TAE buffer solution, 10 μL of a solution of ethidium bromide (3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide) (1 in 100) and 100 mL of water. After dissolving the materials by heating, cool the solution to about 60°C, and prepare gels.

(x) Loading buffer solution (6-fold concentrated): Dissolve 0.25 g of bromophenol blue, 0.25 g of xylene cyanol FF and 1.63 g of disodium dihydrogen ethylenediamine tetraacetate dihydrate in 50 mL of water, and add 30 mL of glycerol and water to make 100 mL.

(xi) PCR primers

For	Primer	
Bacteria	10F	5′-GTTTGATCCTGGCTCA-3′
	800R	5′-TACCAGGGTATCTAATCC-3′
	800F	5′-GGATTAGATACCCTGGTA-3′
	1500R	5′-TACCTTGTTACGACTT-3′
Fungi	ITS1F	5′-GTAACAAGGT(T/C)TCCGT-3′
	ITS1R	5′-CGTTCTTCATCGATG-3′

(xii) Polyoxyethylene(10)octylphenyl ether: A pale yellow, viscous liquid.

Rapid Counting of Microbes using Fluorescent Staining <*G4-8-152*>

This chapter provides rapid methods using fluorescence staining for the quantitative estimation of viable microorganisms. Incubation on an agar medium has been widely used for quantitative estimation of viable microorganisms, but a number of environmental microorganisms of interest are not easy to grow in culture under usual conditions, thus new microbial detection methods based on fluorescence or luminescence have been developed. In the fluorescence staining method, microorganisms are stained with fluorescent dye, and can easily be detected and counted with various sorts of apparatus, such as a fluorescence microscope or flow cytometer. Methods are available to detect total microorganisms, including both dead and viable cells, or to detect only cells with a specified bioactivity by choosing the dye reagent appropriately. Nucleic acid staining reagents, which bind with DNA or RNA, detect all cells containing

nucleic acids, whether they are live or dead. This technique is the most fundamental for the fluorescence staining method. On the other hand, fluorescent vital staining methods target the respiratory activity of the microorganism and the activity of esterase, which is present universally in microorganisms. In the microcolony method, microcolonies in the early stage of colony formation are counted. The CFDA-DAPI double staining method and the microcolony method are described below. These methods can give higher counts than the other techniques, because these rapid and accurate techniques provide quantitative estimation of viable microorganisms based on a very specific definition of viability, which may be different from that implicit in other methods. The procedures of these methods described here may be changed as experience with the methods is accumulated. Therefore, other reagents, instruments and apparatus than those described here may also be used if there is a valid reason for so doing.

1. CFDA-DAPI double staining method

Fluorescein diacetate (FDA) reagents are generally used for the detection of microorganisms possessing esterase activity. These reagents are hydrolyzed by intracellular esterase, and the hydrolyzed dye exhibits green fluorescence under blue excitation light (about 490 nm). Modified FDAs such as carboxyfluorescein diacetate (CFDA) are used because of the low stainability of gramnegative bacteria with FDA. The principle of the CFDA-DAPI double staining method, which also employs a nucleic acid staining reagent, 4′,6-diamidino-2-phenylindole (DAPI), is as follows. The nonpolar CFDA penetrates into cells and is hydrolyzed to fluorescent carboxyfluorescein by intracellular esterase. The carboxyfluorescein is accumulated in the living cells due to its polarity, and therefore green fluorescence due to carboxyfluorescein occurs when cells possessing esterase activity are illuminated with blue excitation light. No fluorescent carboxyfluorescein is produced with dead cells, since they are unable to hydrolyze CFDA. On the other hand, DAPI binds preferentially to the adenine and thymine of DNA after penetration into both viable and dead microorganisms, and consequently all of the organisms containing DNA exhibit blue fluorescence under ultraviolet excitation light. Therefore, this double staining method enables to count specifically only live microorganisms possessing esterase activity under blue excitation light, and also to determine the total microbial count (viable and dead microorganisms) under ultraviolet excitation light.

1.1. Apparatus

1.1.1. Fluorescence microscope or fluorescence observation apparatus

Various types of apparatus for counting fluorescencestained microorganisms are available. Appropriate filters are provided, depending on the fluorescent dye reagents used. A fluorescence microscope, laser microscope, flow cytometer, and various other types of apparatus may be used for fluorescence observation.

1.2. Instruments

(i) Filtering equipment (funnels, suction flasks, suction pumps)

(ii) Membrane filters made of polycarbonate (poresize: 0.2 μm); A suitable filter that can trap particles on the surface can be used other than polycarbonate filter.

(iii) Glass slide

(iv) Cover glass

(v) Ocular micrometer for counting (with 10 × 10 grids)

1.3. Procedure

An example of the procedure using a fluorescence microscope is described below.

1.3.1. Preparation of samples

Prepare samples by ensuring that microbes are dispersed evenly in the liquid (water or buffer solution).

1.3.2. Filtration

Set a membrane filter made of polycarbonate (poresize: 0.2 μm) on the funnel of the filtering equipment. Filter an appropriate amount of a sample to trap microbes in the sample on the filter.

1.3.3. Staining

Pour sufficient amount of buffer solution for CFDA staining, mixed to provide final concentration of 150 μg/mL of CFDA and 1 μg/mL of DAPI, into the funnel of the filtering equipment and allow staining in room temperature for 3 minutes, then filter the liquid by suction. Pour sufficient amount of aseptic water in the funnel, filter by suction, and remove excess fluorescent reagent left on the filter. Thoroughly dry the filter.

1.3.4. Slide preparation

Put one drop of immersion oil for fluorescence microscope on the glass slide. Place the air dried filter over it, with the filtering side on the top. Then put one drop of immersion oil for fluorescence microscope on the surface of the filter, place a cover glass to enclose the filter. Put another drop of immersion oil for fluorescence microscope on the cover glass when using an oilimmersion objective lens.

1.3.5. Counting

Observe and count under a fluorescence microscope, with 1000 magnification. In case of CFDA-DAPI double staining method, count the microorganisms (with esterase activity) exhibiting green fluorescence under the blue excitation light first to avoid color fading by the ultraviolet light, then count the microorganisms (with DNA) exhibiting blue fluorescence under the ultraviolet excitation light in the same microscopic field. Count the organisms exhibiting fluorescence on more than 20 randomly selected fields among 100 grids observed through an ocular micrometer of the microscope, and calculate the total number of organisms using the following formula. The area of the microscopic field should be previously determined with the ocular and objective micrometers. The amount of the sample to be filtered must be adjusted so that the cell number per field is between 10 and 100. It might be necessary to reprepare the sample in certain instances. (In such case that the average count number is not more than 2 organisms per field, or where more than 5 fields are found which have no organism per field, it is assumed that the microorganism count is below the detection limit.)

$$\text{Number of microbes (cells/mL)} = \{(\text{average number of microbes per visual field}) \times (\text{area of filtration})\}/\{(\text{amount of sample filtered}) \times (\text{area of one microscopic field})\}$$

1.4. Reagents and test solutions

(i) Aseptic water: Filter water through a membrane filter with 0.2 μm pore size, then sterilize it by heating in an autoclave at 121°C for 15 minutes. Water for injection may be used.

(ii) CFDA solution, 10 mg/mL: Dissolve 50 mg of CFDA in dimethylsulfoxide to prepare a 5 mL solution. Store at −20°C in light shielded condition.

(iii) Buffer solution for CFDA staining: Dissolve 5 g of sodium chloride with 0.5 mL of 0.1 mol/L disodium dihydrogen ethylenediamine tetraacetate TS and diluted disodium hydrogen phosphate TS (1 in 3) to prepare 100 mL of solution. Add sodium dihydrogen phosphate dihydrate solution (1 in 64) to adjust the pH level to 8.5. Filter the solution through a membrane filter with a pore size of 0.2 μm.

(iv) DAPI solution, 10 μg/mL: Dissolve 10 mg of DAPI

in 100 mL of aseptic water. Dilute this solution 10 times with aseptic water and filter through a membrane filter with a pore size of 0.2 μm. Store at 4°C in light shielded condition.

(v) Immersion oil for fluorescence microscope

2. Microcolony method

Microcolonies, which are in early stages of colony formation, are fluorescently stained, then observed and counted under a fluorescence microscope or other suitable systems. This method enables to count the number of proliferative microorganisms, with short incubation time. In this method, the organisms are trapped on a membrane filter, the filter is incubated on a medium for a short time, and the microcolonies are counted. By this method, even colonies which are undetectable with the naked eye can be identified, so viable organisms can be counted rapidly and with high precision. Various nucleic acid staining reagents can be used for staining of microcolonies.

2.1. Apparatus

2.1.1. Fluorescence microscope or fluorescence observation apparatus

Various types of apparatus for counting fluorescencestained microorganisms are available. Appropriate filters are provided, depending on the fluorescence dye reagents used. A fluorescence microscope, laser microscope and various other types of apparatus may be used for fluorescence observation.

2.2. Instruments

(i) Filtering equipment (funnels, suction flasks, suction pumps)

(ii) Membrane filters made of polycarbonate (pore size: 0.2 μm); A suitable filter that can trap particles on the surface can be used other than polycarbonate filter.

(iii) Glass slide

(iv) Cover glass

(v) Filter paper (No. 2)

(vi) Ocular micrometer for counting (with 10 × 10 grids)

2.3. Procedure

An example of the procedure using a fluorescence microscope is described below.

2.3.1. Preparation of samples

Prepare samples by ensuring that microbes are dispersed evenly in the liquid (water or buffer solution).

2.3.2. Filtration

Set a membrane filter made of polycarbonate (pore size: 0.2 μm) on the funnel of the filtering equipment. Filter an appropriate amount of sample to trap microbes in the sample on the filter.

2.3.3. Incubation

Remove the filter from the filtering equipment and place it with filtering side facing up on a culture medium avoiding formation of air bubbles between the filter and the medium. Incubate at a suitable temperature for appropriate hours in a dark place. It should be noted that the appropriate incubation conditions (such as medium, incubation temperature and/or incubation time) are different, depending on the sample.

2.3.4. Fixation

Soak a filter paper with an appropriate amount of neutral buffered formaldehyde solution, then place the filter that has been removed from the culture medium on top with filtering side up, and allow to remain at room temperature for more than 30 minutes to fix the microcolonies.

2.3.5. Staining

Soak a filter paper with an appropriate amount of staining solution (such as 1 μg/mL of DAPI, 2% polyoxyethylenesorbitan monolaurate), then place the filter on top with filtering side up, and then leave at room temperature, light shielded for 10 minutes to stain microcolonies. Wash the filter by placing it with the filtering side facing up on top of a filter paper soaked with aseptic water for 1 minute. Thoroughly air dry the filter.

2.3.6. Slide preparation

Put one drop of immersion oil for fluorescence microscope on the slide glass. Place an air dried filter over it, with the filtering side on the top. Then, put one drop of immersion oil for fluorescence microscope on top, place a cover glass to enclose the filter.

2.3.7. Counting

Count the organisms exhibiting fluorescence on more than 20 randomly selected fields among the 100 grids observed through an ocular micrometer of the microscope with 400 or 200 magnification, and calculate the total number of organisms using the following formula. The area of the microscopic field should be previously determined with the ocular and objective micrometers. In such case that the average count number is not more than 2 microcolonies per field, or where more than 5 fields are found which have no microcolony per field, it is assumed that the microorganism count is below the detection limit.

Number of microcolonies (cells/mL)
= {(average number of microcolonies per visual field) × (area of filtration)}/{(amount of sample filtered) × (area of one microscopic field)}

2.4. Reagents and test solutions

(i) Aseptic water: Filter water through a membrane filter with 0.2 μm pore size, and sterilize it by heating in an autoclave at 121°C for 15 minutes. Water for injection may be used.

(ii) Staining solution: Dissolve 10 mg of DAPI in 100 mL of aseptic water. Dilute the solution 10 times with aseptic water and filter through a membrane filter with pore size of 0.2 μm. Store at 4°C in light shielded condition. Dissolve polyoxyethylene sorbitan monolaurate to the final concentration of 2%, when using.

(iii) Neutral buffered formaldehyde solution (4w/v% formaldehyde solution; neutrally buffered).

(iv) Immersion oil for fluorescence microscope

Disinfection and Decontamination Methods <*G4-9-170*>

This chapter describes procedures for reducing the number of microorganisms to a predetermined level using chemical agents during hygiene control of structures and facilities in clean areas or aseptic processing areas that require cleanliness control, as well as of personnel involved in manufacturing control and manufacturing operations in those areas, in pharmaceutical product manufacturing plants.

Appropriate action should be taken based on this chapter when performing microbiological tests specified in the monographs, when taking measures needed to prevent microbial contamination of products and materials used in the manufacture of pharmaceutical products, and when microbial control is needed in pharmacies.

1. Terms and Definitions

The terms used in this chapter are defined as follows.

• Microorganisms: Generally it is a term for bacteria, fungi, protozoa, viruses, and the like. In this chapter, the term means bacteria and fungi.

• Disinfection: Generally it is a term for harmful microorganisms such as pathogens are removed, killed, or detoxified. In this chapter, the term means microorganisms on an object or in local areas such as the surface of an object are reduced.

• Decontamination: To reduce microorganisms in structures and facilities such as work space and work rooms to a predetermined microbial count level.

• Logarithmic reduction value: The difference in log values for microbial count before and after certain treatments.

• Disinfectant rotation: A disinfection program for when microorganisms that are resistant to a using disinfectant are discovered. In which a disinfectant with different efficacy is used until those microorganisms are no longer detected, or disinfectants with different mechanisms of action are alternately used for certain periods of time in turn. The effectiveness of this method should be evaluated before its implementation.

2. Disinfection Methods

This includes methods such as wiping, spraying, or immersion using chemical agents to reduce microorganisms on equipment, floors, walls, or containers that are carried into clean areas or aseptic processing areas, and the local surfaces such as the wrapping materials of environmental monitoring media. The disinfectants in Table 1 are commonly used, in single or in combination, upon due consideration of the nature of the surface to which the method is applied, such as corrosion by disinfectants, as well as the extent of contamination, such as the types and counts of microorganisms. Although this method does not kill or remove all microorganisms on an object or local surface, disinfectants of proven efficacy should be used when this method is employed. The effects of chemical agents used as disinfectants on microorganisms will differ depending on factors such as the applied concentration, temperature, contact time, and level of surface contamination. When using this method, attention should be paid to disinfectant expiration dates, microbial contamination, the effect of chemical residue on pharmaceutical product quality, and deterioration such as discoloration, deformation and corrosion of the materials being treated.

2.1. Disinfectants

Table 1 presents examples of commonly used disinfectants and their concentrations, and the microbial effects of these disinfectants. Disinfectants and concentrations proven to be safe and effective other than those shown here can also be used.

Table 1 Types of disinfectants, concentrations, and mechanisms of action

Classification	Types of Disinfectant	Concentration	Mechanism of action
Oxidant	Peracetic acid	0.3 w/v%	Oxidizing action
	Hydrogen peroxide	3 w/v%	
	Sodium hypochlorite	0.02 to 0.05%	
Alcohol-based	Isopropanol	50 to 70%	Protein and nucleic acid denaturation
	Ethanol	76.9 to 81.4 vol%	
Surfactant-based	Benzalkonium chloride Benzethonium chloride	0.05 to 0.2%	Protein denaturation
	Alkyldiaminoethylglycine hydrochloride	0.05 to 0.5%	Cell membrane function impairment, protein coagulation/denaturation
Biguanide-based	Chlorhexidine gluconate	0.05 to 0.5%	Interruption of bacterial enzyme or alteration/disruption of cytoplasmic membrane

Table 2 Test microorganisms

Classification	Test microorganism
Bacteria	*Escherichia coli* ATCC 8739, NBRC 3972 *Staphylococcus aureus* ATCC 6538, NBRC 13276 *Pseudomonas aeruginosa* ATCC 9027, NBRC 13275 *Bacillus subtilis* ATCC 6633, NBRC 3134
Fungi	*Candida albicans* ATCC 10231, NBRC 1594 *Aspergillus brasiliensis* ATCC 16404, NBRC 9455

2.2. Evaluation Methods

When disinfection methods are applied to clean areas and aseptic processing areas, the effectiveness of the conditions should be checked upon due consideration of factors such as the disinfectant concentration, contact time, material of the surface being disinfected, and type of microorganisms that are to be reduced with the disinfectant. Examples of evaluation methods are provided below. Methods other than those shown in the examples may be used if they can be demonstrated to be scientifically appropriate.

2.2.1. Test Microorganism Suspension Method

The diluent that is actually used (purified water, tap water, etc.) should be used to adjust the disinfectant to the actually used concentration. Inoculate 1 mL of the prepared disinfectant with 10^5 to 10^6 CFU test microorganisms. Allow the disinfectant to take effect for the prescribed time (usually 5 to 15 minutes), then dilute or remove (filter) the disinfectant. In the diluent or filtered wash solution, neutralize[1)] the disinfectant using a solution containing an inactivator such as lecithin, polysorbate 80, or sodium thiosulfate as needed. Count the number of the microorganisms used for inoculation and the number after disinfection under conditions meeting the requirements in Microbiological Examination of Non-sterile Products <*4.05*> I. 3.4 Suitability of the counting method in the presence of product. Calculate the logarithmic decrement from the test microorganism counts before and after disinfectant treatment. A 3 log or greater decrease in bacteria or fungi and a 2 log or greater decrease in spores will indicate that the disinfection of each target microorganism is effective. The required test microorganism species for evaluating efficacy should be selected with reference to Table 2. These test microorganisms should be used in the evaluation by being cultured and diluted under the conditions described in Microbiological Examination of Non-sterile Products <*4.05*>. However, *Bacillus subtilis* should be used in the evaluation after a spore suspension has been prepared with reference to Microbial Assay for Antibiotics

Table 3 Examples of materials to be disinfected

Material	Example of application
Stainless steel	Workbenches, tanks, machines
Glass	Windows, screens
Polycarbonate	Screens, containers
Decorative calcium silicate board	Walls, ceiling
Epoxy resin coating	Floors
Vinyl chloride	Floors, curtains, vinyl bags
Rigid urethane rubber	Floors
Nitrile rubber	Gloves

<*4.02*>. Species that are suitable for the purpose of the test can be used.

2.2.2. Hard Surface Carrier Method

Prepare each type of surface material carrier (approximately 5 cm × 5 cm) in a quantity resulting in the appropriate precision. Inoculate a broad range of carriers with 10^5 to 10^6 CFU test microorganisms, allow to dry, and then add disinfectant dropwise in the actually used concentration. Note that the number of the inoculated microorganisms may decrease depending on the conditions of drying the inoculated microorganisms which result in the disinfection effect not being evaluated appropriately. Allow the disinfectant to take effect for the prescribed time (usually 5 to 15 minutes), then collect the test microorganisms on the carriers by diluting in the collected solution. In the collected solution, neutralize[1)] the disinfectant using a solution containing an inactivator such as lecithin, polysorbate 80, or sodium thiosulfate as needed.

The stomach method, shaking method, swab method, or the like can be used as the method of collection with reference to JIS T11737-1.[2)] Count the number of the test microorganisms used for inoculation and the number of the recovered microorganisms under test conditions meeting the requirements in Microbiological Examination of Non-sterile Products <*4.05*> I. Microbiological Examination of Non-sterile Products: Microbial Enumeration Tests, 3.4 Suitability of the counting method in the presence of product. Calculate the logarithmic decrement from the test microorganism counts before and after disinfectant treatment. Conditions showing the same results as the decreases specified in 2.2.1 Test Organism Suspension Method will indicate that the disinfection of each target microorganism is effective. In addition to the selection of the required test microorganism species for evaluating efficacy with reference to Table 2, one or two representative microorganisms which are frequently isolated in environmental monitoring should be added. Species that are suitable for the purpose of the test can be used. The test microorganisms should be cultured and diluted, etc., as specified in 2.2.1 Test Microorganism Suspension Method. Examples of various surface materials used in clean areas or aseptic processing areas are given in Table 3, but other materials can be added for evaluation as needed depending on the circumstances of actual use.

3. Decontamination Methods

In these methods, decontamination is achieved, for example, by vaporizing or spraying chemical agents to reduce the number of microorganisms to a predetermined level in isolators and RABS (Restricted Access Barrier Systems) employed in sterile pharmaceutical product manufacturing processes, or structures and facilities such as work spaces and work rooms in clean areas or aseptic processing areas.

When this method is applied to structures and facilities for manufacturing sterile pharmaceutical products, the efficacy of the decontaminants and decontamination conditions must be validated, and worker safety must be ensured.

3.1. Decontaminants

Commonly used decontaminants are shown below. Decontaminants proven to be safe and effective other than those shown here can also be used.

3.1.1. Hydrogen Peroxide

Decontamination is achieved when hydrogen peroxide (30) is volatilized and allowed to spread. This is a method in which hydrogen peroxide that has been vaporized using a heater is allowed to spread inside an isolator or work room to kill microorganisms through the oxidative power of hydrogen peroxide. When a high degree of microbiological cleanliness must be achieved, as when decontaminating the interior of an isolator for sterilization operations, conditions should ensure a 6 log or greater decrease in the spores of the biological indicator, and when work rooms are decontaminated, conditions should ensure a 3 log or greater decrease. Although the method can be used at ambient temperature, the suitability of the method must be investigated beforehand because the potent oxidative power of hydrogen peroxide may cause deterioration such as discoloration, deformation, and corrosion of the materials exposed, depending on the nature of the materials, and the hydrogen peroxide itself may be degraded by the contact of the materials. If surfaces that are in contact with the product exist inside the isolator, it will be necessary to simultaneously decontaminate the interior and ensure the sterility assurance of the surfaces that are in contact with the product. In such cases, the pre-sterilization bioburden, parameters, utilities, and the like should be controlled in terms of the sterilization method with reference to the chapter ''Sterilization Methods and Sterilization Indicators <*G4-10-162*>.''

3.1.2. Peracetic Acid

Decontamination can be achieved, for example, when 0.2% peracetic acid aqueous solution is sprayed in the form of a mist and is allowed to spread. This method is used to clean work rooms, with conditions ensuring at least a 3 log decrease in the spores of the biological indicator. This is a method in which microorganisms are killed through the oxidative power of peracetic acid. Although the method can be used at ambient temperature, the suitability of the method must be investigated beforehand because the potent oxidative power of peracetic acid may result in deterioration such as discoloration, deformation, or corrosion of some materials.

3.1.3. Formaldehyde

Decontamination is achieved when formalin, an aqueous solution containing 36.0 to 38.0% formaldehyde, is vaporized by being heated, or when paraformaldehyde is sublimated by being heated, and allowed to spread. This is a method in which microorganisms are killed through the denaturation of protein by the aldehyde group (-CHO) in the formaldehyde molecule. This method is used to clean work rooms, with conditions ensuring at least a 3 log decrease in the spores of the biological indicator. As formaldehyde is harmful to the human body and has been designated a deleterious substance in the Poisonous and Deleterious Substances Control Act, it must be handled in work spaces equipped with a power exhaust device. It must also be detoxified when disposing of chemical waste.

3.2. Evaluation Methods

Methods using biological indicators to evaluate the effects of decontamination are generally used. Biological indicators that are resistant to decontaminants are commonly placed in

various locations in structures and facilities such as work spaces and work rooms prior to decontamination. After decontamination, the biological indicators are commonly collected and are cultured to check for survivor microorganisms. In addition to culturing, faster methods, for example, that are equal to or greater than culturing can be used. When the decontamination of an isolator with hydrogen peroxide needs to be verified by inactivating 6 log or greater spore count after the use of 10^6 CFU biological indicators, it is not necessary to demonstrate complete destruction of the spores in the isolator after decontamination. Decontamination conditions suitable for a 6 log reduction in spores can also be established by statistical analysis or a method for evaluating the effects of decontamination by collecting the biological indicator and counting the number of survivor microorganisms by culturing to calculate the logarithmic decrement of the biological indicator.

The spores of *Geobacillus stearothermophilus* ATCC 7953 and 12980 are known to be resistant to hydrogen peroxide and formaldehyde, and can thus be used as indicator organisms. As representative environmental microorganisms, the spores of *Bacillus atrophaeus* ATCC 9372 can also be used as a biological indicator for the decontamination of work rooms.

4. Points to Consider

4.1. Worker Safety

Disinfectants and decontaminants often have an effect on the human body. That is, they are toxic. Therefore, when they are used, the method and amounts used must be strictly observed, protective gear must be properly used as needed, and the residue level must be checked.

4.2. Selection of Disinfectants and Decontaminants Used in Pharmaceutical Product Manufacturing Environments

When selecting disinfectants and decontaminants to be used in pharmaceutical product manufacturing environments, the following should be taken into consideration to select the appropriate ones depending on the purpose for which they are being used. The following items (1) through (13) must also be taken into consideration in order to ensure safer and more appropriate use of disinfectants and decontaminant.

(1) Type and number of microorganisms to be treated
(2) Antimicrobial spectrum
(3) Method of use, concentration, contact time, and expiration period of chemical agents
(4) Method for preparing decontaminant, including sterilization, when used in aseptic processing areas
(5) Suitability of materials being treated with disinfectants and decontaminants (such as extent of deterioration)
(6) Effects in the presence of organic substances such as protein
(7) Effective time dulation
(8) Effect on human body (safety)
(9) Suitability with cleansers
(10) Necessity of disinfectant rotation, and the method, if needed
(11) Necessary procedures for preventing contamination of pharmaceutical products by chemical agents (such as method of inactivation and checking residue level)
(12) Ease of waste disposal (neutralization, inactivation)
(13) Environmental effects of waste disposal

5. References

1) US Pharmacopeia 37 (2014), <1072> Disinfectants and Antiseptics
2) JIS T 11737 – 1: 2013, Sterilization of medical devices—Microbiological methods, Part 1: Determination of a population of microorganisms on products (ISO 11737 – 1: 2006)

Sterilization and Sterilization Indicators <*G4-10-162*>

Sterilization refers to the destruction or removal of all forms of viable microorganisms in items. This reference information applies to cases where sterilization is required as well as the manufacture of sterile products. When sterilization is applicable, an appropriate sterilization method should be selected in accordance with the items being sterilized (such as products, or equipment, instrumentation, or materials that must be sterilized), including the packaging, after full consideration of the advantages and disadvantages of each sterilization method.

After installation of a sterilizer (including design and development of the sterilization process), an equipment maintenance and inspection program must be established based on qualification evaluation to ensure that the sterilization process is being properly performed as designed on the basis of sufficient scientific evidence. A quality system must also be established for manufacturing in general at manufacturing facilities where sterile pharmaceutical products are manufactured. For example, all operation potentially affecting quality, including sterility after sterilization, must be clearly identified, and any operating procedures that are needed to prevent microbial contamination of products must be established and properly enforced.

In order to establish sterilization conditions and ensure sterility after sterilization, the bioburden before sterilization of the items being sterilized must be evaluated periodically or on the basis of batches. For bioburden test methods, refer to 4.05 Microbiological Examination of Non-sterile Products, etc.

Representative sterilization methods are presented in this reference information, but other sterilization methods can also be used, provided that they meet the following requirements and do not have any deleterious affect on the item being sterilized.

- The mechanism of sterilization is well established
- The critical physical parameters of the sterilization process are clear, controllable, and measurable
- The sterilization procedure can be performed effectively and reproducibly

1. Definitions

The terms used in this text are defined as follows.

- Filter integrity test: A non-destructive test which is demonstrated to correlate with the microbial removal performance data of filters.
- Bioburden: Population of viable microorganisms in an item to be sterilized.
- *D* value: The value represents exposure time (decimal reduction time) to achieve 90% reduction of a population of the test microorganism, and resulted that 10% of the original organisms remain.
- F_H value: The unit of lethality indicating the measure of the microbial inactivation capacity of a process in dry heat sterilization, expressed as the equivalent time (minutes) at 160°C for microbes with a *z* value (the number of degrees that are required for a 10-fold change in the *D* value) of 20°C.
- F_0 value: The unit of lethality indicating the measure of

the microbial inactivation capacity of a process in moist heat sterilization, expressed as the equivalent time (minutes) at 121.1°C for microbes with a z value (the number of degrees that are required for a 10-fold change in the D value) of 10°C.

- Sterility assurance level (SAL): Probability of a single viable microorganism surviving in a product after sterilization, expressed as 10^{-n}.
- Dose of irradiation (absorbed dose): Quantity of ionizing radiation energy imparted per unit mass of the item, expressed in units of gray (Gy).
- Critical parameter: A measurable parameter that is inherently essential to the sterilization process.
- Loading pattern: A specified combination of the numbers, orientation and distribution of the item(s) to be sterilized within the sterilization chamber or irradiation container.

2. Sterilization

2.1. Heat method

In the heat method, microorganisms are killed by heat.

2.1.1. Moist-heat sterilization

Moist-heat sterilization includes widely used saturated steam sterilization and other types of moist-heat sterilization. The control points, utilities, and control devices in moist-heat sterilization are provided as reference in Table 1.

Saturated steam sterilization is a method for killing microorganisms with high pressure saturated steam. Critical parameters in this method are temperature, pressure, and exposure time at the specified temperature. Therefore, the temperature, pressure, and exposure time in routine sterilization process control should be continuously monitored and measured, and measuring equipment for that purpose should be included in the sterilization equipment specifications.

Other types of moist-heat sterilization may include steam pressurization cycles, water dispersion cycles, water immersion cycles, and the like, which are used when the items

Table 1 Control points, utilities, and control devices in moist-heat sterilization (reference)

	Saturated steam sterilization	Other types of moist-heat sterilization
Control point	• Temperature profile (usually indicated by F_0 value) • Temperature (drain or the like as needed) • Pressure (in sterilizer) • Exposure time at specified temperature • Loading pattern of items being sterilized • Steam quality (degree of superheat, dryness, non-condensable gas concentration, and chemical purity, as needed) • Quality of air that is introduced to the sterilizer for vacuum break • Quality of cooling water • Other requirements	• Temperature profile (usually indicated by F_0 value) • Temperature (drain and the like as needed) • Pressure, as needed (in sterilizer) • Exposure time at specified temperature • Loading pattern of item being sterilized • Quality of air that is introduced to the sterilizer for vacuum break • Quality of cooling water • Other requirements
Utilities and control devices that should be controlled	• Steam • Air introduced to the sterilizer for vacuum break • Cooling water • Temperature control device • Pressure control device • Time control device • Other	• Steam • Hot water • Air introduced to the sterilizer for vacuum break • Cooling water • Temperature control device • Pressure control device • Time control device • Conveyor for when a continuous sterilizer is used • Other

Table 2 Control points, utilities, and control devices in dry-heat sterilization (reference)

	Batch-type dry heat sterilizer	Tunnel-type dry heat sterilizer
Control point	• Temperature profile (usually indicated by F_H value) • Temperature • Exposure time at specified temperature • Pressure differential between inside and outside of container • Loading pattern of items being sterilized • Quality of air (heating air, cooling air) • Other requirements	• Temperature profile (usually indicated by F_H value) • Temperature • Belt speed (exposure time) • Pressure differential between inside and outside of equipment • Loading density • Quality of air (heating air, cooling air) • Other requirements
Utilities and control devices that should be controlled	• Air (heating air, cooling air) • Temperature control device • Time control device • Internal differential pressure gage • HEPA filter • Other	• Air (heating air, cooling air) • Temperature control device • Time control device • Internal differential pressure gage • HEPA filter • Cooler (if needed) • Other

being sterilized is sterilized in a hermetically sealed container. Critical parameters in such methods are the temperature in the container and the exposure time at the specified temperature.

2.1.2. Dry-heat sterilization

Dry-heat sterilization is a method for destructing microorganisms with dry heated air. This method is usually conducted in a batch or continuous (tunnel-type) dry heat sterilizer. Attention must be paid to the cleanliness of the air that flows into the sterilizer in either case. The control points, utilities, and control devices in dry-heat sterilization are provided as reference in Table 2. This method is suitable for when the item to be sterilized is highly heat-resistant, such as glass, ceramic or metal, or is thermo-stable, such as mineral oils, fatty oils, or solid pharmaceutical products.

Critical parameters in this method are temperature and the exposure time at the specified temperature (belt speed). Dry-heat sterilization requires higher temperatures and longer exposure times than does moist-heat sterilization even though the sterilization in both methods may be based on the same heating temperature. The temperature and exposure time in routine sterilization process control should be continuously monitored and measured, and measuring equipment for that purpose should be included in the sterilization equipment specifications.

2.1.3. Microwave sterilization

When substances to be sterilized such as drug solutions are exposed to microwaves, the polar molecules of the substance being sterilized vibrate as they attempt to change orientation due to the absorbed microwaves, and energy is released by the friction between the molecules. The method of killing microorganisms by the heat (microwave heat) generated at this time is called the microwave sterilization. A frequency of 2450 ± 50 MHz is ordinarily used.

Microwave devices are composed of a heating irradiation component which produces radiofrequency radiation to generate heat using a magnetron, a component for maintaining the sterilization temperature using an infrared heater or the like, and a cooling component for cooling the item being sterilized. Such devices continuously sterilize the item at ordinary pressure. The control points, utilities, and control devices in microwave sterilization are provided as reference in Table 3.

This method is applied to liquid products or products with high water content in hermetic container, etc.

Critical parameters in this method include the temperature of the items being sterilized and processing time. Therefore, the temperature of the items being sterilized and the processing time in routine sterilization process control should be continuously monitored and measured, and measuring equipment for that purpose should be included in the sterilization equipment specifications.

Microwave heating characteristically allows rapid sterilization at high temperatures to be continuously carried out with excellent thermal efficiency and responsiveness. However, the ease of heat transfer in the items being sterilized sometimes makes it difficult to ensure uniform heating. Attention must also be paid to the pressure resistance of the containers that are used because the heating takes place at ambient pressure, resulting in increases in internal pressure.

Table 3 Control points, utilities, and control devices in microwave sterilization (reference)

Control point	• Temperature profile (usually indicated by F_0 value) • Temperature • Processing time • Configuration of items being sterilized • Other requirements
Utilities and control devices that should be controlled	• High frequency control device • External heater (if needed) • Cooler (if needed) • Temperature monitoring device • Time monitoring device • Other

Table 4 Control points, utilities, and control devices in EO gas sterilization (reference)

Control point	• Pressure increase, injection time, and final pressure for the injection of sterilization gas • Temperature (in sterilizer and items being sterilized) • Humidity • EO gas concentration (gas concentration in sterilizer should be directly analyzed, but the following alternatives are acceptable when direct analysis is not feasible) i) Mass of gas used ii) Volume of gas used iii) Use of conversion formula based on initial reduced pressure and gas injection pressure • Operating time (exposure time) • Loading pattern of items being sterilized • Biological indicator placement points and cultivation results • Preconditioning conditions (temperature, humidity, time, etc.) • Aeration conditions (temperature, time, etc.) • Other requirements
Utilities and control devices that should be controlled	• EO gas • Injected vapor or water • Air replaced after completion of sterilization • Temperature control device • Humidity control device • Pressure control device • Time control device • Other

2.2. Gas method

The gas method kills microorganisms through contact with a sterilization gas or vapor. Microorganisms can be sterilized at lower temperatures than in heat methods, and the items being sterilized generally sustain little thermal damage. This method is therefore often applied to plastic containers and the like which are not very resistant to heat.

In the most common gas sterilization methods, adequate washing and drying are important to prevent contamination and moisture from compromising the sterilization effect. The sterilization effect may also be compromised if the gas is absorbed by the item being sterilized.

2.2.1. Ethylene oxide (EO) gas sterilization

EO gas sterilization kills microorganisms by altering the proteins and nucleic acids of microorganisms. Since EO gas is explosive, it is usually diluted 10 to 30% with carbon dioxide. EO gas is also a strongly reactive alkylating agent and therefore cannot be used to sterilize products which are likely to react with or absorb it.

The sterilization process consists of preconditioning, sterilization cycles, and aeration. EO gas is toxic (mutagenic, for example), and the substance being sterilized must therefore be aerated to ensure that the residual concentration of EO gas or other secondarily generated toxic gases (such as ethylene chlorohydrin) is at or below safe levels. Gas emissions must also be treated in compliance with regulations. The control points, utilities, and control devices in EO gas sterilization are provided as reference in Table 4.

Critical parameters in this method include temperature, humidity, gas concentration (pressure), and time. Therefore, the temperature, humidity, gas concentration (pressure), and time in routine sterilization process control should be continuously monitored and measured, and measuring equipment for that purpose should be included in the sterilization equipment specifications.

2.2.2. Hydrogen peroxide sterilization

Sterilization with hydrogen peroxide is a method for killing microorganisms through the oxidative power of hydrogen peroxide or the oxidation caused by radicals that are produced upon the generation of hydrogen peroxide plasma. Although items can be sterilized at lower temperatures than in heat methods, this method is not suitable for the sterilization of objects that absorb hydrogen peroxides, such as cellulose-based disposable garment and membrane filters because the sterilization effect will be compromised. The control points, utilities, and control devices in hydrogen peroxide sterilization are provided as reference in Table 5.

Critical parameters in this method include the concentration, time, and temperature. The control of a radio frequency device is also important when substances are sterilized with the use of plasma. The residual moisture of the substance being sterilized and the humidity in the sterilization environment may affect sterilization and should therefore be controlled when necessary.

2.3. Radiation method

2.3.1. Radiation sterilization

Radiation sterilization includes γ-ray radiation for killing microorganisms through the exposure of the items that are to be sterilized to γ-rays emitted from ^{60}Co, and electron beam radiation for killing microorganisms through exposure to an electron beam emitted from an electron beam accelerator. To select the method of sterilization, it must first be ensured that it is compatible with the items to be sterilized, including whether the quality of the substance could potentially deteriorate.

In γ-ray radiation sterilization, microorganisms are killed by secondarily produced electrons, whereas in electron beam radiation sterilization, microorganisms are directly killed by electrons. Although this kind of electron-based direct action is available, indirect action is also available, where sterilization is accomplished through the production of radicals and the like and damage to the DNA of microorganisms when γ-rays or electron beams react with water molecules.

Since sterilization can take place at room temperature, both methods can be applied to heat-labile items, and items can be sterilized while packaged because the radiation rays

Table 5 Control points, utilities, and control devices in hydrogen peroxide sterilization (reference)

	Hydrogen peroxide sterilization	Hydrogen peroxide low temperature gas plasma sterilization
Control point	• Concentration (the concentration in the sterilizer should be directly analyzed, but a method based on evidence of sterilizer performance uniformity in the chamber is an acceptable alternative when direct analysis is not feasible) • Time • Temperature • Humidity • Pressure • Quality of hydrogen peroxide • Consumption of hydrogen peroxide • Residual moisture of substance being sterilized • Loading pattern of items being sterilized • Biological indicator placement points and cultivation results • Chemical indicator placement points and results • Other requirements	• Concentration (the concentration in the sterilizer should be directly analyzed, but a method based on evidence of sterilizer performance uniformity in the chamber is an acceptable alternative when direct analysis is not feasible) • Time • Temperature • Humidity • Pressure • Quality of hydrogen peroxide • Consumption of hydrogen peroxide • Residual moisture of substance being sterilized • Loading pattern of items being sterilized • Biological indicator placement points and cultivation results • Chemical indicator placement points and results • Other requirements
Utilities and control devices that should be controlled	• Hydrogen peroxide • Pressure gauge • Hydrogen peroxide injector • Other	• Hydrogen peroxide • Pressure gauge • Hydrogen peroxide injector • High frequency generator • Other

will penetrate the packaging. γ-Ray sterilization is suitable primarily for high density products such as metals, water, and powder because the penetration is better than that of electron beams. Electron beam radiation sterilization has a higher radiation dose per unit time (dose rate) compared with γ-rays and therefore has a shorter processing time. The control points, utilities, and control devices in radiation sterilization are provided as reference in Table 6.

2.4. Filtration method

The filtration method is a method for physically removing microorganisms in liquids or gas using a sterilization filter. It can therefore be applied to items that are unstable against heat or radiation. Filtration sterilization is for microorganisms which can be removed by a 0.2 μm membrane filter, and is not suitable for *Mycoplasma* spp., *Leptospira* spp., or viruses. The control points, utilities, and control devices in filtration sterilization are provided as reference in Table 7.

The critical parameters affecting the removal of microorganisms by the filter in liquid filtration sterilization include filtration time, filtration capacity, filtration flow rate, filtration differential pressure, and temperature. The critical parameters in gas filtration sterilization include filtration differential pressure and temperature. When a liquid is to be sterilized, the removal of microorganisms by a filter will be affected by the physicochemical properties of the liquid that is undergoing filtration (such as viscosity, pH, and surfactant action). The microbial trapping performance of a sterilizing filter can generally be validated when a sterilizing filter challenged with more than 10^7 CFU microorganisms of a strain of *Brevundimonas diminuta* (ATCC 19146, NBRC 14213) or appropriate smaller species, cultured under the appropriate conditions, per square centimeter of effective filter area, provides a sterile effluent.

The bioburden of liquids prior to filtration will affect filtration sterilization performance and should therefore be controlled.

3. Sterilization Indicators

3.1. Biological indicators (BI)

3.1.1. Introduction

A BI is an indicator prepared from the spores of a microorganism resistant to the specified sterilization process, and is used to develop and/or validate a sterilization process.

Indicators are classified based on configuration into the ''paper strip type'', ''the type that is inoculated on or into the surface of metal or the like'', ''liquid type'' and ''the self-contained type in which a medium and paper strip are pre-encapsulated''. They are also classified by carrier, where one type comprises a carrier of paper, glass, stainless steel, plastic or the like that is inoculated with bacterial spores and packaged, and another type comprises the product or simulated product as the carrier, which is inoculated with bacterial spores. Typical examples of indicators by sterilization method are shown in Table 8.

3.1.2. Labeling of commercially available BI

Users of commercially available BI produced in accordance with ISO11138-1 must check the following information provided by the BI manufacturer to users.

- Traceability (microorganism, carrier, labeling, etc.)
- Species name

Table 6 Control points, utilities, and control devices in radiation sterilization (reference)

	γ-Ray radiation sterilization	Electron beam radiation sterilization
Control point	• Absorbed dose • Loading pattern (density) of items being sterilized • Exposure time (conveyor speed or cycle time) • Other requirements	• Absorbed dose • Loading pattern (density) of items being sterilized • Electron beam properties (mean electron beam current, electron beam energy, scanning width) • Other requirements
Utilities and control devices that should be controlled	• Belt conveyor • Dose measurement system • Other	• Electron beam measurement device • Belt conveyor • Dose measurement system • Other

Table 7 Control points, utilities, and control devices in filtration sterilization (reference)

	Liquid filtration sterilization	Gas filtration sterilization
Control point	• Filtration time • Filtration capacity • Filtration flow rate • Filtration differential pressure • Temperature • Filter integrity • In cases involving multiple use: expiration period and number of times the filter can be used for sterilization • Other requirements	• Filtration differential pressure • Temperature, if needed • Filter integrity • Expiration period • Number of sterilizations of times the filter can be used for sterilization • Direction of gas current (for bidirectional flow) • Other requirements
Utilities and control devices that should be controlled	• Pressure gage • Flow rate meter • Integrity tester • Other	• Pressure gage • Flow rate meter • Integrity tester • Other

Table 8 List of typical indicators by sterilization method

Sterilization method	Species	Strain name	*D* value, etc. (reference)
Moist-heat sterilization	*Geobacillus stearothermophilus*	ATCC 7953, NBRC 13737	≥1.5 min (121°C)
Dry-heat sterilization	*Bacillus atrophaeus*	ATCC 9372, NBRC 13721	≥2.5 min (160°C)
EO gas sterilization	*Bacillus atrophaeus*	ATCC 9372, NBRC 13721	≥2.5 min (54°C) ≥12.5 min (30°C) Gas concentration: 600 mg/L ± 30 mg/L; relative humidity: 60% RH
Hydrogen peroxide sterilization	*Geobacillus stearothermophilus*	ATCC 12980, NBRC 12550 or ATCC 7953, NBRC 13737	—

- Nominal bacterial spore count
- Resistance
- Method used
- Storage conditions (temperature, expiration date, etc.)
- Culture conditions (temperature, time, medium, etc.)
- Disposal method

Parameters determining BI performance include ''species,'' ''resistance,'' and ''bacterial count.'' Resistance varies, even for the same species, depending on the nature and configuration of the carrier or packaging, and evaluation must therefore include the packaging.

3.1.3. Control during use of commercially available BI

BI must be handled in accordance with the storage conditions, time to start of culture after sterilization, culturing conditions, disposal method, and the like provided by the BI manufacturer. Because the storage conditions in particular affect BI performance, precautions must be taken to prevent a BI from being allowed to stand for a long period of time until use after being removed from the packaging.

The BI should be set up to enable evaluation of the entire items being sterilized. The BI should be set up in places where the sterilization effect is expected to be low in any given method, such as cold spots in heat sterilization. Care should be taken to avoid damaging the BI packaging or carrier when recovered. Predetermined procedures for preventing microbial contamination should be in place in case bacteria are released or spread if the packaging does end up becoming damaged.

When using a BI that has been purchased, the user should measure the spore count or the like when received as needed to make sure there are no significant differences with the nominal count provided by the BI manufacturer.

3.1.4. Precautions for when sterilization indicators are prepared by the user

The following must be evaluated prior to use when users prepare indicators themselves using the bioburden collected from the items being sterilized or the manufacturing environment rather than purchasing a BI for use.

- Species name
- Bacterial spore count
- Resistance (*D* value at sterilization temperature or sterilization gas concentration)
- Storage conditions (temperature, expiration date, etc.)
- Culture conditions (temperature, incubation time, medium, etc.)

An evaluation program must be established to continuously show that the resistance of picked bacteria is the most resistant of the bioburden.

3.1.5. Precautions when commercially available BI are modified by users

When a BI that has been purchased is removed from the packaging and is used to inoculate an item such as drug solution or materials, the bacterial spore count or resistance will vary and must therefore be assessed prior to use.

ISO11138 or USP <55> can be used for reference for such evaluation. Resistance can be evaluated by using a biological indicator evaluation resistometer (BIER) or the capillary method with oil bath. When such self-assessment is unfeasible, a third-party testing facility can be used.

3.2. Chemical indicator (CI)

A CI is an indicator that chemically or physically changes due to exposure to heat, gas, radiation, or the like. Such indicators are produced by being applied to or printed on a piece of paper, for example. Because the principals involved in such changes will depend on the sterilization method, a CI that is suitable for the intended sterilization method must be used. CI is classified into the following six classes based on the intended application. The classes shown here are unrelated to level of performance.

A CI indicates the progress of a sterilization step or of a number of critical parameters, but is not used to assure sterilization effect or sterility and therefore cannot be used as an alternative to a BI.

Class 1: Process indicators

These are intended to distinguish whether an item being sterilized has passed through a sterilization step. They respond to one or more critical parameters.

Class 2: Indicators for use in specific tests

These are used in tests of the exhaust capacity and vapor penetration of a vacuum-type high-pressure steam sterilizer as specified in the ISO11140 series. They correspond to the Bowie-Dick type.

Class 3: Single-variable indicators

These respond to only one critical parameter. They show exposure in a sterilization step based on a specified value for the designated parameter.

Class 4: Multi-variable indicators

These respond to two or more critical parameters. They show exposure in a sterilization step based on specified values for the designated parameters.

Class 5: Integrating indicators

These respond to all critical parameters. Their performance is equal to or greater than that required of BI in the ISO11138 series.

Class 6: Emulating indicators

These respond to all critical parameters of a specified sterilization cycle. The specifications are critical

Table 9 Types of dosimeters

Type of radiation	Dosimeter
γ-ray	Dyed polymethyl methacrylate dosimeter Clear polymethyl methacrylate dosimeter Ceric-cerous dosimeter Alanine – EPR dosimeter
γ-ray, electron beam	Cellulose acetate dosimeter Radiochromic film dosimeter

parameters of the designated sterilization step.

3.3. Dosimeter

3.3.1. Types of dosimeters

The dosimeter in a radiation process is an instrument or system which reads the absorbed dose based on changes caused by the absorption of the radiation, for which ''reproducibility'' and ''response permitting radiation to be measured'' are required. Most dosimeters are susceptible to environmental conditions (process parameters) such as temperature and dose rate before, during, and after exposure to the facilities being used, and caution is therefore required. The choice of dosimeter and calibration guidelines for radiation processes have been specified (ISO/ASTM 51261) as reference for the selection and use of dosimeters. Dosimeters for measuring the absorbed dose of radiation are shown in Table 9. γ-Ray dosimeters are not normally suitable for sterilization process control involving the use of electron beams of less than 3 MeV energy.

3.3.2. Dosimeter use

Dosimeters are used when dose distribution is measured to determine the conditions of radiation and to evaluate the absorbed dose of an items being sterilized during ordinary radiation sterilization. In the former, dosimeters are set up in advance in the object being sterilized and are then recovered after radiation for measurement in the measurement system to find the absorbed dose at each location. The dosimeters should be arranged in a broad range of vertical and horizontal directions because it is necessary to determine the relationship between minimum/maximum exposure and the process parameters as well as to verify the appropriateness of the packaging configuration based on the variation in radiation penetration and dose. In the latter, there is no need to arrange the dosimeters in the locations characterized by the maximum or minimum dose in the object being sterilized. Control points where dosimeters are easily arranged and recovered should be selected, and the absorbed dose of the object being sterilized should be ensured based on the absorbed dose at the control points. Therefore, in the measurement of dose distribution, the quantitative relationship between the control points and the locations of maximum/minimum exposure should be determined, and the passing dose range at the control points should also be calculated.

Newly purchased dosimeters should be calibrated prior to use, and dosimeters should be calibrated every time a batch is changed and at least once a year.

4. Establishment of Sterilization Conditions

4.1. Half-cycle method

In the half-cycle method, a sterilization time twice as long as that required to inactivate all of the 10^6 CFU bacteria included in the BI is used, regardless of the bioburden count on the object being sterilized or the resistance of the test microorganisms to sterilization. This method is primarily used to establish the conditions of EO or other gas sterilization.

4.2. Overkill method

In the overkill method, a sterilization condition to achieve an SAL of 10^{-6} or better is used, regardless of bioburden count on the object being sterilized or the resistance of the test microorganisms to sterilization.

This means a level of sterilization of 12 D in steam sterilization. However, a level $\geq F_0$ 12 is also referred to as the overkill method.

4.3. Combination of bioburden and BI method

In the combined bioburden/BI method, the maximum bioburden count is determined based on the results of extensive bioburden analysis, and the sterilization time (or radiation dose) is calculated using an appropriate commercially available BI with a test microorganism count $\geq$ the maximum bioburden count based on the target SAL.

When this procedure is used, the bioburden count of the object being sterilized must be tested on a daily basis, and the resistance of the test microorganisms to sterilization must be periodically measured.

If the bioburden testing reveals a microorganism more resistant than the BI microorganism, it should be used as the indicator. The sterilization conditions must also be revised as needed.

$$\text{Sterilization time (or radiation dose)} = D \times \log (N_0/N)$$

D: D value of BI
N: Target sterility assurance level (SAL)
N_0: Maximum bioburden count in object being sterilized

4.4. Absolute bioburden method

In the absolute bioburden method, the sterilization resistance of the microorganisms found in the object being sterilized or environment is measured, and the sterilization conditions are determined, in the case of moist-heat sterilization, by employing the D value of the most resistant microorganism based on the bioburden count of the object being sterilized.

The bioburden count should be determined by extensive bioburden analysis. When this procedure is used, the microorganism count and the resistance of the detected microorganisms to sterilization must be assessed on a daily basis in routine bioburden control.

Radiation sterilization may be performed in accordance with ISO11137–2.

5. References

- ISO 11138-1: 2006, Sterilization of health care products-Biological indicators-Part1: General requirements
- ISO 11137-2: 2013, Sterilization of health care products-Radiation- Part2: Establishing the sterilization dose
- ISO/ASTM 51261: 2013, Guide for selection and calibration of dosimetry systems for radiation processing
- ISO 11140–1: 2014, Sterilization of health care products-Chemical indicators- Part1: General requirements
- US Pharmacopeia 38 (2015), <55> Biological Indicators-Resistance Performance Tests.

G5 Crude Drugs

On the Scientific Names of Crude Drugs listed in the JP *<G5-1-180>*

The notation system of the scientific names for the original plants and animals of crude drugs listed in JP is not nec-

Scientific Names used in the JP and Those being used Taxonomically

Crude Drug	Scientific names used in the JP = Scientific names being used taxonomically (Combined notation, Standard form for author or authors) Scientific names that are different from those written in JP but identical to them taxonomically or being regarded as identical, and typical subclassified groups belonging to their species. The names marked with "*" are those being written together in JP.	Family
Acacia アラビアゴム	*Acacia senegal* Willdenow =*Acacia senegal* (L.) Willd.	*Leguminosae*
	Other species of the same genus	
Achyranthes Root ゴシツ	*Achyranthes bidentata* Blume	*Amaranthaceae*
	Achyranthes fauriei H. Léveillé et Vaniot =*Achyranthes fauriei* H. Lev. & Vaniot	
Agar カンテン	*Gelidium elegans* Kuetzing	*Gelidiaceae*
	Other species of the same genus	
	Red seaweeds of several species	
Akebia Stem モクツウ	*Akebia quinata* Decaisne =*Akebia quinata* (Thunb. ex Houtt.) Decne.	*Lardizabalaceae*
	Akebia trifoliata Koidzumi =*Akebia trifoliata* (Thunb.) Koidz.	
Alisma Tuber タクシャ	*Alisma orientale* Juzepczuk =*Alisma orientale* (Sam.) Juz.	*Alismataceae*
	Alisma plantago-aquatica L. var. *orientale* Sam.	
Aloe アロエ	*Aloe ferox* Miller =*Aloe ferox* Mill.	*Liliaceae*
	Interspecific hybrid between *Aloe ferox* Miller and *Aloe africana* Miller =*Aloe africana* Mill.	
	Interspecific hybrid between *Aloe ferox* Miller and *Aloe spicata* Baker	
Alpinia Officinarum Rhizome リョウキョウ	*Alpinia officinarum* Hance	*Zingiberaceae*
Amomum Seed シュクシャ	*Amomum villosum* Loureiro var. *xanthioides* T. L. Wu et S. J. Chen = *Amomum villosum* Lour. var. *xanthioides* (Wall. ex Baker) T. L. Wu & S. J. Chen	*Zingiberaceae*
	Amomum xanthioides Wallich = *Amomum xanthioides* Wall. ex Baker	
	Amomum villosum Lour. var. *nanum* H. T. Tsai & S. W. Zhao	
	Amomum villosum Loureiro var. *villosum* = *Amomum villosum* Lour. var. *villosum*	
	Amomum villosum Lour.	
	Amomum longiligulare T. L. Wu	
Anemarrhena Rhizome チモ	*Anemarrhena asphodeloides* Bunge	*Liliaceae*
Angelica Dahurica Root ビャクシ	*Angelica dahurica* Bentham et Hooker filius ex Franchet et Savatier =*Angelica dahurica* (Hoffm.) Benth. & Hook. f. ex Franch. & Sav.	*Umbelliferae*

Apricot Kernel キョウニン	*Prunus armeniaca* Linné ＝*Prunus armeniaca* L.	*Rosaceae*
	Prunus armeniaca Linné var. *ansu* Maximowicz ＝*Prunus armeniaca* L. var. *ansu* Maxim.	
	Prunus sibirica Linné ＝*Prunus sibirica* L.	
Aralia Rhizome ドクカツ	*Aralia cordata* Thunberg ＝*Aralia cordata* Thunb.	*Araliaceae*
Areca ビンロウジ	*Areca catechu* Linné ＝*Areca catechu* L.	*Palmae*
Artemisia Capillaris Flower インチンコウ	*Artemisia capillaris* Thunberg ＝*Artemisia capillaris* Thunb.	*Compositae*
Artemisia Leaf ガイヨウ	*Artemisia princeps* Pampanini ＝*Artemisia princeps* Pamp.	*Compositae*
	Artemisia montana Pampanini ＝*Artemisia montana* (Nakai) Pamp.	
Asiasarum Root サイシン	*Asiasarum heterotropoides* F. Maekawa var. *mandshuricum* F. Maekawa ＝*Asiasarum heterotropoides* (F. Schmidt) F. Maek. var. *mandshuricum* (Maxim.) F. Maek.	*Aristolochiaceae*
	Asarum heterotropoides F. Schmidt var. *mandshuricum* (Maxim.) Kitag.	
	Asiasarum sieboldii F. Maekawa ＝*Asiasarum sieboldii* (Miq.) F. Maek.	
	Asarum sieboldii Miq. *Asarum sieboldii* Miq. var. *seoulense* Nakai	
Asparagus Root テンモンドウ	*Asparagus cochinchinensis* Merrill ＝*Asparagus cochinchinensis* (Lour.) Merr.	*Liliaceae*
Astragalus Root オウギ	*Astragalus mongholicus* Bunge	*Leguminosae*
	Astragalus membranaceus (Fisch.) Bunge var. *mongholicus* (Bunge) Hsiao	
	Astragalus membranaceus Bunge ＝*Astragalus membranaceus* (Fisch.) Bunge	
Atractylodes Lancea Rhizome ソウジュツ	*Atractylodes lancea* De Candolle ＝*Atractylodes lancea* (Thunb.) DC.	*Compositae*
	Atractylodes chinensis Koidzumi ＝*Atractylodes chinensis* (Bunge) Koidz.	
	Interspecific hybrid between above species	
Atractylodes Rhizome ビャクジュツ	*Atractylodes japonica* Koidzumi ex Kitamura ＝*Atractylodes japonica* Koidz. ex Kitam.	*Compositae*
	Atractylodes macrocephala Koidzumi ＝*Atractylodes macrocephala* Koidz.	
	* *Atractylodes ovata* De Candolle ＝*Atractylodes ovata* (Thunb.) DC.	
Bear Bile ユウタン	*Ursus arctos* Linné ＝*Ursus arctos* L.	*Ursidae*
	Other animals of the related genus	
Bearberry Leaf ウワウルシ	*Arctostaphylos uva-ursi* Sprengel ＝*Arctostaphylos uva-ursi* (L.) Spreng.	*Ericaceae*
Beef Tallow 牛脂	*Bos taurus* Linné var. *domesticus* Gmelin ＝*Bos taurus* L. var. *domesticus* Gmelin	*Bovidae*

Yellow Beeswax ミツロウ	*Apis mellifera* Linné ＝*Apis mellifera* L.	*Apidae*
	Apis cerana Fabricius	
Belladonna Extract ベラドンナコン	*Atropa belladonna* Linné ＝*Atropa belladonna* L.	*Solanaceae*
Benincasa Seed トウガシ	*Benincasa cerifera* Savi	*Cucurbitaceae*
	Benincasa hispida (Thunb.) Cogn.	
	Benincasa cerifera Savi forma *emarginata* K. Kimura et Sugiyama ＝*Benincasa cerifera* Savi f. *emarginata* K. Kimura & Sugiyama	
Benzoin アンソッコウ	*Styrax benzoin* Dryander ＝*Styrax benzoin* Dryand.	*Styracaceae*
	Other species of the same genus	
Bitter Cardamon ヤクチ	*Alpinia oxyphylla* Miquel ＝*Alpinia oxyphylla* Miq.	*Zingiberaceae*
Bitter Orange Peel トウヒ	*Citrus aurantium* Linné ＝*Citrus aurantium* L.	*Rutaceae*
	Citrus aurantium Linné var. *daidai* Makino ＝*Citrus aurantium* L. var. *daidai* Makino	
	Citrus aurantium L. ‘Daidai’	
Brown Rice コウベイ	*Oryza sativa* Linné ＝*Oryza sativa* L.	*Gramineae*
Bupleurum Root サイコ	*Bupleurum falcatum* Linné ＝*Bupleurum falcatum* L.	*Umbelliferae*
	Bupleurum chinense DC. *Bupleurum scorzonerifolium* Willd.	
Burdock Fruit ゴボウシ	*Arctium lappa* Linné ＝*Arctium lappa* L.	*Compositae*
Cacao Butter カカオ脂	*Theobroma cacao* Linné ＝*Theobroma cacao* L.	*Sterculiaceae*
Calumba コロンボ	*Jateorhiza columba* Miers	*Menispermaceae*
Camellia Oil ツバキ油	*Camellia japonica* Linné ＝*Camellia japonica* L.	*Theaceae*
Capsicum トウガラシ	*Capsicum annuum* Linné ＝*Capsicum annuum* L.	*Solanaceae*
Cardamon ショウズク	*Elettaria cardamomum* Maton	*Zingiberaceae*
Carnauba Wax カルナウバロウ	*Copernicia cerifera* Martius ＝*Copernicia cerifera* Mart.	*Palmae*
Cassia Seed ケツメイシ	*Cassia obtusifolia* Linné ＝*Cassia obtusifolia* L.	*Leguminosae*
	Cassia tora Linné ＝*Cassia tora L.*	
Castor Oil ヒマシ油	*Ricinus communis* Linné ＝*Ricinus communis* L.	*Euphorbiaceae*
Catalpa Fruit キササゲ	*Catalpa ovata* G. Don	*Bignoniaceae*
	Catalpa bungei C. A. Meyer ＝*Catalpa bungei* C. A. Mey.	

Cherry Bark オウヒ	*Prunus jamasakura* Siebold ex Koidzumi ＝*Prunus jamasakura* Siebold ex Koidz.	*Rosaceae*
	Prunus verecunda Koehne ＝*Prunus verecunda* (Koidz.) Koehne	
Chrysanthemum Flower キクカ	*Chrysanthemum indicum* Linné ＝*Chrysanthemum indicum* L.	*Compositae*
	Chrysanthemum morifolium Ramatuelle ＝*Chrysanthemum morifolium* Ramat.	
Cimicifuga Rhizome ショウマ	*Cimicifuga dahurica* Maximowicz ＝*Cimicifuga dahurica* (Turcz.) Maxim.	*Ranunculaceae*
	Cimisifuga heracleifolia Komarov ＝*Cimisifuga heracleifolia* Kom.	
	Cimicifuga foetida Linné ＝*Cimicifuga foetida* L.	
	Cimicifuga simplex Turczaninow ＝*Cimicifuga simplex* (DC.) Turcz.	
Cinnamon Bark ケイヒ	*Cinnamomum cassia* J. Presl ＝*Cinnamomum cassia* (L.) J. Presl	*Lauraceae*
Cinnamon Oil ケイヒ油	*Cinnamomum cassia* J. Presl ＝*Cinnamomum cassia* (L.) J. Presl	*Lauraceae*
	Cinnamomum zeylanicum Nees	
Cistanche Herb ニクジュヨウ	*Cistanche salsa* G. Beck ＝*Cistanche salsa* (C.A.Mey.) Beck	*Orobanchaceae*
	Cistanche deserticola Y. C. Ma ＝*Cistanche deserticola* Ma	
	Cistanche tubulosa Wight	
Citrus Unshiu Peel チンピ	*Citrus unshiu* Marcowicz ＝*Citrus unshiu* (Swingle) Marcow.	*Rutaceae*
	Citrus reticulata Blanco 'Unshiu'	
	Citrus reticulata Blanco	
Clematis Root イレイセン	*Clematis mandshurica* Ruprecht ＝*Clematis mandshurica* Rupr.	*Ranunculaceae*
	Clematis chinensis Osbeck	
	Clematis hexapetala Pallas ＝*Clematis hexapetala* Pall.	
Clove チョウジ Clove Oil チョウジ油	*Syzygium aromaticum* Merrill et Perry ＝*Syzygium aromaticum* (L.) Merr. & L. M. Perry	*Myrtaceae*
	* *Eugenia caryophyllata* Thunberg ＝*Eugenia caryophyllata* Thunb. *Eugenia caryophyllus* (Spreng.) Bullock & S. G. Harrison	
Cnidium Monnieri Fruit ジャショウシ	*Cnidium monnieri* Cusson ＝*Cnidium monnieri* (L.) Cusson	*Umbelliferae*
Cnidium Rhizome センキュウ	*Cnidium officinale* Makino	*Umbelliferae*
Coconut Oil ヤシ油	*Cocos nucifera* Linné ＝*Cocos nucifera* L.	*Palmae*
Codonopsis Root トウジン	*Codonopsis pilosula* Nannfeldt ＝*Codonopsis pilosula* Nannf.	*Campanulaceae*
	Codonopsis tangshen Oliver ＝*Codonopsis tangshen* Oliv.	

Coix Seed ヨクイニン	*Coix lacryma-jobi* Linné var. *mayuen* Stapf ＝*Coix lacryma-jobi* L. var. *mayuen* (Rom. Caill.) Stapf	*Gramineae*
Condurango コンズランゴ	*Marsdenia cundurango* Reichenbach filius ＝*Marsdenia cundurango* Rchb. f.	*Asclepiadaceae*
Coptis Rhizome オウレン	*Coptis japonica* Makino ＝*Coptis japonica* (Thunb.) Makino	*Ranunculaceae*
	Coptis japonica (Thunb.) Makino var. *dissecta* (Yatabe) Nakai *Coptis japonica* (Thunb.) Makino var. *japonica* *Coptis japonica* (Thunb.) Makino var. *major* (Miq.) Satake	
	Coptis chinensis Franchet ＝*Coptis chinensis* Franch.	
	Coptis deltoidea C. Y. Cheng et Hsiao	
	Coptis teeta Wallich ＝*Coptis teeta* Wall.	
Corn Oil トウモロコシ油	*Zea mays* Linné ＝*Zea mays* L.	*Gramineae*
Cornus Fruit サンシュユ	*Cornus officinalis* Siebold et Zuccarini ＝*Cornus officinalis* Siebold & Zucc.	*Cornaceae*
Corydalis Tuber エンゴサク	*Corydalis turtschaninovii* Besser forma *yanhusuo* Y. H. Chou et C. C. Hsu ＝*Corydalis turtschaninovii* Besser f. *yanhusuo* (W. T. Wang) Y. H. Chou & C. C. Hsu	*Papaveraceae*
	Corydalis yanhusuo W. T. Wang	
Crataegus Fruit サンザシ	*Crataegus cuneata* Siebold et Zuccarini ＝*Crataegus cuneata* Siebold & Zucc.	*Rosaceae*
	Crataegus pinnatifida Bunge var. *major* N. E. Brown ＝*Crataegus pinnatifida* Bunge var. *major* N. E. Br.	
Curcuma Rhizome ガジュツ	*Curcuma zedoaria* Roscoe	*Zingiberaceae*
	Curcuma phaeocaulis Valeton	
	Curcuma kwangsiensis S. G. Lee et C. F. Liang	
Cyperus Rhizome コウブシ	*Cyperus rotundus* Linné ＝*Cyperus rotundus* L.	*Cyperaceae*
Digenea マクリ	*Digenea simplex* C. Agardh ＝*Digenea simplex* (Wulfen) C. Agardh	*Rhodomelaceae*
Dioscorea Rhizome サンヤク	*Dioscorea japonica* Thunberg ＝*Dioscorea japonica* Thunb.	*Dioscoreaceae*
	Dioscorea batatas Decaisne ＝*Dioscorea batatas* Decne.	
	Dioscorea opposita Thunb.	
Dolichos Seed ヘンズ	*Dolichos lablab* Linné ＝*Dolichos lablab* L.	*Leguminosae*
Eleutherococcus Senticosus Rhizome シゴカ	*Eleutherococcus senticosus* Maximowicz ＝*Eleutherococcus senticosus* (Rupr. & Maxim.) Maxim.	*Araliaceae*
	* *Acanthopanax senticosus* Harms ＝*Acanthopanax senticosus* (Rupr. & Maxim.) Harms	
Ephedra Herb マオウ	*Ephedra sinica* Stapf	*Ephedraceae*
	Ephedra intermedia Schrenk et C. A. Meyer ＝*Ephedra intermedia* Schrenk & C. A. Mey.	
	Ephedra equisetina Bunge	

Epimedium Herb インヨウカク	*Epimedium koreanum* Nakai	*Berberidaceae*
	Epimedium grandiflorum Morren var. *thunbergianum* Nakai ＝*Epimedium grandiflorum* Morr. var. *thunbergianum* (Miq.) Nakai	
	Epimedium pubescens Maximowicz ＝*Epimedium pubescens* Maxim.	
	Epimedium brevicornu Maximowicz ＝*Epimedium brevicornu* Maxim.	
	Epimedium wushanense T. S. Ying	
	Epimedium sagittatum Maximowicz ＝*Epimedium sagittatum* (Siebold & Zucc.) Maxim.	
	Epimedium sempervirens Nakai	
Eucalyptus Oil ユーカリ油	*Eucalyptus globulus* Labillardiere ＝*Eucalyptus globulus* Labill.	*Myrtaceae*
	Allied species	
Eucommia Bark トチュウ	*Eucommia ulmoides* Oliver ＝*Eucommia ulmoides* Oliv.	*Eucommiaceae*
Evodia Fruit ゴシュユ	*Euodia officinalis* Dode	*Rutaceae*
	* *Evodia officinalis* Dode *Evodia rutaecarpa* (A. Juss.) Benth. var. *officinalis* (Dode) Huang	
	Euodia bodinieri Dode	
	* *Evodia bodinieri* Dode *Evodia rutaecarpa* (A. Juss.) Benth. var. *bodinieri* (Dode) Huang	
	Euodia ruticarpa Hooker filius et Thomson ＝*Euodia ruticarpa* (A. Juss.) Hook. f. & Thomson	
	* *Evodia rutaecarpa* Bentham ＝*Evodia rutaecarpa* (A. Juss.) Benth. *Tetradium ruticarpum* (A. Juss.) Hartley	
Fennel ウイキョウ	*Foeniculum vulgare* Miller ＝*Foeniculum vulgare* Mill.	*Umbelliferae*
Fennel Oil ウイキョウ油	*Foeniculum vulgare* Miller ＝*Foeniculum vulgare* Mill.	*Umbelliferae*
	Illicium verum Hooker filius ＝*Illicium verum* Hook. f.	*Illiciaceae*
Forsythia Fruit レンギョウ	*Forsythia suspensa* Vahl ＝*Forsythia suspensa* (Thunb.) Vahl	*Oleaceae*
Fritillaria Bulb バイモ	*Fritillaria verticillata* Willdenow var. *thunbergii* Baker ＝*Fritillaria verticillata* Willd. var. *thunbergii* (Miq.) Baker	*Liliaceae*
	Fritillaria thunbergii Miq.	
Gambir アセンヤク	*Uncaria gambir* Roxburgh ＝*Uncaria gambir* (Hunter) Roxb.	*Rubiaceae*
Gardenia Fruit サンシシ	*Gardenia jasminoides* Ellis	*Rubiaceae*
	Gardenia jasminoides Ellis f. *longicarpa* Z. W. Xie & Okada	
Gastrodia Tuber テンマ	*Gastrodia elata* Blume	*Orchidaceae*
Gentian ゲンチアナ	*Gentiana lutea* Linné ＝*Gentiana lutea* L.	*Gentianaceae*

Geranium Herb ゲンノショウコ	*Geranium thunbergii* Siebold et Zuccarini ＝*Geranium thunbergii* Siebold & Zucc.	*Geraniaceae*
Ginger ショウキョウ	*Zingiber officinale* Roscoe	*Zingiberaceae*
Ginseng ニンジン	*Panax ginseng* C. A. Meyer ＝*Panax ginseng* C. A. Mey.	*Araliaceae*
	* *Panax schinseng* Nees	
Glehnia Root ハマボウフウ	*Glehnia littoralis* Fr. Schmidt ex Miquel ＝*Glehnia littoralis* F. Schmidt ex Miq.	*Umbelliferae*
Glycyrrhiza カンゾウ	*Glycyrrhiza uralensis* Fischer ＝*Glycyrrhiza uralensis* Fisch.	*Leguminosae*
	Glycyrrhiza glabra Linné ＝*Glycyrrhiza glabra* L.	
Hedysarum Root シンギ	*Hedysarum polybotrys* Handel-Mazzetti ＝*Hedysarum polybotrys* Hand.-Mazz.	*Leguminosae*
Hemp Fruit マシニン	*Cannabis sativa* Linné ＝*Cannabis sativa* L.	*Moracea*
Honey ハチミツ	*Apis mellifera* Linné ＝*Apis mellifera* L.	*Apidae*
	Apis cerana Fabricius	
Houttuynia Herb ジュウヤク	*Houttuynia cordata* Thunberg ＝*Houttuynia cordata* Thunb.	*Saururaceae*
Immature Orange キジツ	*Citrus aurantium* Linné var. *daidai* Makino ＝*Citrus aurantium* L. var. *daidai* Makino	*Rutaceae*
	Citrus aurantium L. ‘Daidai’	
	Citrus natsudaidai Hayata	
	Citrus aurantium Linné ＝*Citrus aurantium* L.	
	Citrus aurantium L. subsp. *hassaku* (Tanaka) Hiroe ＝*Citrus hassaku* hort. ex Tanaka	
Imperata Rhizome ボウコン	*Imperata cylindrica* Beauvois ＝*Imperata cylindrica* (L.) P. Beauv.	*Gramineae*
	Imperata cylindrica (L.) P. Beauv. var. *major* (Nees) C. E. Hubb.	
Ipecac トコン	*Cephaelis ipecacuanha* A. Richard ＝*Cephaelis ipecacuanha* (Brot.) A. Rich.	*Rubiaceae*
	Cephaelis acuminata Karsten ＝*Cephaelis acuminata* H. Karst.	
Japanese Angelica Root トウキ	*Angelica acutiloba* Kitagawa ＝*Angelica acutiloba* (Siebold & Zucc.) Kitag.	*Umbelliferae*
	Angelica acutiloba Kitagawa var. *sugiyamae* Hikino ＝*Angelica acutiloba* (Siebold & Zucc.) Kitag. var. *sugiyamae* Hikino	
Japanese Gentian リュウタン	*Gentiana scabra* Bunge	*Gentianaceae*
	Gentiana scabra Bunge var. *buergeri* (Miq.) Maxim.	
	Gentiana manshurica Kitagawa ＝*Gentiana manshurica* Kitag.	
	Gentiana triflora Pallas ＝*Gentiana triflora* Pall.	
	Gentiana triflora Pall. var. *japonica* Hara	

Japanese Valerian カノコソウ	*Valeriana fauriei* Briquet ＝*Valeriana fauriei* Briq.	*Valerianaceae*
	Valeriana fauriei Briq. f. *yezoensis* Hara	
Japanese Zanthoxylum Peel サンショウ	*Zanthoxylum piperitum* De Candolle ＝*Zanthoxylum piperitum* (L.) DC.	*Rutaceae*
	Zanthoxylum piperitum (L.) DC. f. *inerme* Makino	
Jujube タイソウ	*Ziziphus jujuba* Miller var. *inermis* Rehder ＝ *Ziziphus jujuba* Mill. var. *inermis* (Bunge) Rehder	*Rhamnaceae*
Jujube Seed サンソウニン	*Ziziphus jujuba* Miller var. *spinosa* Hu ex H. F. Chow ＝ *Ziziphus jujuba* Mill. var. *spinosa* (Bunge) Hu ex H. F. Chow	*Rhamnaceae*
Koi コウイ	*Zea mays* Linné ＝*Zea mays* L.	*Gramineae*
	Manihot esculenta Crantz	*Euphorbiacaea*
	Solanum tuberosum Linné ＝*Solanum tuberosum* L.	*Solanaceae*
	Ipomoea batatas Poiret ＝*Ipomoea batatas* (L.) Poir.	*Convolvulaceae*
	Ipomoea batatas (L.) Lam.	
	Oryza sativa Linné ＝*Oryza sativa* L.	*Gramineae*
Purified Lanolin 精製ラノリン	*Ovis aries* Linné ＝*Ovis aries* L.	*Bovidae*
Lard 豚脂	*Sus scrofa* Linné var. *domesticus* Gray ＝*Sus scrofa* L. var. *domesticus* Gray	*Suidae*
Leonurus Herb ヤクモソウ	*Leonurus japonicus* Houttuyn ＝*Leonurus japonicus* Houtt.	*Labiatae*
	Leonurus sibiricus Linné ＝*Leonurus sibiricus* L.	
Lilium Bulb ビャクゴウ	*Lilium lancifolium* Thunberg ＝*Lilium lancifolium* Thunb.	*Liliaceae*
	Lilium brownii F. E. Brown var. *colchesteri* Wilosn ＝*Lilium brownii* F. E. Br. var. *colchesteri* (Van Houtte) E. H. Wilson ex Elwes	
	Lilium brownii F. E. Brown var. *viridulum* Baker	
	Lilium brownii F. E. Brown ＝*Lilium brownii* F. E. Br.	
	Lilium pumilum De Candolle ＝*Lilium pumilum* DC.	
Lindera Root ウヤク	*Lindera strychnifolia* Fernandez-Villar ＝*Lindera strychnifolia* (Siebold & Zucc.) Fern.-Vill.	*Lauraceae*
	Lindera aggregata (Sims) Kosterm.	
Lithospermum Root シコン	*Lithospermum erythrorhizon* Siebold et Zuccarini ＝*Lithospermum erythrorhizon* Siebold & Zucc.	*Boraginaceae*
Longan Aril リュウガンニク	*Euphoria longana* Lamarck ＝*Euphoria longana* Lam.	*Sapindaceae*
	Dimocarpus longan Lour.	
Lonicera Leaf and Stem ニンドウ	*Lonicera japonica* Thunberg ＝*Lonicera japonica* Thunb.	*Caprifoliaceae*

Loquat Leaf ビワヨウ	*Eriobotrya japonica* Lindley ＝*Eriobotrya japonica* (Thunb.) Lindl.	*Rosaceae*
Lycium Bark ジコッピ	*Lycium chinense* Miller ＝*Lycium chinense* Mill.	*Solanaceae*
	Lycium barbarum Linné ＝*Lycium barbarum* L.	
Lycium Fruit クコシ	*Lycium chinense* Miller ＝*Lycium chinense* Mill.	*Solanaceae*
	Lycium barbarum Linné ＝*Lycium barbarum* L.	
Magnolia Bark コウボク	*Magnolia obovata* Thunberg ＝*Magnolia obovata* Thunb.	*Magnoliaceae*
	* *Magnolia hypoleuca* Siebold et Zuccarini ＝*Magnolia hypoleuca* Siebold & Zucc.	
	Magnolia officinalis Rehder et Wilson ＝*Magnolia officinalis* Rehder & E. H. Wilson	
	Magnolia officinalis Rehder et Wilson var. *biloba* Rehder et Wilson ＝*Magnolia officinalis* Rehder & E. H. Wilson var. *biloba* Rehder & E. H. Wilson	
Magnolia Flower シンイ	*Magnolia biondii* Pampanini ＝*Magnolia biondii* Pamp.	*Magnoliaceae*
	Magnolia heptapeta Dandy ＝*Magnolia heptapeta* (Buchoz) Dandy	
	* *Magnolia denudata* Desrousseaux ＝*Magnolia denudata* Desr.	
	Magnolia sprengeri Pampanini ＝*Magnolia sprengeri* Pamp.	
	Magnolia salicifolia Maximowicz ＝*Magnolia salicifolia* (Siebold & Zucc.) Maxim.	
	Magnolia kobus De Candolle ＝*Magnolia kobus* DC.	
Mallotus Bark アカメガシワ	*Mallotus japonicus* Müller Argoviensis ＝*Mallotus japonicus* (Thunb.) Müll. Arg.	*Euphorbiaceae*
Malt バクガ	*Hordeum vulgare* Linné ＝*Hordeum vulgare* L.	*Gramineae*
Mentha Herb ハッカ *Mentha Oil* ハッカ油	*Mentha arvensis* Linné var. *piperascens* Malinvaud ＝*Mentha arvensis* L. var. *piperascens* Malinv.	*Labiatae*
	Mentha haplocalyx Briq.	
	Hybrid originated from *Mentha arvensis* L. var. *piperascens* Malinv. as the mother species	
Moutan Bark ボタンピ	*Paeonia suffruticosa* Andrews	*Paeoniaceae*
	* *Paeonia moutan* Sims	
Mulberry Bark ソウハクヒ	*Morus alba* Linné ＝*Morus alba* L.	*Moraceae*
Nelumbo Seed レンニク	*Nelumbo nucifera* Gaertner ＝*Nelumbo nucifera* Gaertn.	*Nymphaeaceae*
Notopterygium Rhizome キョウカツ	*Notopterygium incisum* Ting ex H. T. Chang	*Umbelliferae*
	Notopterygium forbesii Boissieu	

Nuphar Rhizome センコツ	*Nuphar japonica* De Candolle ＝*Nuphar japonica* DC.	*Nymphaeaceae*
	Nuphar pumila De Candolle ＝*Nuphar pumila* (Timm) DC.	
Nutmeg ニクズク	*Myristica fragrans* Houttuyn ＝*Myristica fragrans* Houtt.	*Myristicaceae*
Nux Vomica ホミカ	*Strychnos nux-vomica* Linné ＝*Strychnos nux-vomica* L.	*Loganiaceae*
Olive Oil オリブ油	*Olea europaea* Linné ＝*Olea europaea* L.	*Oleaceae*
Ophiopogon Root バクモンドウ	*Ophiopogon japonicus* Ker-Gawler ＝*Ophiopogon japonicus* (L. f.) Ker Gawl.	*Liliaceae*
Orange Oil オレンジ油	*Citrus species*	*Rutaceae*
Oriental Bezoar ゴオウ	*Bos taurus* Linné var. *domesticus* Gmelin ＝*Bos taurus* L. var. *domesticus* Gmelin	*Bovidae*
Oyster Shell ボレイ	*Ostrea gigas* Thunberg ＝*Ostrea gigas* Thunb.	*Ostreidae*
Panax Japonicus Rhizome チクセツニンジン	*Panax japonicus* C. A. Meyer ＝*Panax japonicus* C. A. Mey.	*Araliaceae*
Peach Kernel トウニン	*Prunus persica* Batsch ＝*Prunus persica* (L.) Batsch	*Rosaceae*
	Prunus persica Batsch var. *davidiana* Maximowicz ＝*Prunus persica* (L.) Batsch var. *davidiana* (Carrière) Maxim.	
	Prunus davidiana (Carrière) Franch.	
Peanut Oil ラッカセイ油	*Arachis hypogaea* Linné ＝*Arachis hypogaea* L.	*Leguminosae*
Peony Root シャクヤク	*Paeonia lactiflora* Pallas ＝*Paeonia lactiflora* Pall.	*Paeoniaceae*
Perilla Herb ソヨウ	*Perilla frutescens* Britton var. *crispa* W. Deane ＝*Perilla frutescens* (L.) Britton var. *crispa* (Thunb.) W. Deane	*Labiatae*
Peucedanum Root ゼンコ	*Peucedanum praeruptorum* Dunn	*Umbelliferae*
	Angelica decursiva Franchet et Savatier ＝*Angelica decursiva* (Miq.) Franch. & Sav.	
	* *Peucedanum decursivum* Maximowicz ＝*Peucedanum decursivum* (Miq.) Maxim.	
Pharbitis Seed ケンゴシ	*Pharbitis nil* Choisy ＝*Pharbitis nil* (L.) Choisy	*Convolvulaceae*
Phellodendron Bark オウバク	*Phellodendron amurense* Ruprecht ＝*Phellodendron amurense* Rupr.	*Rutaceae*
	Phellodendron amurense Rupr. var. *sachalinense* F. Schmidt *Phellodendron amurense* Rupr. var. *japonicum* (Maxim.) Ohwi *Phellodendron amurense* Rupr. var. *lavallei* (Dode) Sprangue	
	Phellodendron chinense Schneider ＝*Phellodendron chinense* C. K. Schneid.	
Picrasma Wood ニガキ	*Picrasma quassioides* Bennet ＝*Picrasma quassioides* (D. Don) Benn.	*Simaroubaceae*
Pinellia Tuber ハンゲ	*Pinellia ternata* Breitenbach ＝*Pinellia ternata* (Thunb.) Breitenb.	*Araceae*

Plantago Herb シャゼンソウ	*Plantago asiatica* Linné ＝*Plantago asiatica* L.	*Plantaginaceae*
Plantago Seed シャゼンシ	*Plantago asiatica* Linné ＝*Plantago asiatica* L.	*Plantaginaceae*
Platycodon Root キキョウ	*Platycodon grandiflorus* A. De Candolle ＝ *Platycodon grandiflorus* (Jacq.) A. DC.	*Campanulaceae*
Pogostemon Herb カッコウ	*Pogostemon cablin* Bentham ＝*Pogostemon cablin* (Blanco) Benth.	*Labiatae*
Polygala Root オンジ	*Polygala tenuifolia* Willdenow ＝*Polygala tenuifolia* Willd.	*Polygalaceae*
Polygonatum Rhizome オウセイ	*Polygonatum kingianum* Collett et Hemsley ＝*Polygonatum kingianum* Collett & Hemsl.	*Liliaceae*
	Polygonatum sibiricum Redouté	
	Polygonatum cyrtonema Hua	
	Polygonatum falcatum A. Gray	
Polygonum Root カシュウ	*Polygonum multiflorum* Thunberg ＝*Polygonum multiflorum* Thunb.	*Polygonaceae*
Polyporus Sclerotium チョレイ	*Polyporus umbellatus* Fries ＝*Polyporus umbellatus* (Pers.) Fries	*Polyporaceae*
Poria Sclerotium ブクリョウ	*Wolfiporia cocos* Ryvarden et Gilbertson ＝*Wolfiporia cocos* (Schw.) Ryv. & Gilbn.	*Polyporaceae*
	* *Poria cocos* Wolf ＝*Poria cocos* (Schw.) Wolf	
Powdered Opium アヘン末	*Papaver somniferum* Linné ＝*Papaver somniferum* L.	*Papaveraceae*
Prepared Glycyrrhiza シャカンゾウ	*Glycyrrhiza uralensis* Fischer ＝*Glycyrrhiza uralensis* Fisch.	*Leguminosae*
	Glycyrrhiza glabra Linné ＝*Glycyrrhiza glabra* L.	
Processed Aconite Root ブシ	*Aconitum carmichaeli* Debeaux	*Ranunculaceae*
	Aconitum japonicum Thunberg ＝*Aconitum japonicum* Thunb.	
Processed Ginger カンキョウ	*Zingiber officinale* Roscoe	*Zingiberaceae*
Prunella Spike カゴソウ	*Prunella vulgaris* Linné var. *lilacina* Nakai ＝*Prunella vulgaris* L. var. *lilacina* Nakai	*Labiatae*
Pueraria Root カッコン	*Pueraria lobata* Ohwi ＝*Pueraria lobata* (Willd.) Ohwi	*Leguminosae*
Quercus Bark ボクソク	*Quercus acutissima* Carruthers ＝*Quercus acutissima* Carruth.	*Fagaceae*
	Quercus serrata Murray	
	Quercus mongholica Fischer ex Ledebour var. *crispula* Ohashi ＝*Quercus mongholica* Fisch. ex Ledeb. var. *crispula* (Blume) Ohashi	
	Quercus variabilis Blume	
Rape Seed Oil ナタネ油	*Brassica napus* Linné ＝*Brassica napus* L.	*Cruciferae*
	Brassica rapa Linné var. *oleifera* De Candolle ＝*Brassica rapa* L. var. *oleifera* DC.	

Red Ginseng コウジン	*Panax ginseng* C. A. Meyer ＝*Panax ginseng* C. A. Mey.	*Araliaceae*
	* *Panax schinseng* Nees	
Rehmannia Root ジオウ	*Rehmannia glutinosa* Liboschitz var. *purpurea* Makino ＝*Rehmannia glutinosa* Libosch. var. *purpurea* Makino	*Scrophulariaceae*
	Rehmannia glutinosa Liboschitz ＝*Rehmannia glutinosa* Libosch.	
Rhubarb ダイオウ	*Rheum palmatum* Linné ＝*Rheum palmatum* L.	*Polygonaceae*
	Rheum tanguticum Maximowicz ＝*Rheum tanguticum* Maxim.	
	Rheum officinale Baillon ＝*Rheum officinale* Baill.	
	Rheum coreanum Nakai	
	Interspecific hybrid between above species	
Rose Fruit エイジツ	*Rosa multiflora* Thunberg ＝*Rosa multiflora* Thunb.	*Rosaceae*
Rosin ロジン	Several plants of *Pinus genus*	*Pinaceae*
Royal Jelly ローヤルゼリー	*Apis mellifera* Linné ＝*Apis mellifera* L.	*Apidae*
	Apis cerana Fabricius	
Safflower コウカ	*Carthamus tinctorius* Linné ＝*Carthamus tinctorius* L.	*Compositae*
Saffron サフラン	*Crocus sativus* Linné ＝*Crocus sativus* L.	*Iridaceae*
Salvia Miltiorrhiza Root タンジン	*Salvia miltiorrhiza* Bunge	*Labiatae*
Saposhnikovia Root ボウフウ	*Saposhnikovia divaricata* Schischkin ＝*Saposhnikovia divaricata* (Turcz.) Schischk.	*Umbelliferae*
Sappan Wood ソボク	*Caesalpinia sappan* Linné ＝*Caesalpinia sappan* L.	*Leguminosae*
Saussurea Root モッコウ	*Saussurea lappa* Clarke ＝*Saussurea lappa* (Decne.) C. B. Clarke	*Compositae*
	Aucklandia lappa Decne.	
Schisandra Fruit ゴミシ	*Schisandra chinensis* Baillon ＝*Schisandra chinensis* (Turcz.) Baill.	*Schisandraceae*
Schizonepeta Spike ケイガイ	*Schizonepeta tenuifolia* Briquet ＝*Schizonepeta tenuifolia* Briq.	*Labiatae*
Scopolia Rhizome ロートコン	*Scopolia japonica* Maximowicz ＝*Scopolia japonica* Maxim.	*Solanaceae*
	Scopolia carniolica Jacquin ＝*Scopolia carniolica* Jacq.	
	Scopolia parviflora Nakai ＝*Scopolia parviflora* (Dunn) Nakai	
Scutellaria Root オウゴン	*Scutellaria baicalensis* Georgi	*Labiatae*

Senega セネガ	*Polygala senega* Linné ＝*Polygala senega* L.	*Polygalaceae*
	Polygala senega Linné var. *latifolia* Torrey et Gray ＝*Polygala senega* L. var. *latifolia* Torr. & A. Gray	
Senna Leaf センナ	*Cassia angustifolia* Vahl	*Leguminosae*
	Cassia acutifolia Delile	
Sesame ゴマ Sesame Oil ゴマ油	*Sesamum indicum* Linné ＝*Sesamum indicum* L.	*Pedaliaceae*
Sinomenium Stem ボウイ	*Sinomenium acutum* Rehder et Wilson ＝*Sinomenium acutum* (Thunb.) Rehder & E. H. Wilson	*Menispermaceae*
Smilax Rhizome サンキライ	*Smilax glabra* Roxburgh ＝*Smilax glabra* Roxb.	*Liliaceae*
Sophora Root クジン	*Sophora flavescens* Aiton	*Leguminosae*
Soybean Oil ダイズ油	*Glycine max* Merrill ＝*Glycine max* (L.) Merr.	*Leguminosae*
Sweet Hydrangea Leaf アマチャ	*Hydrangea macrophylla* Seringe var. *thunbergii* Makino ＝*Hydrangea macrophylla* (Thunb.) Ser. var. *thunbergii* (Siebold) Makino	*Saxifragaceae*
Swertia Herb センブリ	*Swertia japonica* Makino ＝*Swertia japonica* (Shult.) Makino	*Gentianaceae*
Toad Cake センソ	*Bufo gargarizans* Cantor	*Bufonidae*
	＝*Bufo bufo gargarizans* Cantor	
	Bufo melanostictus Schneider ＝*Duttaphrynus melanostictus* Schneider	
Tragacanth トラガント	*Astragalus gummifer* Labillardiére ＝*Astragalus gummifer* Labill.	*Leguminosae*
Tribulus Fruit シツリシ	*Tribulus terrestris* Linné ＝*Tribulus terrestris* L.	*Zygophyllaceae*
Trichosanthes Root カロコン	*Trichosanthes kirilowii* Maximowicz ＝*Trichosanthes kirilowii* Maxim.	*Cucurbitaceae*
	Trichosanthes kirilowii Maximowicz var. *japonica* Kitamura ＝*Trichosanthes kirilowii* Maxim. var. *japonica* (Miq.) Kitam.	
	Trichosanthes bracteata Voigt ＝*Trichosanthes bracteata* (Lam.) Voigt	
Turmeric ウコン	*Curcuma longa* Linné ＝*Curcuma longa* L.	*Zingiberaceae*
Turpentine Oil テレビン油	Several plants of *Pinus genus*	*Pinaceae*
Uncaria Hook チョウトウコウ	*Uncaria rhynchophylla* Miquel ＝*Uncaria rhynchophylla* (Miq.) Miq.	*Rubiaceae*
	Uncaria sinensis Haviland ＝*Uncaria sinensis* (Oliv.) Havil.	
	Uncaria macrophylla Wallich ＝*Uncaria macrophylla* Wall.	

Wood Creosote 木クレオソート	Several plants of *Pinus* genus	*Pinaceae*
	Several plants of *Cryptomeria* genus	*Taxodiaceae*
	Several plants of *Fagus* genus	*Fagaceae*
	Several plants of *Afzelia (Intsia)* genus	*Leguminosae*
	Several plants of *Shorea* genus	*Dipterocarpaceae*
	Several plants of *Tectona* genus	*Verbenaceae*

When ''Other species of the same genus'' is included as its original plants the scientific name is not written in Monograph, however, it is written in this table.

Reference

Terabayashi S. *et al.*: Pharmaceutical and Medical Device Regulatory Science, 41(5), 407 – 418 (2010).

essary the same as the taxonomic system used in the literature. The reason for this is that the JP is not an academic text, but an ordinance. The relationship between the scientific names used in the JP and those generally used taxonomically is indicated in the following table, to avoid misunderstanding by JP users owing to differences in the notation system.

Notification for the Quantitative Marker Constituents of Crude Drugs and Crude Drug Preparations

〈G5-2-170〉

One of the highly distinctive features of the crude drugs and preparations containing crude drugs as main ingredient (crude drug preparations) is the fact that they are multicomponent systems composed of a prodigious number of compounds. As an example, glycyrrhiza, one of the most important crude drugs worldwide has been demonstrated that they harbor more than 100 kinds of secondary metabolites, and this number could be counted up to more than 1000 when the intermediates occurred in the biosynthetic pathways were included. The whole members of the compounds are supposed to be integrated to reveal the effects of glycyrrhiza.

A wide diversity of constituents in the drugs made from botanical raw materials is fundamentally derived from a wide variety of secondary metabolites occurred in plants. However, there are more reasons for the diversity. The species of plants used as raw materials for drugs are specified in the approval documents of the drugs, though, a variation of their composition derived from the genetic variation of the plants will be produced since each botanical species includes a significant genetic divergence. The secondary metabolites of plants will be further differentiated in their quality and quantity according to the environmental factors such as soil (soil texture, water retentivity, pH, etc.) and weather (amount of precipitation, temperature, humidity, etc.) conditions. Furthermore, the constituents will change depending on wild or cultivation, cultivation method and harvest period. Also crude drugs are finally processed by excluding periderm, steaming, roasting, etc. and consequently the constituents are different according to the process method.

The stipulation on the contents (quantitative values) of marker constituents in crude drugs and crude drug preparations has very important implications for standardization of the quality of pharmaceutical natural products. The stipulations on crude drugs generally provide the minimum requisite amount of marker compounds. For example, glycyrrhiza of the Japanese Pharmacopoeia is ordered to contain not less than 2.0% of glycyrrhizic acid. Crude drugs are known to exhibit large difference in the content of secondary metabolites among individual plants that caused from multiple reasons described in the previous paragraph. It is reported that the glycyrrhizic acid content of glycyrrhiza in one plant can contain some 10 times the content of another plant when they are grown together in one farm field and harvested simultaneously. Accordingly, it is hard to standardize the ranges of content for marker constituents of crude drugs on a policy of the efficient use of natural resources for crude drugs. However, the crude drug preparations as finished products are required to contain a certain amount of active ingredients from a perspective of the reproducibility of medical treatments. Consequently, the marker constituents of the Kampo formulations and conventional crude drug preparations as the final stage of crude drug preparations are generally specified by the range of content values. Crude drugs containing different amount of marker constituents are appropriately blended to prepare the finished products with a particular amount of marker constituents.

It should be noted that the marker constituents of crude drugs for regulations include a variety of types. Followings are the examples of some different types. Sennosides, the marker constituents of senna leaf are the obvious active ingredients that accounts for the laxative effects of senna leaf to some extent. Other compounds of the anthraquinone-type found in senna leaf, such as rhein and aloe-emodin, are also potent as laxative, though, because of their large difference in content in senna leaf, a medicinal effect of senna leaf can be standardized by managing the content of sennosides. However, sennosides are likely to be broken down into anthraquinones by heating and other factors that not only sennosides but also the increased rhein may be targeted for standardization in cases of the Kampo formula extracts including rhubarb, one of the sennoside-containing crude drugs, as a component. On the other hand, glycyrrhizic acid, a very famous bioactive component of glycyrrhiza, is an active ingredient that contributes to some parts of the medical effect of glycyrrhiza. Though, many components other than glycyrrhizic acid are also known to play a part in the medical effects of glycyrrhiza. Consequently, glycyrrhizic acid should be regarded as one of the multiple active ingredients of glycyrrhiza that being specified its content as a marker constituent. In other cases of the marker constituents that seemed to have less biological effects such as 10-hydroxy-2-(*E*)-decenoic acid in royal jelly or (*E*)-cinnamic acid in some extracts of Kampo formula, their content are standardized as a distinctive compound of each pharmaceutical product. Referring to the biosyntheses of natural products, the con-

tent of these compounds specified as marker constituents are unlikely to be independently changed to stand out from others. The specification values of crude drugs and crude drug preparations are stipulated in order to control the appropriate production process based on the strategy that every crude drug and crude drug preparation will be standardized to a certain level through the content control of marker constituents.

An instance of the relationship between the active ingredient and the marker compound of senna leaf may be a special case among crude drugs and pharmaceutical natural products made from crude drugs. The medicinal effects of the majority of crude drugs and pharmaceutical natural products made from crude drugs are achieved by the cooperative performance of their entire constituents. These drugs are standardized with the quantitative specification of particular constituents designated as the quantitative marker constituents, and this is owing to the fact that the standardization of every constituent consisting the multicomponent system is impossible. Taken all together, it requires particular consideration to the fact that the quantitative marker compounds specified for crude drugs and crude drug preparations are not directly same as the active ingredients in the chemical drugs that absolutely accounts for the medical effects of the drugs.

Thin-layer Chromatography for Crude Drugs and Crude Drug Preparations <*G5-3-170*>

Thin-layer chromatography is a method to separate each component by developing with a mobile phase, using a thin-layer consisting of an appropriate stationary phase, and is used for identification, purity test, etc. of substances.

Thin-layer chromatography for crude drugs and preparations containing crude drugs as main ingredient (crude drug preparations) is used for identifying whether characteristic constituents or groups of constituents in crude drugs and extracts based on Kampo formulae are included or not.

1. Instruments and equipment:

Generally, the following instruments and equipment are used.

(i) Thin-layer plate: A smooth and uniformly thick glass plate is coated in advance with an uniform powder of carrier listed in Solid Supports/Column Packings for Chromatography <*9.42*>. It is classified into two types. The stationary phase of thin-layer chromatography plates (TLC plates) has a particle size of 10 – 15 μm, and that of high-performance thin-layer chromatography plates (HPTLC plates) has a particle size of 5 – 7 μm. In a case where separation requirements given that the quality of the chromatogram indicated in the individual monograph is ensured, it is possible to use a thin-layer plate with a preadsorbent zone which has been coated in advance and the home-made plate. Alternatively, such thin-layer plates are also available that use plate-like or sheeted hard aluminum and polyester for support medium instead of glass plate. Thin-layer plates are kept while avoiding humidity.

(ii) Application of samples: The sample solution(s) or standard solution(s) at the prescribed volume in the individual monograph are applied with sample applicators of constant volume, which is generally a special capillary or microsyringe, at a position around 20 mm distance from the lower edge as a starting line of and release at least 10 mm from side to side edges of the thin-layer plate, in the form of circular spots (spot-like) of 2 – 6 mm in diameter or narrow linearly bands (band-shaped) of 4 – 10 mm in width with an appropriate interval of at least about 10 mm and then allowed to dry in air. In a case where separation requirements given that the quality of the chromatogram indicated in the individual monograph is ensured, it is possible to modify the position of a starting line and sample spots application interval.

(iii) Chromatographic chamber: Generally, a chromatographic chamber made of inert, transparent material and having a lid is used: a flat-bottom or twin trough. Unless otherwise specified, attach a filter paper along with the inside wall of the chamber, and moisten the filter paper with the developing solvent. In the chamber, the developing solvent is placed up to about 10 mm in height from the bottom, seal the chamber closely, and allow it to stand for 1 hour at ordinary temperature. Place a thin-layer plate in the chamber so as only the upper end of the plate is touched to the wall of the chamber, seal the chamber closely, and perform the development at ordinary temperature. A chromatographic chamber shall be of a size appropriate for the thin-layer plate and the developing solvent to be poured into shall be of a volume not to immerse spot(s) or band(s) of samples applied to the thin-layer plate in advance.

(iv) Device for coloring: A glass mister sprayer or an electric mister sprayer is used for spraying a visualization reagent. Drying the thin-layer plate after development, visualization of components to be tested on the chromatogram is performed by an evenly sprayed visualization reagent directly on the thin-layer plate to work the test reagent. The ways to discharge the visualization reagent include air supply of compressed gas either by manually or electrically. Further, in case of a heating device, components to be tested which have been separated on the chromatogram are heated after spraying a visualization reagent and derivatized for visualization. It is preferable to use a hot plate at a constant temperature to heat a thin-layer plate after spraying visualization reagent. In case of using a thermostatic oven, thin-layer plate is heated on the metal plate heated to a constant temperature in advance. A flat-bottom trough chamber, twin trough chamber and desiccator, could be used during the immersion visualization and the fumigation (exposure to reagent vapor) visualization.

(v) Detection device: It is a camera obscura equipped with visible light, ultraviolet light of wavelength 254 nm and 365 nm, and wide-range wavelength ultraviolet light, and corresponding filter, a dark box or room. The light source is required to meet the requirements for the tests prescribed in the individual monograph. Photographing device to be added to the detecting device is used for taking photographs to be recorded and requires adequate sensitivity, resolution and reproducibility enough to perform the tests.

(vi) Record of TLC images: TLC images are taken by a camera and recorded/stored in a format of film image or electronic image. Except for detection after exposure to ultraviolet radiation, it is preferable to take pictures of color samples for reference concurrently in case of recording color tones of chromatogram detected under a visible light. Further, it should be noted that color tones identified visually and those recorded are different in some cases when recording fluorescent spots caused by irradiation with a wavelength of 365 nm. It is also possible to use an image scanner with sufficient resolution to record chromatograms detected under visible light. A TLC scanning device is capable of detecting absorption of light or fluorescence of chromatograms and spots or bands of samples to be tested and recording ab-

sorption and fluorescence spectra corresponding to components to be tested.

(vii) TLC scanning device: The device measures absorption by ultraviolet or visible light or fluorescence by excitation light on a developed thin-layer plate and stores records of development patterns by converting them into chromatograms (peak information). Scanning data obtained from chromatograms is used for quantitative analyses.

2. Detection and visualization

Generally, pulling out a thin-layer plate and drying it after development, detection of spots separated on a chromatogram is visually confirmed under visible light directly or after visualized. It is detected as a spot in a form close to circle when applied in a circular form (spot-like) and as a linear band when applied in a narrow linear form (band-shaped). In case of components to be tested having ultraviolet absorptivity, detection is performed using a thin-layer plate containing fluorescent agent (fluorescent indicator) by ultraviolet irradiation with a dominant wavelength of 254 nm. While fluorescent indicator contained in the thin-layer plate emits greenish fluorescence excited by irradiation with a dominant wavelength of 254 nm, spots or bands of components to be tested reduce radiation light emission by absorbing irradiation light to reduce excitation of fluorescent indicator resulting in an observation as raspberry (dark purple) spots or bands. With a property to produce fluorescence on itself excited under ultraviolet irradiation, spots or bands of components to be tested produce fluorescence excited on the thin-layer plate by irradiation of ultraviolet with a dominant wavelength of 365 nm even without using fluorescent indicator. High illumination light source with stable radiation intensity at around 365 nm within the ultraviolet wavelength range includes lamps having a line spectrum with a narrow width at 365 nm and having a line spectrum at 366 nm (within a range from 364 to 367 nm) with more intense radiated signal. Even though light source and wavelength described in specification differs depending on the lamps to be used, light source lamp with a wavelength of 366 nm also includes a light source lamp with a wavelength of 365 nm making it possible to handle as a description of irradiation with a dominant ultraviolet wavelength of 365 nm.

Derivatization reaction based on spraying, immersion and fumigation of an appropriate coloring reagent visualizes spots or bands of components to be tested. In case of some visualization reagents, such derivatization reaction is further visualized by subsequent heating after spraying reagents. In some cases, characteristic fluorescence may be produced by irradiation with a dominant wavelength of 365 nm after spraying or after spraying and heating as well.

3. Operation methods

Generally, unless otherwise specified, operation method shall comply with the following methods. Prepared sample solution and standard solution which is prescribed in the individual monograph shall be applied on the starting line of a thin-layer plate by an indicated volume. Confirming that applied circular form or linear spots or bands are not immersed in the developing solvent and placing a thin-layer plate in a developing container, developments are initiated after closing the lid of the chromatographic chamber. Sending up the developing solvent to a required development distance, the thin-layer plate is taken out to be allowed to dry in air. In addition, starting line (starting point) and mobile phase front are marked before and after development. Then, based on visualization of chromatogram on the thin-layer plate, color tone or *R*f value of circular spots or linear bands of components to be tested is determined (Fig. 1). *R*f value is obtained

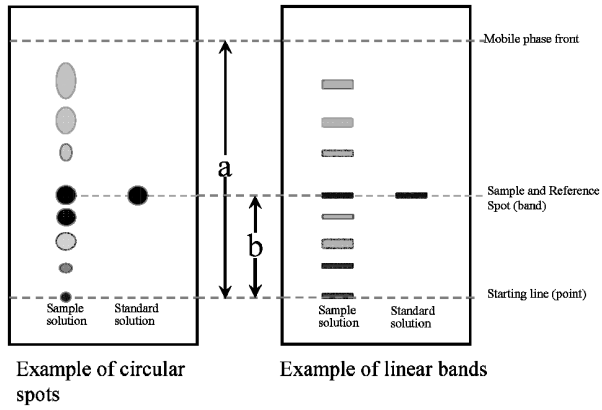


Fig. 1 Pattern diagram of TLC chromatogram

by the following formula.

$$Rf = \frac{\text{Distance from starting line to center of spot or band}}{\text{Distance from starting line to mobile phase front}} = \frac{b}{a}$$

Development operation and visualization shall be performed in an apparatus such as a draft chamber in which solvent vapor is efficiently removed with sufficient air ventilation.

4. Confirmation and purity test

When using this test method for an identification test, it is confirmed in general that color tone and *R*f value of components to be tested in sample solution is equal to those in standard solution. In case of the identification test of multicomponent system sample solution, it is possible to confirm by color tone and *R*f value of spots when components to be tested are recognized as a single spot clearly showing characteristic fluorescence and coloring. Alternatively, it is also possible to identify by the patterns of spots and bands. Moreover, this test method in combination with spectroscopic method (such as Ultraviolet-visible Spectrophotometry <*2.24*>, Nuclear Magnetic Resonance Spectrophotometry <*2.21*>) and Mass Spectrometry <*2.62*> makes it possible to perform further reliable confirmation.

In case of using this test method for purity test, a standard solution with a concentration corresponding to the limit of impurities in the sample solution is used in general, and purity is confirmed by whether any spot of components to be tested derived from sample solution is detected or whether the magnitude of the spot is lighter than that of standard solution.

5. Semi-quantitative and quantitative measurement

Presumptive quantitative measurement can be made by observation of spots or bands of identical *R*f value and about equal magnitude obtained, respectively, with sample solution and standard solution or indicative component solution by applying the same volume, chromatographed on the same thin-layer plate. A visual comparison of the size or intensity of the spots or bands may serve for semi-quantitative estimation. Quantitative measurements are possible by means of densitometry.

6. Confirmation of suitability of lamp

Objectives of confirmation of suitability are sensitivity, resolution and reproducibility required for securing quality of chromatogram and satisfying separation requirements specified in the individual monograph. Confirmation of suitability in this test method is performed mainly for radia-

tion intensity of line light source used for ultraviolet irradiation. In other words, the test is performed if specified spot (or band) is not recognized by irradiation with a wavelength of line light source specified in the individual monograph or specification of irradiation system was changed. Generally, in case of irradiation with a dominant wavelength of 254 nm to a thin-layer plate containing fluorescent agent, it is confirmed whether the thin-layer plate produce a green fluorescence. Alternatively, in case of irradiation with a dominant wavelength of 365 nm (366 nm), whether blue-white fluorescence is produced is confirmed by 2 μL spotting the 0.5 μg/mL scopoletin for thin-layer chromatography solution on a thin-layer plate.

In case of automated sample application equipment and TLC scanning device using densitometry, specifications of system suitability in Liquid Chromatography <*2.01*> is applied as required.

7. Point to consider regarding test conditions

Among tests prescribed in individual monograph, in identification tests using reagents of the Reference Standard or components to be tested (such as reagents for thin-layer chromatography), it is possible to modify developing temperature, developing distance, composition of developing solvent, developing rate, coloring reagent composition, heating temperature and duration of thin-layer plate within a range of better accuracy and precision than those prescribed is secured. However, such semi-quantitative identification tests are excluded that judgment criteria is based on size and strength of spots. On the other hand, in identification tests which do not use reagents of the Reference Standard or components to be tested, it is possible to modify developing distance, heating temperature and duration of thin-layer plate within a range where separation, *R*f value and color tone prescribed in individual monograph is observed. Further, even in case of identification tests without specification for the Reference Standard or components to be tested, it is possible to make a confirmation based on conformity of color tone for reference and *R*f value using the Reference Standard or components to be tested.

8. Reference

1) MHLW Notification No. 65 of March 24, 2011, The Japanese Pharmacopoeia Sixteenth Edition/General Test Procedures/2. Physical Test Procedures/2.01 Liquid Chromatography/2.03 Thin-layer Chromatography
2) EP 8.0 (2014), 2.2.27/Thin-Layer Chromatography
3) USP 37 (2014), <621> Chromatography, <201> Thin-Layer Chromatographic Identification Test
4) Pharmacopoeia of the People's Republic of China (2010), A-42 Appendix VI B Thin-Layer Chromatography (English version)
5) Y. Goda: The Japanese Journal of Pharmacognosy, 66, 63 (2012).

Aristolochic Acid <*G5-4-141*>

Aristolochic acid, which occurs in plants of *Aristolochiaceae*, is suspected to cause renal damage. It is also reported to be oncogenic (see References).

Aristolochic acid toxicity will not be a problem if crude drugs of the origin and parts designated in the JP are used, but there may be differences in crude drug nomenclature between different countries, and it is known that crude drug preparations not meeting the specifications of the JP are circulating in some countries. Consequently, when crude drugs or their preparations are used, it is important that the materials should not include any plant containing aristolochic acid.

Since Supplement I to JP14, the test for aristolochic acid I was added to the Purity under Asiasarum Root, which consists of the rhizome and root. Because the aerial part of the plant may contain aristolochic acid and may have been improperly contaminated in Asiasarum Root.

It is considered that Akebia Stem, Sinomenium Stem and Saussurea Root do not contain aristolochic acid, unless plants of origin other than that designated in the JP are used. However, contamination of aristolochic acid might occur, as mentioned above. In this case, the test described in the Purity under Asiasarum Root is useful for checking the presence of aristolochic acid.

1. References

1) Drug & Medical Device Safety Information, No. 161, July, 2000, https://www.pmda.go.jp/safety/info-services/drugs/calling-attention/safety-info/0092.html#10 (Reference: 2019-7-18).
2) J. L. Nortier, *et al.*, *N. Engl. J. Med.*, 342, 1686 – 1692 (2000).
3) A. Kohara, *et al.*, *Mutation Research*, 515, 63 – 72 (2002).

Quantitative Analytical Technique Utilizing Nuclear Magnetic Resonance (NMR) Spectroscopy and its Application to Reagents in the Japanese Pharmacopoeia <*G5-5-170*>

1. Marker Compounds for the Assay of Crude Drugs in the JP and Establishment of Reference Substances for Quantitative Analyses

When the quantitative assay values are specified in the monographs of crude drugs and extracts of Kampo formulations in the JP, it is more difficult to establish and prepare their JP Reference Standards than those for synthetic chemical pharmaceutical substances, because the marker compounds for their assay are derived from natural sources.

Unlike the synthetic chemical pharmaceutical substances, crude drugs and extracts of Kampo formulations are mixtures of a great deal of compounds. Although it is necessary to choose a substance contained at the level of 0.1% to several % in the crude drugs and the extracts of Kampo formulations as the marker compounds for their quantitative assay, the synthesis of such compounds is not so easy in most cases. Therefore, the marker compound would be separated from natural materials and be isolated to have sufficient purity. However, the preparation of the reference substance in such a way would require high economical cost and a great deal of effort. In addition, the composition of impurities contained in the reference substance prepared in such a way would be different batch by batch according to the difference of raw materials and their processes of extraction, isolation and purification. Accordingly, the difference among batches of reference materials is much larger than that of synthetic substances, and the control of their purity as the official reference standards is very difficult. Furthermore, in many cases of substances of natural origin, the greatest impurity would be water. For determining water

contents precisely, it is necessary to use Karl Fischer method, and as the result, a large quantity of the valuable reference standard would be consumed.

Because there are such bottlenecks mentioned above in many cases of monographs of crude drugs and extracts of Kampo formulations, the establishment of the JP Reference Standard is difficult. Instead, reagents, which are commercially available or ready to put into the market, are designated as the reference substances for the quantitative assay, and the method and the content specification using the reagent are specified in monographs of crude drugs and extracts of Kampo formulations. In these cases, the specifications of their marker substances are defined in the section of Reagents and Test Solutions of the JP. However, in a strict sense, since the assay values obtained in this manner are not certified metrologically, the reliability of the analytical value obtained by using them is somewhat ambiguous.

2. Application of Quantitative NMR to Reference Substances Used in the Assay of Crude Drugs and Extracts of Kampo Formulations

The application of quantitative NMR can solve the issue on the purity of reagents derived from natural source. These reagents are used as the reference substances with metrological traceability, when the precise contents of these reagents are determined metrologically by using quantitative NMR based on the idea shown in 10.1 Principle of Quantitative Analytical Technique Utilizing Nuclear Magnetic Resonance (NMR) Spectroscopy under <*5.01*> Crude Drugs Test.

Currently, the quantitative NMR is being carried out for these reagents defined for the quantitative assay of crude drugs in the JP and a report in which the points to practically consider at determination of absolute purities of the reagents by using quantitative NMR are discussed has been published.[1)] In addition, a validation study of quantitative NMR has also been performed using the substances which will be used with high possibility as the reference substances for HPLC quantitative analysis. For the analyte compound having molecular mass of around 300, when about 10 mg of the compound was used for the quantitative NMR measurement, it was demonstrated that an accuracy of 2 significant digits for the determined value was achieved at the ordinary laboratory level, even when the error among the NMR instruments used was included.[2)] Usually, the contents of marker compounds for the quantitative assay of crude drugs are several % at the maximum, and the minimum unit for the content specification is at the level of 0.1%. Therefore, when variability of content in crude drugs is considered, the assurance of 2 significant digits for accuracy seems sufficient for the reference substances, which are used for the quantitative assay of crude drugs.

When discussion above is considered, the ambiguity of analytical values obtained by the use of the reagents derived from natural source as the reference substances for the quantitative assay of crude drugs can be avoided practically, by using the reagents certified by quantitative NMR as the reference substances in HPLC, etc., and by incorporating the certified purity of such reagents into the calculation of the quantitative value of the sample. For example, for Gardenia Fruit in the JP, the content of geniposide is specified at not less than 3.0% based on the HPLC analysis. The report cited above[1)] demonstrated that the absolute purity of geniposide used as the reference substance in the quantitative assay of Gardenia Fruit is determined to be about 92% by quantitative NMR. Therefore, in the case that the quantitative value of 3.0% in Gardenia Fruit sample is obtained as a result of HPLC analysis by using this reagent as the reference substance assuming its purity as 100%, the true value for the sample is evaluated to be 2.8% taking it into consideration of the absolute purity determined by quantitative NMR with the assurance of metrological traceability.

3. Supply of Certified Reagents by Using Quantitative NMR

Currently, in the accreditation system of the International Accreditation Japan (IA Japan), the National Institute of Technology and Evaluation (ASNITE), a feasibility study how the accreditation should be given to the organization which performs the assay certification of the reagents using calibrated NMR apparatus has been in progress. In addition, in the IA Japan, addition of''Quantitative NMR''to the test method categories is scheduled. Therefore, in the near future, the reagent manufacturers will become able to perform the assay certification of the reagent after having this accreditation. Under such situation, the user of the reagent would not be required to perform qualitative NMR individually to obtain the purity value with SI traceability. Furthermore, the inter-institutional errors (including inter-instrumental errors) would become negligible, and we will be able to carry out more precise and accurate quantitation assay of the sample by incorporating the labeled certified value on the reagent into the calculation of the quantitative value of the sample.

The certified reference materials (NMIJ CRM) to be used for the SI traceable metrological determination of the internal reference compounds are supplied from the National Metrology Institute of Japan, National Institute of Advanced Industrial Science and Technology (NMIJ AIST).

4. Management of Instrument Performance for Quantitative NMR

Quantitative NMR used to determine the purity of reagents for the JP, is an internal standard method that an analyte compound and a SI traceable reference material in a NMR tube are measured at the same time.[3)] In this method, the number of nuclei are measured using NMR phenomenon, which means that the molar quantity of an analyte compound in a sample solution is directly calibrated with a reference material.

In the management of instrument performance for quantitative NMR measurements, it should be confirmed that integral value of the targeted signals can be determined correctly within the spectrum where the signals are measured (in general, 0 – 10 ppm). The important point here is not to include the signals derived from impure substances in the quantitative spectrum when integrate. Therefore, to manage instrument performance, a high-purity compound of already known purity (determined by quantitative NMR and not less than 99.0% is preferable) should be used. In addition, signals derived from simpler spin system should be selected and integrated, and the ratio of theoretical number of nuclei among signals should be accurate (for example, when each of the two signals is derived from 1H, the ratio of the integrated values of the both is 0.995 – 1.005).

Considering the excitation bandwidth of a NMR pulse, when an instrument of 800 MHz is used under the following conditions; center of spectrum window around 5 ppm, spectral width at 20 ppm (the conditions stipulated in the assay using qNMR in the section of reagents), with a 90° pulse width of 10 microseconds, the excitation efficiency of the pulse in the range of 0 – 10 ppm, where signals of an analyte compound are observed, is usually not less than 99.95%. Thus, the instrument, when the probe is well tuned and shim is adjusted properly, can assure an accuracy of 2 significant digits at routine level of measurement. Furthermore, when

an instrument of 400 MHz is used, similar excitation efficiency can be obtained up to 20 microseconds for 90° pulse width, so quantitative NMR can be measured sufficiently with a standard probe.

5. Reference

1) J. Hosoe, *et al.*, *Pharmaceutical and Medical Device Regulatory Science*, 41, 960 – 970 (2010)
2) J. Hosoe, *et al.*, *Pharmaceutical and Medical Device Regulatory Science*, 43, 182 – 193 (2012)
3) J. Hosoe, *et al.*, *Pharmaceutical and Medical Device Regulatory Science*, 45, 243 – 250 (2014)

Purity Tests on Crude Drugs using Genetic Information

⟨*G5-6-172*⟩

The first step in the quality assurance of natural products is the use of raw materials from the right part of the right origin. Therefore, it is clearly stated in Article 4 of the General Rules For Crude Drugs that the source of a crude drug is an approval or rejection criterion. There are various methods for differentiating the sources of crude drugs, such as morphological methods, organoleptic tests, and chemical methods, and appropriate methods for each are described in the individual monographs. Morphological methods, organoleptic tests, and chemical methods are discrimination methods for species that are based on the phenotypic characteristics of the crude drugs. On the other hand, together with recent progress in molecular biology techniques and the accumulation of genetic information on plants, differentiating methods of crude drugs based on genotypes is being established. Unlike morphological and other methods that are based on phenotypic characteristics, the genotypic methods are not affected by environmental factors. Also, the methods have several advantages, such as specialized expertise and skill for classification are not needed, and objective results are easily obtained.

The evolution of living organisms is accomplished by genetic mutation, and differences among the nucleotide sequences of genes of closely related species reflect the strain relationships between the species. Based on this theory, methods that classify species phylogenetically using the nucleotide sequence of rDNA that codes for ribosomal RNA (rRNA) on the nuclear genome have recently been adopted for the classification of microorganisms. In the same way, the sequence of this rDNA is also most often used in the classification of higher plants based on the genotype. In particular, it is very easy to classify closely related species using the internal transcribed spacer (ITS) region of the rDNA, since nucleotide substitution is more often undertaken by comparison with the coded gene region. Furthermore, since the genes on the nuclear genome originate from the parents' genomes, there is an advantage that interspecies hybrids can be detected. Higher plants also have mitochondrial genes and chloroplastic genes. Although the genes on these genomes are also often used for classification, interspecies hybrids cannot be confirmed because the genes are normally uniparental inheritance.

The three methods presented here are, 1) the purity test of Atractylodes Rhizome for Atractylodes Lancea Rhizome, 2) the purity test of Saposhnikovia Root and Rhizome for *Peucedanum ledebourielloides*, which are developed based on the difference of the gene sequence of the ITS region of rDNA recently reported[1-4], and the inter-laboratory validation study have been completed.

The plant sources for Atractylodes Lancea Rhizome stipulated in the individual monographs are *Atractylodes lancea* De Candolle and *A. chinensis* Koidzumi (*Compositae*), while those for Atractylodes Rhizome are *A. japonica* Koidzumi ex Kitamura and *A. macrocephala* Koidzumi (*Compositae*). The approval or rejection of the both sources is, in principle, determined by the description of the crude drug, including microscopy, together with thin-layer chromatography in identification tests. In the above scientific paper, it was shown that these 4 plant species can be clearly classified by comparing the nucleotide sequences of the ITS region mentioned above, and that the species can be easily classified without performing sequence analysis by performing PCR using a species-specific primer pair or by using a restriction enzyme which recognizes species-specific sequence.

Likewise, the plant source of Saposhnikovia Root and Rhizome is stipulated as *Saposhnikovia divaricata* Schischkin (*Umbelliferae*), and the approval or rejection of the source is determined by the description of the crude drug and thin-layer chromatography in identification tests. According to the report[4], crude drugs treated as Saposhnikovia Root and Rhizome in Shaanxi and Shanxi Provinces are frequently derived from *Peucedanum ledebourielloides*, and it was shown that the differentiation of the both is possible by using the nucleotide sequence in the ITS region of the rDNA.

In purity tests on crude drugs using genetic information, the simplicity of the test is given maximum consideration. We established methods that observe PCR amplification bands using species-specific primer pair (Mutant Allele Specific Amplification: Method 1) and methods that observe DNA fragments produced by restriction enzyme treatment of the PCR products, which are prepared using a primer pair common to each plant source (PCR—Restriction Fragment Length Polymorphism: Method 2), without nucleotide sequence analyses. In these methods based on PCR, an extremely small amount of template DNA is amplified to billions to hundreds of billions times. Therefore, when using them as identification tests for powdered crude drugs, the target DNA fragment can be observed even if the vast majority of the crude drug for analysis is not appropriate plant species and there is only a minute amount of powder from a crude drug derived from a suitable plant. Consequently, in identification tests, either a cut or a whole crude drug must be used, as long as one is careful to avoid contamination by powder originating from other crude drugs. On the other hand, when used as a purity test, the form of the crude drug is irrelevant as long as the gene amplification is performed properly and the target gene is not polymorphic, so if DNA fragments of an inappropriate plant to be examined are confirmed in the purity test, regardless of the form of the crude drug, it becomes clear there is contamination by an inappropriate crude drug to be examined.

The methods shown here are general information and at the present stage results obtained using the methods do not affect the approval or rejection of the crude drug in each monograph. Furthermore, by performing the sequence analysis outlined in the previous paper for a crude drug sample derived from a single individual, it goes without saying that more accurate decision concerning the source species can be made.

1. DNA Amplification Equipment

DNA amplification equipment is used to amplify the DNA which is extracted from a crude drug and then purified. Since there are slight differences in the methods of temperature

control, and so on depending on the equipment used, there may be differences in the intensity, etc. of the PCR amplification bands even if PCR is carried out under the stipulated conditions. Therefore, when judging results based solely on the presence or absence of PCR amplification bands as in Methods 1, the use of equipment described in the JAS analytical test handbook: genetically modified food quality, labeling analysis manual for individual products[5] is recommended. When other equipment is used, confirm that only proper amplification bands are obtained by performing PCR using DNA obtained from samples confirmed beforehand to be the source species. If proper amplification bands are not obtained, the PCR temperature conditions should be slightly adjusted. This equipment can be used for the restriction enzyme treatment in Method 2.

2. General precautions

Crude drugs are different from fresh plants in that they are dried products and a certain amount of time has passed since they were harvested. Therefore, in many cases the DNA has undergone fragmentation. Furthermore, various substances that can block or interfere with the PCR reaction may be present in the plant. For these reasons, the extraction and purification of template DNA is the process that should receive the greatest amount of attention. In the case of Atractylodes crude drugs, the periderm should be removed using a clean scalpel or other clean instrument before pulverizing the sample because very often there are inhibitory substances present in the periderm.

The PCR used for this test is the technique that amplify the target DNA more than hundreds of millions times, and a trace of contamination leads an incorrect result. Therefore, careful attention is required to prevent contamination. For treatment to prevent contamination, refer to the prevention of contamination section[6] in the above manual.

3. Purity test of Atractylodes Rhizome for Atractylodes Lancea Rhizome

3.1. Method 1 (Mutant Allele Specific Amplification Method)

Generally, this method is referred to as Mutant Allele Specific Amplification (MASA) or Amplification Refractory Mutation System (ARMS), and it provides nucleotide sequence information of sample-derived template DNA, based upon the presence or absence of DNA amplification in PCR using a species specific primer pair.

3.1.1. Procedure

The following is an example procedure.

3.1.1.1. Preparation of template DNA

There are various methods with which to extract and purify DNA from the samples. It is recommended that commercially available DNA extraction kits be used when considering their advantages of not using any noxious reagents and not requiring any complicated purification procedures. In this case, attention should be paid to the final amount (concentration) of DNA obtained, and the initial amount of initial sample and the volume of liquid to elute the DNA need to be controlled. When extraction and purification are performed using silica gel membrane type kits stipulated in notifications[7] related to inspection methods of the foods produced by recombinant DNA techniques, it is appropriate to use 200 mg of sample, 1 mL of AP1 buffer solution, 2 μL of RNase A, and 325 μL of AP2 buffer solution. Also, the most important things are that the supernatant loaded on the first column is clear and that there is no need to load 1 mL unreasonably. Furthermore, 50 μL is an appropriate volume used in the final elution of the DNA, and normally the initial eluate is used as the DNA sample stock solution.

3.1.1.2. Confirmation of purity of DNA in DNA sample stock solution and assay of DNA

The purity of the DNA in the stock solution can be confirmed by the $OD_{260\,nm}/OD_{280\,nm}$ ratio using a spectrophotometer. A ratio of 1.5 indicates that the DNA has been adequately purified. The amount of DNA is calculated using 1 $OD_{260\,nm}$ = 50 μg/mL. The measurement mentioned above is performed using the appropriately diluted DNA sample stock solution. Based on the results obtained, dilute with water to the concentration needed for the subsequent PCR reactions, dispense the solution into micro tubes as the sample DNA solution, and if necessary store frozen at not over −20°C. The dispensed DNA sample is used immediately after thawing and any remaining solution should be discarded and not refrozen. If the concentration of the DNA sample stock solution does not reach the concentration stipulated in PCR, it is used as a DNA sample solution.

3.1.1.3. PCR

When a commercially available PCR enzyme mentioned in the above notification[8] is used, it is appropriate that 25 μL of a reaction mixture consisting of 2.5 μL of the PCR buffer solution containing magnesium, dNTP (0.2 mmol/L), 5′ and 3′primer (0.4 μmol/L), Taq DNA polymerase (1.25 units), and 5 μL of 10 ng/μL sample DNA solution (50 ng of DNA) is prepared on ice. Among them, the PCR buffer solution and dNTP are provided as adjuncts to the enzyme. When conducting purity tests on Atractylodes Lancea Rhizome in Atractylodes Rhizome, the primer sets used are C and D (C is positive with *A. lancea*, D is positive with *A. chinensis*) as described in the paper[1] mentioned above, however, when primer sets A and B are used, it is possible to confirm the source species of each of the respective specimens. In order to confirm that the DNA has been extracted correctly, the reaction solution containing the positive control primer pair (Pf and Pr) as shown below should be prepared. In addition, the negative control solutions which are not containing DNA sample or either of the primer pair should be prepared and simultaneously conduct PCR.

Pf: 5′-CAT TGT CGA AGC CTG CAC AGC A-3′
Pr: 5′-CGA TGC GTG AGC CGA GAT ATC C-3′

The PCR reaction is performed under the following conditions: starting the reaction at 95°C for 10 minutes, 30 cycles of 0.5 minutes at 95°C and 0.75 minutes at 68°C (69°C only when using the primer set C), terminate the reaction at 72°C for 7 minutes, and store at 4°C. The resulting reaction mixture is used for the following process as PCR amplification reaction solution.

3.1.1.4. Agarose gel electrophoresis and detection of PCR products

After completion of the PCR reaction, mix 5 μL of the PCR amplification reaction solution with an appropriate volume of gel loading buffer solution, add the mixture to the wells of 2 w/v% agarose gel, and then perform electrophoresis using 1-fold TAE buffer solution (refer to General Information, ''Rapid Identification of Microorganisms Based on Molecular Biological Method <*G4-7-160*>''). Carry out the electrophoresis together with an appropriate DNA molecular marker. Electrophoresis is terminated when the bromophenol blue dye in the gel loading buffer has advanced to a point corresponding to 1/2 to 2/3 the length of the gel.

Stain the gel after electrophoresis when not using gel stained in advance with ethidium bromide. Place the gel that has undergone electrophoresis and staining in a gel image analyzer, irradiate with ultraviolet light (312 nm), and detect its electrophoresis pattern. Compare this to the DNA molecular marker and determine the absence or presence of the

target amplification band.

3.1.2. Judgment

Confirm at first that a 305 bp band is found with the reaction solution to which the positive control primer pair has been added, and confirm there are no bands in a solution with no primer sets and a solution with no sample DNA solution. Next, if a 226 bp band is confirmed when the primer set C is added or if a 200 bp band is confirmed when the primer set D is added, the sample is judged to be Atractylodes Lancea Rhizome (in the case of cut crude drug, contamination of Atractylodes Lancea Rhizome is observed) and it is rejected. The sample is judged not to be Atractylodes Lancea Rhizome (in the case of cut crude drug, there is no contamination of Atractylodes Lancea Rhizome) and the purity test is acceptable if a 305 bp band is confirmed with the positive control primer pair, bands are not observed in the reaction solution without primer and the reaction solution without DNA sample solution, and a 226 bp band is not observed with the primer set C and a 200 bp band is not observed with the primer set D. If a band is not observed with the positive control primer pair, it is to be concluded that the DNA extraction failed and the procedure should be started over again from the DNA extraction step. If bands are confirmed in reaction solutions without primer sets or without DNA sample solution, it should be assumed that there was an error in the PCR procedure and therefore the procedure should be repeated again from the step 3.1.1.3. PCR.

3.2. Method 2 (PCR—Restriction Fragment Length Polymorphism)

Generally, this method is referred to as PCR—Restriction Fragment Length Polymorphism (RFLP), and it provides nucleotide sequence information of sample-derived template DNA, based upon the DNA fragment pattern produced by restriction enzyme treatment of the PCR products, which are amplified by using a primer pair common to the DNA sequence of the objective plant.

The test is performed with 25 samples randomly taken from a lot, and each sample is designated with a number from 1 to 25. Differentiation of the sources is performed by individual PCR—RFLP measurement of the samples, and decision of the acceptability of the purity is dependent on how many nonconforming samples are present in the first 20 samples, taken in numerical order, for which judgment is possible as described below.

3.2.1. Procedure

The following is an example procedure.

3.2.1.1. Preparation of template DNA

There are various methods with which to extract and purify DNA from the samples. It is recommended that commercially available DNA extraction kits be used, when considering their advantages of not using noxious reagents and not requiring complicated purification procedures. Recently, PCR reagents that inhibit the effect of PCR enzymeinhibiting substances present in samples have become commercially available, and by using these reagents, it is possible to prepare the template DNA from the sample simply by incubating the sample with the DNA extraction reagent. Here, a recommended DNA preparing procedure using such PCR reagents is described for the convenience of experimenters.

Cut 20 mg of the sample into small pieces with a clean knife, add 400 μL of the DNA extraction reagent, and incubate at 55°C overnight (16 – 18 hours). Then heat at 95°C for 5 minutes to inactivate the enzyme in the reagent. Centrifuge to precipitate the sample, and use 50 μL of the supernatant liquid as the template DNA solution. The DNA solution prepared in this method can not be used for concentration measurement based on $OD_{260\,nm}$, because it contains many foreign substances affecting $OD_{260\,nm}$ value from the sample.

The composition of the DNA extraction reagent is as follows:

2-Amino-2-hydroxymethyl-1,3-propanediol-hydrochloric acid (pH 8.0)	20 mmol/L
Ethylenediamine tetraacetate	5 mmol/L
Sodium chloride	400 mmol/L
Sodium dodecyl sulfate	0.3%
Proteinase K	200 μg/mL

3.2.1.2. PCR

In the method using the PCR enzyme and PCR reagent as described[3], the reaction mixture is prepared on an ice bath in a total volume of 20 μL of a solution containing 10.0 μL of 2-fold concentrated PCR reagent, 5′- and 3′-primers (0.5 μmol/L), Taq DNA polymerase (0.5 units) and 0.5 μL of template DNA solution.

The PCR reaction is performed under the following conditions: 95°C for 10 minutes, 40 cycles of 95°C for 0.5 minute, 65°C for 0.25 minute, and 72°C for 0.25 minute and 72°C for 7 minutes. Store the solution at 4°C, and use this solution as the PCR amplification reaction solution. A negative control (containing water instead of the template DNA solution) must be included in the procedure.

The sequence of each primer is as follows:

5′-primer: 5′-GGC ACA ACA CGT GCC AAG GAA AA-3′

3′-primer: 5′-CGA TGC GTG AGC CGA GAT ATC C-3′

3.2.1.3. Restriction enzyme treatment

The treatment is performed on individual reaction solutions using two enzymes, *Fau* I and *Msp* I. In the case of *Fau* I, to an appropriate amount of the reaction solution, composed of a reaction buffer containing 1.0 unit of enzyme, add 3.0 μL of PCR products while cooling in an ice bath to make 15.0 μL. In the case of *Msp* I, to an appropriate amount of the reaction solution, composed of a reaction buffer containing 20.0 units of enzyme, add 3.0 μL of PCR products while cooling in an ice bath to make 15.0 μL. Incubate these solutions at the temperature recommended by the manufacturer for 2 hours, and then inactivate the enzyme by heating at 72°C for 10 minutes. The negative control of the PCR reaction is also treated in the same manner.

3.2.1.4. Agarose gel electrophoresis and detection of DNA fragments

After the restriction enzyme treatment, mix the total amount of the reaction solution and an appropriate amount of the gel loading buffer solution, place it in a 4 w/v% agarose gel well, and carry out electrophoresis with 1-fold concentrated TAE buffer solution (see ''Rapid Identification of Microorganisms Based on Molecular Biological Methods <*G4-7-160*>'' under General Information). Carry out the electrophoresis together with appropriate DNA molecular markers. Stop the electrophoresis when the bromophenol blue included in the loading buffer solution has moved about 2 cm from the well. The 4 w/v% agarose gel is sticky, difficult to prepare and hard to handle, so that it is better to use a commercially available precast gel.

After the electrophoresis, stain the gel, if it is not already stained, with ethidium bromide, and observe the gel on an illuminating device under ultraviolet light (312 nm) to confirm the electrophoretic pattern.

3.2.2. Judgment

3.2.2.1. Judgment of each sample

Confirm that no band is obtained with the negative control of the PCR, other than the primer dimer (about 40 bp) band. A sample treated with *Fau* I, showing bands of about 80 bp

and 60 bp, or that treated with *Msp* I, showing bands of about 90 bp and 50 bp, is judged as Atractylodes Lancea Rhizome. A sample not showing any band other than a band at about 140 bp and the primer dimer band is judged as Atractylodes Rhizome. If a sample does not show any band other than the primer dimer band, it is considered that PCR products were not obtained, and judgment is impossible for the sample.

3.2.2.2. Judgment of the purity

Judgment of the purity is based on the result of the judgment of each sample. If there is no sample that is judged as Atractylodes Lancea Rhizome among 20 samples taken in order of the numbering, excluding any sample for which judgment is impossible, the lot is acceptable for purity. When there is one sample that is judged as Atractylodes Lancea Rhizome among the 20 samples, perform the same test with 25 newly taken samples from the lot, and if there is no sample that is judged as Atractylodes Lancea Rhizome, the lot is acceptable for purity. When there is a sample that is judged as Atractylodes Lancea Rhizome in the second test, or there is more than one sample that is judged as Atractylodes Lancea Rhizome in the first test, the lot is not acceptable for purity.

4. Purity test of Saposhnikovia Root and Rhizome for *Peucedanum ledebourielloides*

4.1. Method 1

Similarly as 3.1., this method provides nucleotide sequence information of sample-derived template DNA, based upon the presence or absence of DNA amplification band in PCR using a species specific primer pair.

4.1.1. Procedure

The following is an example procedure.

4.1.1.1. Preparation of template DNA

For Atractylodes crude drugs, a preparation procedure using a silica gel membrane type kit is adopted, however for the test of Saposhnikovia Root and Rhizome, and *Peucedanum ledebourielloides*, the simple preparation procedure shown below is adopted for the convenience of experimenters, because it was confirmed that the PCR product is stably obtained in using a DNA sample solution prepared by the simple preparation procedure shown in 3.2.1.1. as a template.

Cut 10 mg of the sample into small pieces with a clean knife, add 400 μL of the DNA extraction reagent, and incubate at 55°C overnight (16 – 18 hours). Then heat at 95°C for 5 minutes to inactivate the enzyme in the reagent. Centrifuge to precipitate the sample, and use 50 μL of the supernatant liquid as the template DNA solution. The DNA solution prepared in this method can not be used for concentration measurement based on OD_{260nm}, because it contains many foreign substances affecting OD_{260nm} value from the sample.

The composition of the DNA extraction reagent is as follows:

2-Amino-2-hydroxymethyl-1,3-propanediol-hydrochloric acid (pH 8.0)	20 mmol/L
Ethylenediamine tetraacetate	5 mmol/L
Sodium chloride	400 mmol/L
Sodium dodecyl sulfate	0.3%
Proteinase K	200 μg/mL

4.1.1.2. PCR

In the method using the PCR enzyme and PCR reagent as described[3], the reaction mixture is prepared on an ice bath in a total volume of 20 μL of a solution containing 10.0 μL of 2-fold concentrated PCR reagent, 5′- and 3′-primers (0.5 μmol/L), Taq DNA polymerase (0.5 units) and 0.5 μL of template DNA solution.

When the purity test of Saposhnikovia Root and Rhizome for *Peucedanum ledebourielloides* is performed, the reaction solution containing the positive control primer pair as shown below should be prepared besides the reaction solution containing a species specific primer pair in order to confirm that the DNA has been extracted correctly. In addition, the negative control solutions which are not containing the DNA sample solution should be prepared and simultaneously conduct PCR.

The PCR reaction is performed under the following conditions: 95°C for 10 minutes, 45 cycles of 95°C for 0.5 minute, 62°C for 0.5 minute, and 72°C for 0.75 minute and 72°C for 7 minutes. Store the solution at 4°C, and use this solution as the PCR amplification reaction solution. The sequence of each primer is as follows. The positive control 3′-primer for PCR and the species specific 3′-primer for PCR have the same sequence.

5′-primer for positive control PCR: 5′-GCG TGG GTG TCA CGC ATC G-3′

3′-primer for positive control PCR: 5′-GTA GTC CCG CCT GAC CTG-3′

5′-primer for species specific PCR: 5′-CTG AGA AGT TGT GCC CGG-3′

3′-primer for species specific PCR: 5′-GTA GTC CCG CCT GAC CTG-3′

4.1.1.3. Agarose gel electrophoresis and detection of PCR products

After completion of PCR reaction, mix 5 μL of the PCR amplification reaction solution with an appropriate volume of gel loading buffer solution, add the mixture to the wells of 2 w/v% agarose gel, and then perform electrophoresis using 1-fold TAE buffer solution (refer to General Information, ''Rapid Identification of Microorganisms Based on Molecular Biological Method <*G4-7-160*>''). Carry out the electrophoresis together with an appropriate DNA molecular marker. Electrophoresis is terminated when the bromophenol blue dye in the gel loading buffer has moved about 2 cm from the well.

Stain the gel after electrophoresis when not using gel stained in advance with ethidium bromide. Place the gel that has undergone electrophoresis and staining in a gel image analyzer, irradiate with ultraviolet light (312 nm), and confirm its electrophoresis pattern. Compare this to the DNA molecular marker and determine the absence or presence of the target amplification band.

4.2. Judgment

Confirm at first that a 250 bp band is found with the reaction solution to which the positive control primer pair has been added, and confirm there are no bands other than the primer dimer (about 40 bp) in a solution with no sample DNA solution. Next, if a 200 bp band is confirmed when the species specific primer pair is added, the sample is judged to be contaminated with *Peucedanum ledebourielloides* and it is rejected. The sample is judged not to be contaminated with *Peucedanum ledebourielloides* and the purity test is acceptable if a 250 bp band is confirmed with the positive control primer pair, bands are not observed in the reaction solution without DNA sample solution, and a 200 bp band is not observed with the species specific primer pair. If a band is not observed with the positive control primer pair, it is to be concluded that the DNA extraction failed and the procedure should be started over again from the DNA extraction step. If bands are confirmed in the reaction solution without DNA sample solution, it should be assumed that there was an error in the PCR procedure and therefore the procedure should be repeated again from the step 4.1.1.2. PCR.

5. Reference

1) Y. Guo, *et al.*, *J. Nat. Med.*, 60, 149-156 (2006).
2) K. Kondo, *et al.*, *J. Jpn. Bot.*, 84, 356-359 (2009).
3) T. Maruyama, *et al.*, *Shoyakugaku Zasshi* 64, 96-101 (2010).
4) T. Maruyama, *et al.*, *J. Nat. Med.*, in press.
5) JAS analytical test handbook: genetically modified food quality, labeling analysis manual for individual products, ver. 3, I Fundamental procedure 4.4.1 PCR, (September 24, 2012). Incorporated Administrative Agency Food and Agricultural Materials Inspection Center
6) JAS analytical test handbook: genetically modified food quality, labeling analysis manual for individual products, ver. 3, IV Contamination prevention, (September 24, 2012). Food and Agricultural Materials Inspection Center Independent Administrative Organization
7) Notification No. 110, Director of Food Health Department, March 2001; Partial Amendment: Notification No. 0629002, 2.2.1.2, Director of Food Safety Department, June 2006.
8) Notification No. 0629002, 2.1.3.1.1, Director of Food Safety Department, June 2006.

Analytical Methods for Aflatoxins in Crude Drug and Crude Drug Preparations <*G5-7-170*>

Aflatoxins are carcinogenic secondary metabolites produced by some fungal strains[1]. They are found in agricultural products such as cereals, tree nuts and spices. Many countries including Japan have set regulatory limits on aflatoxins in foods[2,3]. Not only foods but also crude drugs may be contaminated by aflatoxins because aflatoxin contamination in the ingredients of botanical products has been reported in some foreign countries[4-6]. Therefore, aflatoxin testing in crude drugs and preparations containing crude drugs as main ingredient (crude drug preparations) is required to be performed. Concerning the risk of aflatoxin contamination, it is necessary to consider the presence of producing bacteria and the possibility of contamination in processes of processing and manufacturing.

In this connection, the regulatory limit for aflatoxins (sum of B_1, B_2, G_1 and G_2) has been set at 10 μg/kg for all foods in Japan[3].

1. Summary

The methods based on HPLC with fluorescence detection and LC-MS are used for aflatoxins instrumental analysis[7-10]. Aflatoxin standards should be handled with care because they are highly toxic compounds. (Refer to 4. Points to note) Simple measurement equipment and quantitative test kits are also used for the simple analysis of aflatoxin and some of them enable aflatoxin determination without using aflatoxin standards.

Aflatoxins are generally purified from the samples by cartridge-type columns such as immunoaffinity column and multifunctional column. An immunoaffinity column is useful for purification of aflatoxin from crude drug preparations, but in some cases, a multifunctional column is effective.

In this document, the analytical methods for aflatoxins using simple measurement kits, which are useful for screening, and HPLC with fluorescence detection are described. The methods should be selected according to the characteristics and aflatoxin contamination levels of the samples. Method optimization and validation are required to be performed. As references, an official method of Japan for aflatoxin analysis in food[7,11], analytical methods described in European and US Pharmacopeia, and WHO guidelines can be used[8-10].

2. Analytical methods

2.1. An analytical method using a qualitative kit

The kit can detect the presence or absence of aflatoxin in the sample by using an antigen-antibody interaction. A kit for the detection of total aflatoxins can be used for qualitative test. The following method can be used for detecting aflatoxins in some extracts (Orengedokuto, Kakkonto, Shoseiryuto, Hachimijiogan, Goshajinkigan, Daiokanzoto and Mukoi-Daikenchuto) listed in Japanese Pharmacopoeia at cut-off levels of 4 ppb[12]. In order to quantify aflatoxins in the positive samples, instrumental analysis is required to be done.

(i) Preparation for sample solution

Weigh accurately about 1 g of the powdered sample, add exactly 4 mL of a mixture of acetonitrile, water and methanol (6:4:1) and then shake for 30 minutes. After centrifugation, dilute exactly 2 mL of the supernatant to 50 mL with phosphate buffered saline (PBS) containing 4% of polysorbate 20. Apply the diluted extract to an immunoaffinity column, which is preequilibrated with PBS. Wash the column with 10 mL of PBS containing 0.01% of polysorbate 20 followed by 10 mL of water. Apply 1 mL of acetonitrile on the column and collect eluate. Wait 5 minutes and apply 2 mL of acetonitrile. Collect applied elution solvent. Evaporate the eluate to dryness under nitrogen. Dissolve the residue in exactly 0.5 mL of diluted methanol (7 in 10), and use this as the sample solution. In this method, 0.5 mL of the sample solution is equivalent to 0.5 g of the sample matrix.

(ii) Measurement and evaluation

Prepare the test strips, the microwells with the inner bottom covered by gold colloid, and the assay diluent attached with the kit. Add exactly 50 μL of the assay diluent to each microwell. Dissolve the coating conjugate in the microwell by pipetting. Add exactly 50 μL of sample extracts to each microwell and mix the content in each well by pipetting it up and down. Put one test strip into one well and allow the test strip to develop color for 5 minutes. Interpret test results from the lines formed in the test zone and the control zone. If the two lines are visible, this indicates the sample contains total aflatoxin less than 4 ppb (negative sample).

2.2. An instrumental method of analysis

Aflatoxins can be detected by a fluorescence detector because they are fluorescent substances. In order to enhance the fluorescent intensities of AFB_1 and AFG_1, the intensities of which are weak in polar solvents, a derivatization step is performed. Precolumn derivatization with trifluoroacetic acid and postcolumn derivatization with a photochemical reactor or an electrochemical cell are known. AFB_2 and AFG_2 are not derivatized by the above methods. The following method can be used for quantification of aflatoxins in some extracts listed in Japanese Pharmacopoeia[12]. This method is an example and the other method can also be used.

(i) Preparation for sample solution

Weigh accurately about 1 g of the powdered sample and add exactly 4 mL of a mixture of acetonitrile, water and methanol (6:4:1) and then shake for 30 minutes. After centrifugation, dilute exactly 2 mL of the supernatant to 50 mL with phosphate buffered saline (PBS) containing 4% of

polysorbate 20. Apply the diluted extract to an immunoaffinity column, which is preequilibrated with PBS. Wash the column with 10 mL of PBS containing 0.01% of polysorbate 20 followed by 10 mL of water. Apply 1 mL of acetonitrile on the column and collect eluate. Wait 5 minutes and apply 2 mL of acetonitrile. Collect applied elution solvent. Evaporate the eluate to dryness under nitrogen. Redissolve the residue in exactly 0.5 mL of 70% methanol in water. In this method, 0.5 mL of the sample solution is equivalent to 0.5 g of the sample matrix.

(ii) Measurement and evaluation

Use an ODS column for separation. Aflatoxins can be detected by an HPLC equipped with a fluorescence detector because they are fluorescent (excitation λ = 365 nm, emission λ = 430 nm). Use trifluoroacetic acid (TFA) for derivatization of aflatoxins. Aflatoxins elute in the order of AFG_{2a} (a derivative of AFG_1), AFB_{2a} (a derivative of AFB_1), AFG_2 and AFB_2 when a mixture of acetonitrile, water and methanol (6:3:1) is used as a mobile solvent. Postcolumn derivatization with a photochemical reactor is useful when performing continuous monitoring of aflatoxins. In that case, aflatoxins elute in the order of AFG_2, AFG_{2a}, AFB_2 and AFB_{2a}. Prepare some standard solutions in which aflatoxins are present at the concentrations from 0.5 to 20 μg/L, and verify linearity in the range.

3. Reagents and solutions

Some reagents and solutions prescribed in Japanese Pharmacopoeia and those listed below are used.

(i) PBS containing 0.01% or 4% of polysorbate 20 Dissolve 8.0 g of NaCl, 0.2 g of KCl, 2.9 g of $Na_2HPO_4.12H_2O$, 0.2 g of KH_2PO_4 and 0.1 g (0.01%) or 40 g (4%) of polysorbate 20 in 900 mL of water and adjust to pH 7.4 with 0.1 mol/L hydrochloric acid TS or dilute sodium hydroxide TS, and add water to make 1000 mL. Keep the solution at 2 – 8°C.

(ii) Aflatoxin solution Dilute the aflatoxin standard stock solution with acetonitrile or methanol. Use the commercially available standard stock solution which is precisely prepared in concentration.

4. Points to note

(i) Aflatoxins are highly cardiotoxic compounds, and sufficient caution is required when handling them. Especially in cases when handling a high concentration of aflatoxins, pay maximum attention. Wear a protect coat, gloves, a mask and goggles. All handling should be performed in a fume hood.

(ii) Soak the used labware in 0.5 – 1.0% sodium hypochlorite (NaClO) solution for more than 2 hours before discarding and washing it. Commercially available sodium hypochlorite solutions for disinfection or for food additive may also be used after adjusting the concentration.

(iii) Keep the aflatoxin solution in a dark and cool place. Keep the commercially available standard stock solution under specified conditions.

(iv) Aflatoxins may be absorbed by a glass vessel. In order to avoid absorption, using silanized glass vials may be effective. The vial should be washed with 20% acetonitrile and air-dried before use.

(v) Be cautious that there are no big air bubbles or cracks in the gel of the immunoaffinity column. If there are bubbles or cracks, remove them by applying pressure from the upper part of the column.

(vi) Confirm the performance of the immunoaffinity column by performing a spike and recovery test if necessary.

(vii) In case of using a multifunctional column for preparing the sample solution, it is necessary to assess its performance beforehand by performing a spike and recovery test.

5. References

1) IARC, ''IARC Monographs on the Evaluation of Carcinogenic Risks to Humans'', Vol. 82 (2002).
2) FAO, ''FAO Food and Nutrition Paper 81, Worldwide Regulations for Mycotoxins in Food and Feed in 2003'' (2004).
3) Notification No. 0331-5, Director of Food Safety Department, Pharmaceutical and Food Safety Bureau, MHLW, March 31, 2011, ''Management of Food Containing Aflatoxin''
4) M. Trucksess, *et al.*, *J. AOAC Int.*, 89 (3), 624 – 630 (2006).
5) C. Bircan, *Int. J. Food Science and Technology*, 40, 929 – 934 (2005).
6) H. Tosun and R. Arslan, The Scientific World Journal, 2013, Article ID 874093 (2013).
7) Notification No. 0816-1, Director of Food Safety Department, Pharmaceutical and Food Safety Bureau, MHLW, August 16, 2011, ''Method for testing total aflatoxins.''
8) European Pharmacopoeia 8.0 (2013), 2.8.18. Determination of aflatoxin B1 in herbal drugs.
9) US Pharmacopeia 37 (2014), <*561*> Articles of Botanical Origin.
10) WHO: WHO Guidelines for Assessing Quality of Herbal Medicines with Reference to Contaminants and Residues.
11) Notification No. 0816-7, Director of Inspection and Safety Division of Food Safety Department, Pharmaceutical and Food Safety Bureau, MHLW, August 16, 2011, ''Test Methods Related to Aflatoxin Contained in Corns.''
12) H. Sakuma, *et al. Shoyakugaku Zasshi*, 68 (2), 53 – 57 (2014).

Radioactivity Measurements Method for Crude Drugs <*G5-8-180*>

Crude drugs are natural products produced by harvesting cultivated plants/reared animals or collecting wild resources and processing them through washing and drying. This General Information describes the radioactivity measurement method of crude drugs that can be applied when there is a concern about the contamination of radioactive materials in more amounts exceeding that from natural origin.

The measurement methods described here are procedures to measure radioactivity by γ-ray spectrometry, and its target nuclides are ^{131}I, ^{134}Cs and ^{137}Cs.

1. Principle[1)]

In order to measure the radioactivity of a radionuclide in a sample, radioactive materials are identified based on the energy of radiation by measuring α-rays being helium nuclei, β-rays being electrons and γ-rays being photons, emitted when radionuclides decayed, and radioactivity is determined from the number of radiations counted per unit time. Radiations have different penetrating powers depending on their kind and energy. Generally, α-rays have the weakest penetrating power and are shieldable by papers. β-Rays have stronger penetrating power than α-rays, being shieldable by a light metal plate with a few millimeters thickness, and are classified to the weak penetrating power radiation. On the

other hand, γ-rays have strong penetrating power and require substances such as lead which has high atomic number and large density ranging from several to 10 centimeters to be shielded.

Difference in penetration of radiation is an important factor in the measurement of radiation/radioactivity. γ-Rays are usually used to determine radionuclides. α- and β-rays are susceptible to self-shielding (absorption) because of their weak penetrating radiation and are suitable for the measurement of surface contamination, etc. However, the identification of radionuclides by their spectroscopies is not easy because specialized techniques such as sample preparation are required. On the other hand, most γ-rays do not lose their energy when penetrating a substance even in emission from inside the substance, and the information of the emitted γ-ray energy is obtained from the measured spectrum. Since γ-ray energy emitted from a radionuclide is determined for each radionuclide, it is relatively easy to identify the radionuclide based on the obtained energy spectrum. For the measurement of radioactivity concentration in crude drugs, it is necessary to identify the radionuclide contained in the crude drug and to measure the concentration of the radionuclide, therefore measurement methods by γ-ray spectroscopy are recommended.

Semiconductor detectors and scintillators are known as detectors used for the radioactivity measurement methods by γ-ray spectroscopy. By injection of radiation, the former produce electron-hole pairs and the latter emit a light. Scintillators exhibit scintillation (flash and fluorescence), but the intensity of the light is very weak. Therefore, it is used with the combination of a photomultiplier tube, etc. which amplify an electric signal converted from photon. A germanium semiconductor detector (hereinafter referred to as ''Ge detector''), one of semiconductors, has the highest performance as a detector that can measure the radionuclide in crude drugs. In addition, a thallium activated sodium iodide scintillation detector (hereinafter referred to as ''NaI (Tl) detector'') is easy to handle and can measure the radioactivity of crude drugs.

1.1. Target radionuclide

The target nuclides are ^{131}I, ^{134}Cs and ^{137}Cs.

1.1.1. Ge detector

Radiation data necessary for the γ-ray spectrometry radiometry using a Ge detector is shown in Table 1.

Table 1 Radiation data of target nuclides for a Ge detector[1)]

Nuclide	Half-life	Energy	γ-Ray Emission Rate	γ-Ray that require correction of summing effect (γ-Ray Emission Rate)
^{131}I	8.021 days	364.5 keV	0.817	284.3 keV (0.061), 637.0 keV (0.072), etc.
^{134}Cs	2.065 years	604.7 keV	0.976	569.3 keV (0.154), 801.9 keV (0.087)*, etc.
		795.9 keV	0.855	
^{137}Cs	30.17 years	661.7 keV	0.851	no (single γ-ray)

* When resolution is not high, the peaks of 795.9 keV and 801.9 keV can be treated as one peak (0.942).

1.1.2. NaI (Tl) detector

Radiation data necessary for the γ-ray spectrometry radiometry using a NaI detector is shown in Table 2. In the measurement using a NaI detector, radiocesium is treated as the sum of ^{134}Cs and ^{137}Cs because it is difficult to accurately distinguish and quantify the nuclides.

Table 2 Radiation data of target nuclides for a NaI(Tl) detector[1)]

Nuclide	Half-life	Energy	γ-Ray Emission Rate	γ-Ray that require correction of summing effect (γ-Ray Emission Rate)
^{131}I	8.021 days	364.5 keV	0.817	284.3 keV (0.061), 637.0 keV (0.072), etc.
^{134}Cs	2.065 years	604.7 keV	0.976	the peaks of 795.9 keV and 801.9 keV are treated as one peak (0.942).
		795.9 keV 801.9 keV	0.855 0.087	
^{137}Cs	30.17 years	661.7 keV	0.851	no (single γ-ray)

2. Apparatus

The system configuration of a γ-ray spectrometer is shown in Figure 1. The apparatus generally consists of a detector, a circuit part for measuring such as an amplifier, and an analysis part (personal computer: PC) (Figure 1). In some commercially available apparatuses, a circuit part for measuring such as a high voltage power supply, amplifier and multichannel analyzer is integrated with a detector, and the resultant detection part which include a shielding body are combined with a PC for analysis. Details will be described later.

The Ge detector has a cooling system using liquid nitrogen.

3. Sampling, preparation, storage and transport

3.1. Sampling

3.1.1. Sampling container, tool and label

Fresh polyethylene bags are used for sampling containers.

Auxiliary tools for sampling are made of stainless, polyethylene, or their equivalent materials. Parts which contact with samples should be protected with polyethylene bags to prevent contamination during transport. Because auxiliary tools are used at the sampling sites, pay attention not to contaminate samples from these tools when sampling are conducted at multiple sites.

Fill out immediately the following items on sampling containers before or after sampling.

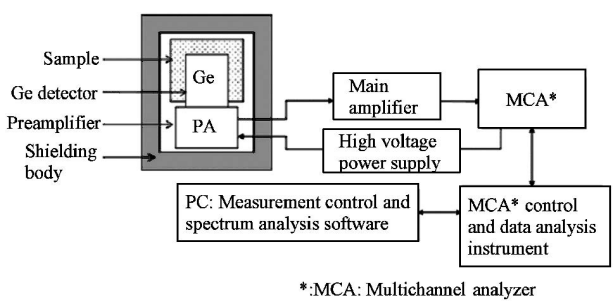


Figure 1 The system configuration of a γ-ray spectrometer[2)]

① Sample number (lot)
(When collecting a same sample in multiple sample containers, each should be distinguished.)
② Sample name
③ Production area of sample
④ Sampling date
⑤ Sampler name
⑥ Special notes
⑦ Others necessary for evaluation

3.1.2. Sampling and handling of samples

Random sampling is performed to collect samples representing a unit for measurement, and collected samples are homogenized by through mixing. As a general rule, one sample is measured per one unit for measurement.

In situation where direct sampling is difficult, samples are collected with a shovel, and transferred to sampling containers using a funnel or the like, if necessary.

3.1.3. Amount of sampling

It is desirable to collect about twice the amount required for testing.

3.2. Preparation of sample

If necessary, prepare to the size of samples appropriate for each apparatus. Crude drugs are derived from various parts of plants, minerals, animals, and so on, and have various sizes, shapes and solids. Therefore, they are cut and crushed according to their characteristics. Procedure that affect test results, such as washing, must not be done after sampling.

3.3. Storage and transport of sample

Test immediately after sampling. Make sure that sampling containers are not broken and samples do not leak from sampling containers. When testing is not performed immediately, store samples avoiding moisture and insect damage.

4. Measurement of sample

An example of analysis by a γ-ray spectrometer is shown in Figure 2.

4.1. Measurement using a Ge spectrometer

4.1.1. Characteristic of the measurement method

Because the γ-ray spectrometry radiometry using a Ge detector has a very high energy resolution, it can determine energy accurately to identify a radionuclide easily and certainly, and clearly analyze the energy by separately from the other γ-rays with close energy. Moreover, because the spreading of γ-ray peaks is small and the ratio of the background to the peak is low, it is suitable for low level radioactivity.

4.1.2. Apparatus, tool and so on

4.1.2.1. Configuration of apparatus

(1) Ge detector

The relative efficacy of a detector should be not less than 20%.

The energy resolution is generally 1.8 to 2.0 keV as a half width.

(2) Shielding body

Shielding a detector is very important in the measurement of low level radioactivity. γ-Rays derived from natural nuclides (^{40}K, nuclides of the uranium and thorium series) should be sufficiently shielded.

A lead shielding body with 10 to 15 cm thick is generally used around a detector.

The size of the inner space of the shielding body must be enough to put a sample container.

There are vertical (dipstick) and L-type (with cryostats on the side of a liquid nitrogen container) detectors. The shielding bodies differ in structure depending on both the shape of a detector and the connection between a detector and a liquid nitrogen container.

4.1.2.2. Tools and so on

(1) Sample container

Sample containers should have good sealing performance, high mechanical strength, resistance to acid and heat, and the internal sample should be visible. Sample containers include Marinelli containers with an internal volume of 1 to 2 L and cylindrical containers with an internal volume of 100 to 500 mL. Sample containers should be selected based on sample volume.

(2) Energy calibration source

Select some energy calibration sources to cover from 100 to 2000 keV such as ^{22}Na (511 keV, 1275 keV), ^{54}Mn (835 keV), ^{60}Co (1173 keV, 1332 keV), ^{88}Y (898 keV, 1836 keV), ^{137}Cs (662 keV) and ^{139}Ce (166 keV). Each radioactivity should be 1000 to 3000 becquerel (Bq).

(3) Efficiency calibration source

Efficiency calibration sources are commercially available standard samples containing ^{137}Cs and so on, whose container and medium volumes are equal to those of the sample. Each radioactivity should be 1000 to 3000 Bq.

(4) Software for spectral analysis

The software which can search peaks, identify nuclides, calculate peak areas and statistical uncertainties of count, is used. In addition, it is desirable to be able to correct self-absorption and summing effect.

4.1.3. Apparatus calibration

4.1.3.1. Energy calibration

For energy calibration sources, correspondence relationship between γ-ray energy and a peak center channel is obtained as a linear equation according to the following procedure.

(1) Attach an energy calibration source to the regular position of a detector, and measure the spectrum until the peak area of a main γ-ray reaches several thousand counts.

(2) Assuming that γ-ray energy (E) and a peak center channel (p) are in linear relationship, the following formula is obtained using spectral analysis software.

$$E = a + b \times p$$

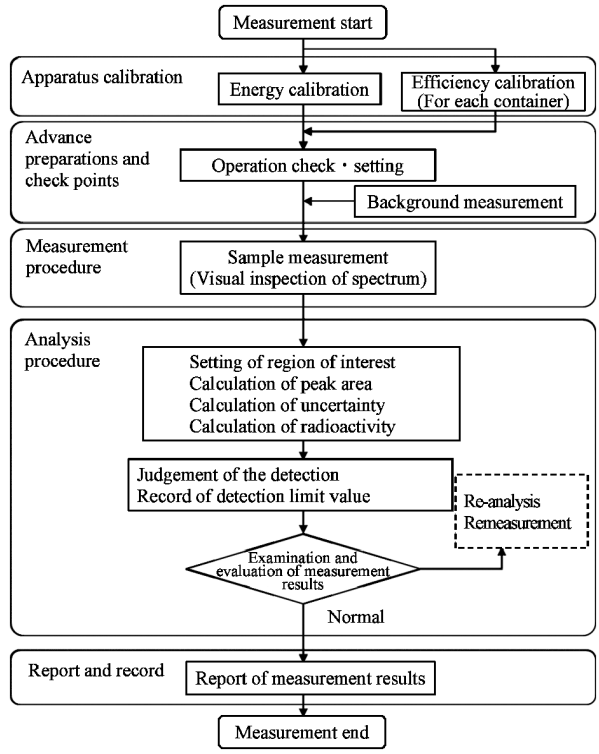


Figure 2 An example of analysis flow

By setting the energy range of γ-rays to 0 to 2000 keV and the channel full scale of a multichannel analyzer to 4000 ch, the region of interest can be set easily even when the count value is low, and in this case ''a'' in the above formula is close to 0, and ''b'' is as close to 0.500 as possible.

(3) Record and save the above data.

4.1.3.2. Efficiency calibration

In order to determine radioactivity from a measured γ-ray spectrum, counting efficiency relative to a peak (hereinafter referred to as ''peak efficiency'') is necessary, and radioactivity analysis postulates that the peak efficiency is correctly calibrated.

For efficiency calibration, use the efficiency calibration sources whose concentration are known. Usually, standard sources containing various nuclides are measured to obtain a peak efficiency function with energy as a variable so that it can be applied to the energy range of approximately 50 to 2000 keV. Since the peak efficiency varies depending on the sample container, it is necessary to perform efficiency calibration for each sample container when multiple sample containers are used.

4.1.4. Procedure

4.1.4.1. Preparations in advance and points to be checked

(1) Operation check of apparatus and settings

Before measuring a sample, use spectral analysis software to analyze the spectrum of the energy calibration source and confirm that the peak center channel, half width and peak count rate are normal for major γ-rays.

(2) Background measurement

Measure a background under the specified measurement conditions. In principle, the measurement is performed by placing a sample for background measurement (enclosing the same amount of water containing no target radionuclides in the same sample container) that has the same conditions as the sample.

Since the analytical result of a background spectrum measured recently is used for the radioactivity analysis of a sample, when a peak corresponding to the main γ-ray energy of a target nuclide shown in Table 1 is observed, calculate the count rate and the statistical uncertainty of count and save the result in preparation for the measurement. Note that there is 609.3 keV (0.426) emitted from ^{214}Bi of the uranium series in a background spectrum and this spectrum is close to 604.7 keV of ^{134}Cs.

4.1.4.2. Measurement procedure

When filling a sample into a sample container, take care to reduce the void as much as possible and make it uniform. Therefore, pretreat it by cutting or crushing according to the characteristics of a crude drug to be measured, if necessary.

Attach the same container filled with the same amount of a sample as the standard sample used for the efficiency calibration to the center of a detector. At this time, the deviation from the center should be within about 1 cm for Marinelli containers and within about 2 mm for cylindrical containers.

Start measurement after setting the measurement time of the sample so that a target detection limit value can be obtained based on the peak efficiency and the results of background measurement, etc. Note that a peak shape may deteriorate when a count rate is very high.

After the measurement, save the spectrum data.

4.1.4.3. Analysis procedure

(1) Setting of region of interest

After the measurement is completed, set the region of interest (ROI) for the γ-ray of the target nuclide using spectrum analysis software. At this time, if the count is insufficient, the variation of the count for each channel can be leveled by smoothing process.

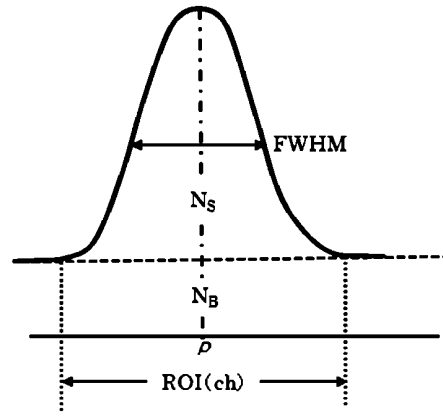


Figure 3 Setting of region of interest (ROI), and calculation of peak area (N_S) and background area (N_B)

From the peak center channel (p) and the full width at half maximum (FWHM), it can be confirmed that the measured spectrum is normal, but for weak peaks, the value may fluctuate.

(2) Calculation of peak area

Peak areas (N_S) are calculated based on peak search in the automatic setting of ROIs by analysis software, but for especially weak peaks, confirm whether the position and width of ROIs are appropriate.

(3) Subtraction of background count rate and calculation of statistical uncertainty of count

In usual γ-ray spectrometry, it is not always necessary to subtract a background count rate n_{BG} (hereinafter referred to as ''BG count rate''), but if a detector and the inside of a shield body are contaminated, it is necessary to subtract the BG count rate. A net count rate $n(s^{-1})$ is obtained by subtracting the BG count rate in the same ROI from the sample count rate ($n_S = N_S/t_S$).

$$n = n_S - n_{BG}$$

The relation of a count error σ_n and a count rate (n) is expressed as the following formula:

$$n \pm \sigma_n = n \pm (n/t)^{1/2}$$

The statistical uncertainty of count to a net count rate, σ_n (s^{-1}), is expressed as the root sum squares of statistical uncertainties (σ_S and σ_{BG}) of each count rate, shown as follows:

$$\sigma_n = (\sigma_S^2 + \sigma_{BG}^2)^{1/2}$$

(4) Calculation of radioactivity

The radioactivity A (Bq) and the radioactive concentration C (Bq/kg) of a sample are obtained by the following formulae:

$$A = \frac{n}{a\varepsilon f_{SUM}} \quad C = \frac{A}{M}$$

In the above formulae, the abbreviations are as follows:

n: Count rate
a: γ-Ray emission rate (Bq^{-1}) shown in Table 1
ε: Peak efficiency
f_{SUM}: Correction factor for summing effect
M: Mass (kg) of a sample in a sample container

However, when the measurement is compared with a standard sample, ''a'', ''ε'', and ''f_{SUM}'' are the same, so there is no need to consider. In other words, when the radioactivity of the standard sample is A_{STD} and the count

rate is n_{STD}, the radioactivity A is obtained by $A = (n/n_{STD})A_{STD}$.

(5) Uncertainty of detection

If it is not necessary to consider the uncertainty of the mass of a sample in a sample container, the statistical uncertainty of count δ_A (Bq) of the radioactivity of the sample and the statistical uncertainty of count δ_C (Bq/kg) of the radioactive concentration are obtained by the following formulae:

$$\delta_A = \frac{\sigma_n}{n}A \quad \delta_C = \frac{\delta_A}{M}$$

If the measured radioactive concentration C exceeds $3\delta_C$, it is considered statistically significant.

(6) Record of detection limit value

If no radioactivity is detected, record $3\delta_C$ of the radioactivity value, which would be measured in the analysis, as the detection limit value.

The detection limit value is affected by a BG count rate, sample measurement time, and sample mass. In radioactivity measurement, methods to calculate radioactivity from the count rate of the background part of the peak to be measured (usually calculated from the baseline region beside the peak), BG count rate, sample measurement time and background measurement time, sample mass, etc. are widely used.

(7) Examination and evaluation of measurement results

Summarize measurement results for each target nuclide, and confirm that it is normal based on the statistical uncertainty of count, peak center channel, FWHM, etc. If there is any doubt in the confirmation of results, remeasure as necessary.

4.1.5. Points to note for measurement

4.1.5.1. Control of background

When the same nuclide as a target nuclide is detected from a background, it is necessary to confirm the cause and to suppress the influence of the background as much as possible. In the case of indoor contamination, the influence can be suppressed by cleaning and checking shielding devices around an apparatus and performing appropriate shielding. If the inside of a shield body or a detector itself is contaminated, decontamination should be generally attempted. However, if decontamination is impossible, it is necessary to subtract the contribution from the background when calculating radioactivity.

4.1.5.2. Contamination prevention of apparatus, tool and so on

Cover a detector with polyethylene bag to prevent from contamination. In the event of a contamination, cope by replacing the polyethylene packaging. If the surface of a detector is contaminated, wipe it off with a neutral detergent or gauze soaked in ethanol. Be careful not to allow dust or other contaminants to enter when opening a shield body.

Use a sample container after simple cleaning. When a sample is placed into a sample container, it is also important to prevent the sample from adhering around the sample container.

Use a disposable container when measuring a high concentration sample or when decontamination is difficult. When a sample container is used repeatedly, it is recommended to apply fluorine coating. It is also effective to use a plastic bag in a sample container.

4.1.5.3. Routine maintenance of apparatus

Regular performance tests of an entire measurement system is very important for the control of apparatuses. In the performance tests, a γ-ray source for checking is placed at a fixed position on a detector, and a peak center channel, FWHM and peak count rate are obtained for low, medium and high energy γ-rays. Save these results as time-series data. The performance tests should be preferably performed daily, at least prior to a series of sample measurement, so that accurate energy calibration can always be used.

In addition, confirm no contamination around a detector and a sample container by regularly performing measurement without sample or by placing an empty container.

One of the detector troubles is vacuum loss in a cryostat. This can be judged from the consumption of liquid nitrogen, a decrease in energy resolution, and visual inspection (the existence of condensation at the neck of the cryostat).

4.2. Measurement using a NaI (Tl) spectrometer

4.2.1. Characteristic of measurement method

Scintillation detectors calculate the energy and number of radiation by converting a weak light emission generated at the time when radiation is incident on a solid crystal called a scintillator, into an electric signal using a photomultiplier. As the advantages, it is relatively inexpensive compared to Ge detectors and can be used at ordinary temperature. Another characteristic of the scintillation detector is that the detection efficiency is determined by the size of crystals because the size of commercially available solid crystals is standardized. NaI and $LaBr_3$, etc. are used as solid crystals[3].

4.2.2. Apparatus, tool and so on

4.2.2.1. Configuration of apparatus

Scintillation spectrometer is generally composed of a scintillation detector, a high-voltage power supply, an amplifier, a multichannel analyzer, and a PC for analysis. Scintillation spectrometer has the analytical function of a γ-ray spectrum and can perform processes from radioactive measurement to quantitative calculation.

(1) Detector

The energy resolution is not more than 8%.

(2) Shielding body

In order to reduce the influence of environmental radiation (background), it is desirable that an apparatus has the lead-shielding structure around the detector. It is more desirable to enclose the entire surface with lead because the reduction effect is low when shielding only the side surface of a detector.

(3) Sample stage

Set the sample stage that a detector and a sample can be always fixed in a fixed spatial position relationship (geometry) in a shield body. In this case, it is desirable to place a beaker sample container perpendicularly just above the detector in terms of the detection efficiency and the stability during measurement. When using a rectangular sample container, it is also possible to attach the side of the container and the sample stage with the detector horizontally.

4.2.2.2. Tool and so on

(1) Sample container

Marinelli containers, plastic bottles, polyethylene tanks, etc. are used as sample containers. In the case of an emergency, quantification is possible by inserting a detector into a bucket containing a sample. However, it is necessary to calculate detection efficiency for each measurement container in advance.

(2) Energy calibration source

Select some energy calibration sources to cover from 100 to 2000 keV such as ^{22}Na (511 keV, 1275 keV), ^{54}Mn (835 keV), ^{60}Co (1173 keV, 1332 keV), ^{88}Y (898 keV, 1836 keV), ^{137}Cs (662 keV), and ^{139}Ce (166 keV). Obtain the relationship between the γ-ray energy and the peak center channel as a linear equation.

Because the energy resolution of NaI spectrometers is low unlike Ge detectors, the mixed radiation source composed of some nuclides with close γ-ray energy is not used.

(3) Efficiency calibration source

For efficiency calibration, use the efficiency calibration source whose radioactivity is known. Since the peak efficiency varies depending on the sample container, it is necessary to perform efficiency calibration for each sample container when multiple sample containers are used. It is desirable to use the nuclide emitting one or two γ-rays, considering the energy resolution of a spectrometer. It is desirable that the source includes target nuclides, ^{134}Cs and ^{137}Cs.

(4) Software for spectral analysis

Even if there is overlapping of peaks attributed to multiple nuclides, the software should be able to separate the peak of interest and calculate its area by peak function fitting, etc. It is desirable to be able to perform peak analysis in accordance with ''Radioactivity measurement series No.7, γ-Ray Spectrometry by Germanium Semiconductor Detector''[4]. In addition, it is desirable to be able to calculate radioactive concentration from the radiation data of a nuclide to be quantified (half-life, γ-ray emission rate) and detection efficiency.

4.2.3. Apparatus calibration

4.2.3.1. Energy calibration

An energy calibration equation is obtained by using several energy calibration sources after the channel width of a multichannel analyzer is set to about 1000 ch and adjusted so that γ-rays up to 2000 keV can be measured.

There is the following relationship between the γ-ray energy (E) and the peak center channel (p):

$$E = a + b \times p$$

In the above equation, ''a'' is desirable to be as close to 0 as possible, and ''b'' is to be as close to 2.0 as possible, considering the number of channels.

4.2.3.2. Efficiency calibration

Because the counting efficiency varies depending on the energy of γ-rays, the efficiency calibration (ε) is obtained as the function of γ-ray energy (E) using an efficiency calibration source composed of some nuclides in known amounts. There is the following relationship in the region of several hundreds to 2000 keV.

$$\log(\varepsilon) = a + b \times \log(E)$$

If there is a calibration source containing ^{134}Cs and ^{137}Cs for quantification, the counting efficiency for a target γ-ray peak can be obtained directly.

4.2.4. Procedure

4.2.4.1. Preparations in advance and points to be checked

(1) Operation check of apparatus and settings

Apply polarity and voltage specified by a manufacturer to a photomultiplier tube. When a source is brought close to a detector, it is desirable to check with an oscilloscope that output waveforms from a preamplifier meet specifications. However, it is also acceptable to refer to an instruction manual for a model to be used. Alternatively, connect a detector to a multichannel analyzer to ensure that no noise signal is present, which is not normally observed, and that a dead time meter does not scale out. The channel width of the multichannel analyzer is set to about 1000 ch. The range of measurement energy is to be about 100 to 2000 keV.

For energy calibration, confirm that reference γ-rays (for example, ^{137}Cs or ^{40}K) can be detected in a channel set in advance at the time of power-on and every day. If there is a significant deviation from the set channel, adjust the gain of the amplifier.

(2) Background measurement

Perform measurement about once a week without sample or with an empty container to ensure that there is no contamination around a detector and a sample container. If a peak is observed in the same channel as a γ-ray to be quantified and decontamination is impossible, the counting rate should be obtained and recorded.

4.2.4.2. Measurement procedure

Since the measurement procedure is basically the same as the method using a Ge detector, follow the operation of a γ-ray spectrometer using a Ge detector.

(1) Setting of measurement time: Determine the measurement time according to a target detection limit and the amount of a sample. To lower the detection limit, the reduction of a background is most effective.

(2) Start and end procedures of measurement, and record of the times.

(3) Store of spectral data: File names should be such that samples and measured dates can be identified.

4.2.4.3. Analysis procedure

Since the analysis procedure is basically the same as the method using a Ge detector, follow the operation of a γ-ray spectrometer using a Ge detector. Points to be noted in this analysis are as follows:

(1) Setting of ROI

Set the ROI where a significant count is obtained against a background as the γ-ray peak used for quantification. At this time, if the variation is large because of an insufficient count and the setting the region is difficult, set the ROI after leveling the count of each channel by smoothing process.

(2) Identification of nuclide

Prepare to convert data by an energy calibration curve in order to determine which channel corresponds to the γ-ray of a nuclide to be quantified. Prepare nuclear data books and environmental radiation spectra, and if an unknown peak is detected, investigate the γ-ray energy, identify the nuclide, and examine possibility of interference with the γ-ray used for quantification.

(3) Calculation of peak area

Subtract the count of a background below a peak from the total count of the peak region. If the peak is too multiple to quantify by this method, the peak area is calculated after the peak separation using a peak function fitting method.

(4) Calculation of radioactivity

In the calculation of radioactivity it is necessary to estimate the calculation result of radioactivity by considering natural radioisotopes contained in a sample and a background. In this case, a net count (n) is obtained by subtracting a count in the region corresponding to the γ-ray peak of a nuclide used for quantification.

The radioactivity A (Bq) and the radioactive concentration C (Bq/kg) of a sample are obtained from the count rate (n), which is obtained by dividing the net count by the measurement time, by the following formulae.

$$A = \frac{n}{a\varepsilon f_{SUM}} \quad C = \frac{A}{M}$$

In the above formula, the abbreviations are as follows.

n: Count rate
a: γ-Ray emission rate (Bq^{-1}) shown in Table 2
ε: Peak efficiency
f_{SUM}: Correction factor for summing effect. The summing effect must be corrected for ^{134}Cs, but if correction is not performed, state that.
M: Mass (kg) of a sample in a sample container

(5) Calculation of detection limit value

The detection limit value is calculated on the assumption that the γ-ray of the target nuclide exists in the channel of a background spectrum. The 3 folds value of the count error in background count of the peak region is expressed as the

detection limit. The detection limit of commercially available spectrometers with a shielding body is about 30 Bq/kg for ^{131}I and ^{137}Cs, but widely differs depending on the detector size, shield thickness and sample volume.

The peak detection limit value in a real sample also highly depends on the spectrum of the sample. When other nuclides coexist in the sample, their Compton background may also affect the detection limit value to result in being larger in some cases.

(6) Examination and evaluation of measurement results

Summarize measurement results for each target nuclide, and confirm that it is normal based on the statistical uncertainty of count, peak center channel, FWHM, etc. If there is any doubt in the confirmation of results, remeasure as necessary.

4.2.5. Points to note for measurement

4.2.5.1. Effect of temperature

In the case of a NaI (Tl) spectrometer, variations in detector ambient temperature can cause the peak center channel to fluctuate. In particular, keep the temperature of the measurement room constant because it fluctuate easily during at nighttime and in winter. If a sample is stored at a lower temperature than the temperature of the measurement room, return it to near the temperature of the measurement room before measurement.

4.2.5.2. Control of background

Apply 4.1.5.1.

4.2.5.3. Contamination prevention of apparatus

Cover a detector with polyethylene bag to prevent from contamination. In the event of a contamination, cope by replacing the polyethylene packaging. If the surface of a detector is contaminated, wipe it off with a neutral detergent or gauze soaked in ethanol. Be careful not to allow dust or other contaminations to enter when opening a shield body.

Use a sample container after simple cleaning, if necessary. A polyethylene bag can be used in a container. When a sample solution is placed into a sample container, prevent the contaminant from adhering around the sample container.

5. Report and record

Examples of items to be described are as follows.

① Information concerning apparatus used: Apparatus name (detector size, resolution), number of measurement channels, analysis software type, processing method

② Sample information: Sample name (number), collection site, collection date and time, collection volume, type of collection container, name of person in charge of collection

③ Measurement conditions: Type of sample container, sample amount, geometry

④ Measurement records: Start date and time of measurement, measurement time (Live Time, Real Time)

⑤ Analysis records: Peak center channel, FWHM, peak area and its statistical uncertainty of count, sample count rate and its statistical uncertainty of count, BG count rate and its statistical uncertainty of count, peak efficiency, attenuation correction coefficient, radioactivity and radioactive concentration and their statistical uncertainties of each count, radioactivity of detection limit or radioactive concentration of detection limit, name of person in charge of measurement/analysis.

For analysis records, report prepared by the analysis software can be used as it is to avoid transcription mistakes. For numerical values, the number of significant digits of radioactivity or radioactive concentration is ''reduced'' based on the number of significant digits of the statistical uncertainty of count.

⑥ Measurement result: Name of nuclide, radioactive concentration (Bq/kg), detection limit value

When contracting a measurement work, in principle, write and report the measurement results in a format specified by the measurement work consignor, and store it together with the original data.

6. References

1) Japan Radioisotope Association, Isotope Notebook the 11th edition, Maruzen Publishing, 2011, ISBN 978-4-89073-211-1.

2) Water Supply Division, Health Service Bureau, The Ministry of Health, Labor and Welfare, Office Memorandum ''Sending of Manual for Radioactivity Measurement of Tap Water'', October 12, 2011.

3) Science and Technology Policy Bureau, The Ministry of Education, Culture, Sports, Science and Technology, ''Radioactivity measurement series No. 6, NaI (Tl) Scintillation spectrometry Instrumental Analysis'', January, 1974.

4) Science and Technology Policy Bureau, The Ministry of Education, Culture, Sports, Science and Technology, ''Radioactivity measurement series No. 7, γ-ray Spectrometry by Germanium Semiconductor Detector'' Revised ver.3, August, 1992.

G6 Drug Formulation

Criteria for Content Uniformity in Real Time Release Testing by Process Analytical Technology

<G6-1-171>

1. Introduction

In recent years, the new criteria for Content Uniformity Test using a large sample size for Real Time Release Testing (RTRT) have become necessary with the rapid development of Process Analytical Technology (PAT). PAT using a non-destructive method such as Near Infrared (NIR) spectrometry enable to measure a large number of samples in real time, resulting in the generation of large amounts of data in a short time, and PAT can improve process control and process capability. However, the current pharmacopoeial criteria for Uniformity of Dosage Unit (the sample size is 10 and 30 for first and second stage respectively) may not be used adequately for large sample sizes over a hundred. For example, zero tolerance criteria has been used for outliers (no unit showing over the 25.0% deviation from label claim must be observed in the 30 samples tested). However, the probability of occurrence of outliers cannot be ignored when sample size was well over a hundred. This document display the consideration about criteria applicable for the large sample size over a hundred in RTRT.

2. Theoretical basis of the criteria

The Content Uniformity Test of the Japanese Pharmacopoeia is a kind of sampling tests, using small picked sample(s) from a large population (batch, lot), used for release of products. Therefore, the quality of estimations (test performance) depends on the sample size. In general, estimate the better the larger the sample size, and it is considered that a large sample size makes it possible to determine

the quality of lots certainly. On the other hand, usage of a large sample size causes consumption of resources. For this reason, compendial tests like the tests in the Japanese Pharmacopoeia use a minimum and optimal sample size accompanying with strict criteria in order not to release bad products. Now a day, as a large sample size (Large-N) has become popular with development of PAT, it needs to set the appropriate criteria for RTRT using Large-N.

In setting of a specification limit, the limit value is determined by the balance between a guaranteed quality limit (acceptable limit) and the severity of a realistically capable test. When the specification limit is too strict, the acceptable quality becomes better, however a stock shortage caused by a low rate of passing the test of actual products occurs and the cost may become abnormally high. In order to maintain an acceptable quality, it is the most reasonable to compare the consumer's risk (risk of poor quality passing the test) and the producer's risk (risk of good quality failing the test) and to determine the severity of the most suitable test. Fig. 1 shows the OC (operating characteristic) curve describes the relationship as above.

The consumer's risk level, an acceptable quality corresponding to pass rate of 5% in release tests, is important to guarantee the quality of the product to be released. This means that possibility of releasing low quality products is considered low ($<5\%$). On the other hand, the producer's risk is important for producers. They should consider how good quality is needed to pass (usually 90 − 95%) the test sufficiently. In spite of the sample size, the lot quality corresponding to 50% of pass rate is almost same the quality on the specification limit. If the sample size is increased without change of the specification limit, the OC curves change as in Fig. 2-A.

That is to say, the quality (x-axis showing variability in unit content) of the 50% acceptance level is unchanged in all the OC curves while the slope of the OC curves become steeper with the larger the sample size. In contrast, if the limit value is changed to more strict without changing the sample size, the OC curves shift to the left at a constant slope (Fig. 2-B). To be constant the consumer's risk level regardless of change of sample size, it is necessary to set the limit value in response to changes in sample size as in Fig. 3-A. In general, the large sample size can have the consumer's risk level maintain to be constant even if the limit value becomes loose.

When products is tested by PAT in a large sample size and then released, they will be subjected to stability tests and survey tests using the usual small sample sizes after releasing. In this case, though the consumer's risk as in Fig. 3-A is at a constant, the producer's risk increase. In order not to increase the producer's risk after releasing, it is necessary to set the test limits so as not to differ very much in producer's risk between the test by PAT and the conventional test. In this case, it is necessary to tighten the test limit in larger sample size, as shown in Fig. 3-B.

Our recommended criteria were determined in consideration of such a point as described above[1]. It should be noted that our criteria are simple and non-parametric criteria that do not depend on the type of distribution of unit content, and also has the same attitude with the Alternate 2[2] of the European Pharmacopoeia (EP) being a standard corresponding to the above mentioned Large-N. In the case of using the Alternate 1 of EP, there could be no problem from the point of view about quality assurance.

3. Criteria for Content Uniformity in sample size equal to or more than 100

The criteria recommended are consisted of two tests by attribute (limits are *C1* and *C2*). The sample sizes and acceptance numbers are shown in Table 1.

Criteria

Select *n* units representing a lot submitted, and assay the units individually using an appropriate analytical method and calculate individual contents expressed by the percentage of label claim. The requirements are met if the number of dosage units outside 15.0% is less than or equal to *C1*, and the number of dosage units outside 25.0% is less than or equal to *C2*. The central point of content bias can be alter to an appropriate value from the label claim if it is needed by quality control issue.

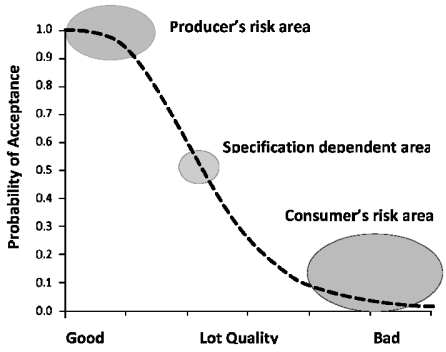
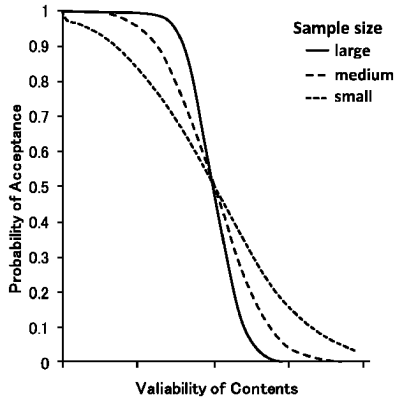


Fig. 1 Consumer's risk and producer's risk areas in an OC curve

A. Effects of sample sizes



B. Effects of specification limits

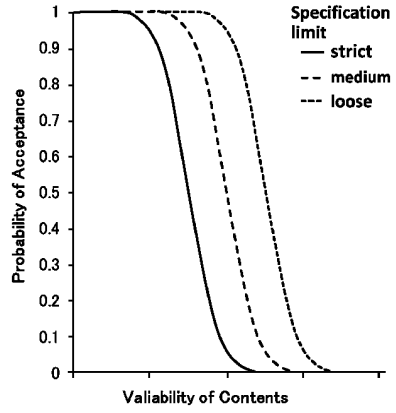
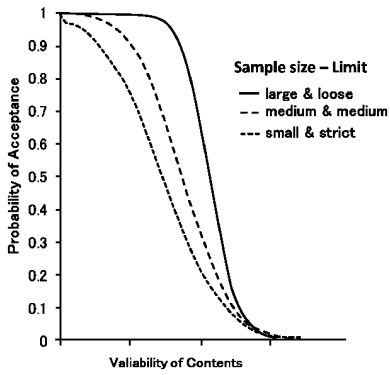


Fig. 2 OC Curves of Content Uniformity Tests — Effects of sample sizes and specification limits

A. Constant risk of consumers



B. Constant risk of producers

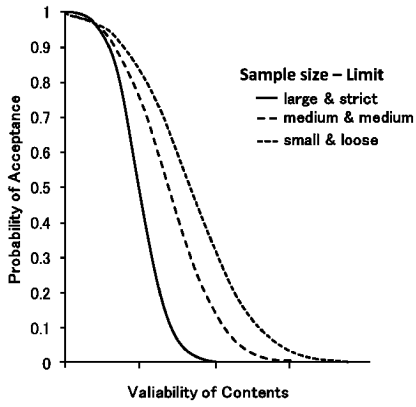


Fig. 3 OC Curves of Content Uniformity Tests — Risks of consumers or producers are constant

4. References

1) N. Katori *et al.*, Sakura Bloom Tablets P2 Mock by MHLW sponsored QbD Drug Product Study Group (Responsible researcher: H. Okuda, Study on Quality Assurance over the Life Cycle of Pharmaceutical Products), (Mar. 2015).
2) European Pharmacopoeia 7.7 (2012), 2.9.47. Demonstration of Uniformity of Dosage Units Using Large Sample Sizes.

Standard Procedure for Mechanical Calibration of Dissolution Apparatus <*G6-2-170*>

This chapter is intended to minimize sources of mechanical variability of apparatus which affect test results and assure the reproducibility of results, for Apparatus 1 (Basket method) and Apparatus 2 (Paddle method), and describes the standard procedure for the mechanical calibration of dissolution apparatus and the recommended specifications. The validity of the mechanical calibration for qualification of dissolution test is confirmed on the meeting of international harmonization of pharmacopeia about dissolution test, and subsequently. the standard practice for qualification of dissolution apparatus[1)] and the guidance of mechanical calibration[2)] were issued in U.S.A.

Requirements for basic quality of materials and sizes, etc., to be required on dissolution test apparatus, and the suitability of the apparatus conform to the statements of Dissolution Test <*6.10*>. The tolerance of some parameters for the mechanical calibration described in this chapter may be specified strictly compared with that specified in Dissolution Test <*6.10*>. The below table shows the comparison of specified values of each calibration parameter. In order to minimize variability of test results by the mechanical calibration, it is recommended to apply this chapter.

Table 1 Criteria for Content Uniformity

Sample size (n)	Acceptance number*	
	*C1*** (± 15.0%)	*C2*** (± 25.0%)
$n < 100$	Criteria of 6.02 Uniformity of Dosage Units	
$100 \leq n < 150$	3	0
$150 \leq n < 200$	4	0
$200 \leq n < 300$	6	1
$300 \leq n < 500$	8	2
$500 \leq n < 1000$	13	4
$1000 \leq n < 2000$	25	8
$2000 \leq n < 5000$	47	18
$5000 \leq n < 10000$	112	47
$10000 \leq n$	217	94

* The requirements are met if the number of outliers is less than or equal to acceptance number.
** Critical acceptance number.

Although qualification of dissolution test results by using Prednisone Tablet RS has been recommended in our country, since it cannot necessarily detect sources of mechanical variability of apparatus, the performance of the mechanical calibration is basically desirable. However the test appropriately performed by using Prednisone Tablet RS is valid for understanding overall factor including degassing state of a test solution and vibration of apparatus undetected by only the calibration of apparatus. Moreover a monitoring by a dissolved oxygen analyzer is valid for confirmation of degassing state of a test solution.

1. Setup of dissolution apparatus and periodic control

Mechanical calibration should be performed on purchase or receipt of dissolution apparatus, after move and after repair which can affect test results, and it is usually desirable to perform the calibration every year. If the instrument is not being used routinely, the mechanical calibration can be performed before performing the first dissolution test after the one year time interval.

2. Procedure of mechanical calibration

2.1. Instruments

Instruments for the mechanical calibration are runout gage, level, centering device and tachometer, etc., as generic ones and it is desirable to use the tools to be traceable to JIS (Japanese Industrial Standards), etc., wherever possible. In addition, special instruments for the mechanical calibration of dissolution apparatus are centering tool, depth gage, plastic ball, etc. Moreover, some dissolution apparatus require the special tools supplied by instrument manufacturers or incorporate automatic mechanical calibration devices within their equipment. These tools and devices may be used provided they follow the general principle of the below procedure.

2.2. Procedure

Perform the mechanical calibration of dissolution apparatus according to the below specified procedure. If each measured value does not meet the specification, repetitive adjustments and measurements may be necessary.

Confirm that the apparatus is horizontal on the installation table in advance. Also confirm the horizontality of the plate which fix the vessel (vessel plate) by placing a bubble level on the stage and confirming the bubble to be within the lines of the level.

2.2.1. Shaft Wobble

A runout gage is placed on top of the vessel plate, and positioned so that the gage probe touches the shaft about 2 cm above the top of the paddle blade or basket. The absolute value of the difference between the maximum and minimum readings is the wobble. The value of total wobble must not exceed 1.0 mm.

2.2.2. Shaft Verticality

Lower the drive unit to where it would be during an actual dissolution test. If necessary, the shaft verticality may be checked by raising the drive unit. Place an accurate bubble level on the front edge of each of the shafts. The bubble should be within the lines of the level. Rotate the level about 90° so that it touches the side of the shaft, and put it on the side of the shaft. The bubble should again be within the lines of the level.

A digital leveling device may also be used to determine the shaft verticality.

The shaft must be not more than 0.5° from vertical.

2.2.3. Basket Wobble

A runout gage is placed on top of the vessel plate and the drive unit is positioned so that the gage probe touches the bottom rim of the basket. The gage is placed so that the probe slightly presses in on the turning shaft. The value of total wobble must not exceed 1.0 mm.

2.2.4. Vessel Centering

Centering inside the vessel is measured by centering tools for dissolution test apparatus or by an alternative procedure.

When measured by centering tools, two centering tools are used to center the paddle or basket shafts in the vessels and to align the vessels so that their sides are vertical.

As an example of the procedure for the paddle method, the bottom of one centering tool is placed 2 mm above the top of the paddle blade and the bottom of the second centering tool is clamped on the shaft 80 mm above the blade with the both probes positioned in the same direction towards the vessel wall. For the basket method, the bottom of one centering tool is placed 2 mm above the top of the basket and the bottom of the second centering tool is placed 60 mm above the top of the basket with the both probes positioned in the same direction towards the vessel wall. Carefully lower the shaft and centering tools into the vessels so that the paddle blade and the bottom of the basket is about 2.5 cm above the bottom of the vessel. Manually rotate the shaft slowly and check the centering at both levels. If the vessel is not centered at either level, adjust the vessel to center it.

An alternative procedure is to use a mechanical or digital centering device that centers the inside wall of the vessel around the shaft or a surrogate shaft. The centering is measured at two positions inside the vessel in the cylindrical portion, one just below the rim of the vessel and one just above the basket or the paddle in the bottom portion of the vessel.

The shaft must be centered within 1.0 mm from the center line.

2.2.5. Vessel Verticality

The vessel verticality can be calculated as the angle of the vertex of the triangle composed of the two points and the vertical line using the two centering measurements in 2.2.4 Vessel Centering and the difference in height between the two measurements. Or it can be determined using a digital leveling device placed on the inside wall of the vessel. The verticality should be determined at two positions 90° apart.

The vessel must be not more than 1.0° from vertical.

After each vessel has been centered and made vertical, each vessel must be positioned in the exact same position and same direction inside the vessel plate opening.

Table Specified values of calibration parameters of Dissolution Test <*6.10*> and this chapter

Calibration Parameter	Dissolution Test <*6.10*>	This chapter
Shaft wobble	rotates smoothly without significant wobble that could affect the results	≦ 1.0 mm total runout
Shaft verticality	—	≦ 0.5° from vertical Bubble should be centered within the lines of the level.
Basket wobble	—	≦ 1.0 mm total runout
Vessel/Shaft centering	≦ 2.0 mm from vertical	≦ 1.0 mm from centerline at upper position and lower position
Vessel verticality	—	≦ 1.0° from vertical
Basket and Paddle depth	25 ± 2 mm	25 ± 2 mm
Rotational speed	± 4% from the specified rate of rotation	± 2% or ± 2 rpm from the specified rate of rotation

2.2.6. Basket and Paddle Depth

The actual distance between the bottom of the vessel and the bottom of the basket or paddle is determined. If the depth of the basket/paddle is adjustable, first a depth gage is used to determine the distance between the bottom of the paddle blade or basket and the inside bottom of the vessel. The depth gage is set at 25 mm and placed on the bottom of the vessel. After each shaft is raised into the apparatus drive module, the drive unit is then lowered to its operating position. The paddle or basket is then lowered into the vessel until it touches the top of the depth gage. Instead of a depth gage, sink a plastic ball with a diameter 25 mm ± 2 mm on the bottom of the vessel and the paddle or basket can be lowered into the vessel until it touches the ball. The shafts are locked into this height. Usually the depth of basket and paddle is 25 mm ± 2 mm.

2.2.7. Rotational Speed

A tachometer is used to measure the rotational speed of the paddle or basket. The shafts should be rotating smoothly with a rate within a larger value of ±2% or ±2 rpm of the specified rate.

3. References

1) ASTM E2503 – 13: 2013, Standard Practice for Qualification of Basket and Paddle Dissolution Apparatus (2013).
2) FDA Guidance for Industry: The Use of Mechanical Calibration of Dissolution Apparatus 1 and 2—Current Good Manufacturing Practice (CGMP), U.S. Depart-

ment of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER), January 2010.

Aerodynamic Particle Size Measurement for Inhalations by Glass Impingers <*G6-3-171*>

This test is used to evaluate the fine particle characteristics of the aerosol clouds generated by preparations for inhalation, and is performed using the following apparatus and test procedures. If justified, modified apparatus or test procedure may be used.

1. Stage mensuration

The most reliable calibration for the separation characteristics of each impaction stage is performed in terms of the relationship between the aerodynamic diameter of particles and droplets passing through it and the stage collection efficiency as an aerosol.

Calibration is usually performed by examination of a property of the jet dimensions, the spatial arrangement of the jet and its collection surface, and the airflow rate passing through it.

Because jets can corrode and wear over time, the critical dimensions of each stage must be measured on a regular basis to confirm them being within required ranges.

Only apparatuses that conform to specifications are used for the aerodynamic particle size measurement for inhalations by glass impingers. An alternate validated and justified method of mensuration may be used.

2. Inter-stage drug losses (wall losses)

Wall losses should be considered in method development and validation. If the wall losses affect the recovery rate (mass balance) of drugs, they should be controlled. Wall losses will be dependent upon a number of factors including the impactor type, operating conditions, formulation type and discharged amount to an impactor. How the wall loss is reflected within the calculation of the aerodynamic diameter of particles should be judged based up on the level and variability of the wall loss. For example, in the cases where wall losses that are low or have a low level of variability, the aerodynamic particle size is calculated by the assay of the drug collected on the stage. In cases where wall losses are high or variable, it may be necessary to collect the wall loss drug separately and take it into account for the calculation of the aerodynamic particle size.

3. Recovery rate of drugs (mass balance)

In addition to the size distribution, good analytical practice dictates that a mass balance be performed in order to confirm that the amount of the drug discharged from the inhaler, which is collected in the mouthpiece adapter and the apparatus, is within an acceptable range around the expected value. The total mass of drug collected in all of the components of the mouthpiece adapter and the apparatus divided by the minimum recommended dose described in the dosage and administration is not less than 75% and not more than 125% of the average delivered dose determined under Uniformity of Delivered Dose for Inhalations <*6.14*>. This mass balance is necessary to ensure that the test results of particle size distributions of inhalations are valid.

4. Glass impinger method

The apparatus used for the glass impinger method is shown in Fig. 1. The apparatus consists of glass parts from the throat (B) to the lower impingement chamber (H) and plastic clips to hold them.

This apparatus is operated based on a collision to a liquid surface and separate the drug discharged from the inhaler to an inhalation part and a non-inhalation part. The drug in the non-inhalation part, which collides with an oral cavity and a pharyngeal region to result in being swallowed, is recovered in the rear of the throat and the upper impingement chamber (collectively stage 1). The drug in the inhalation part, which reaches lungs, is recovered in the lower impingement chamber (stage 2). Because the upper impingement chamber is designed so that the cut-off diameter is 6.4 μm when the test flow rate is 60 L per minute, particles with a diameter of 6.4 μm or less flow down to the lower impingement chamber.

4.1. Procedure for nebulizers

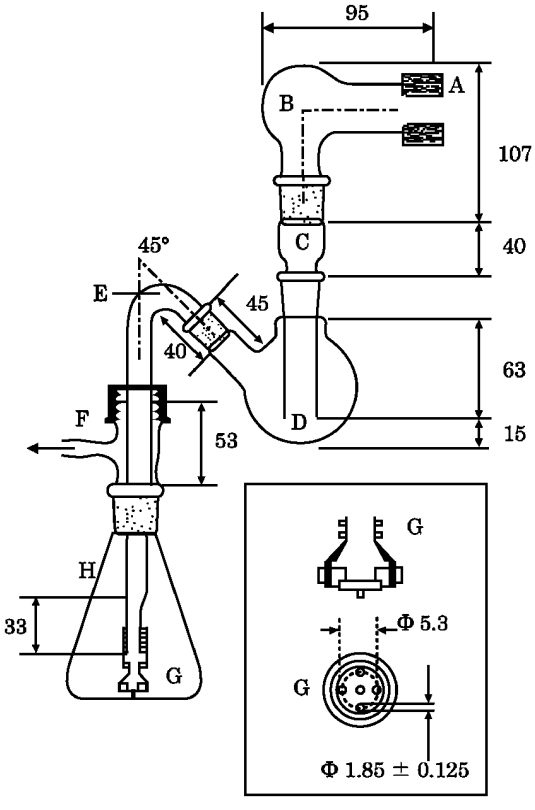
Introduce 7 mL and 30 mL of a suitable solvent into the upper and lower impingement chambers, respectively.

Connect all the component parts. Ensure that the assembly is vertical and adequately supported and that the jet spacer peg of the lower jet assembly just touches the bottom of the lower impingement chamber. Connect a suitable pump fitted with a filter (of suitable pore size) to the outlet of the apparatus. Adjust the air flow through the apparatus, as measured at the inlet to the throat, to 60 ± 5 L per minute.

Introduce the inhalation liquids and solutions into the reservoir of the nebulizer. Fit the mouthpiece and connect it by means of a mouthpiece adapter to the device.

Switch on the pump of the apparatus and after 10 seconds switch on the nebulizer.

After 60 seconds, unless otherwise justified, switch off the nebulizer, wait for 5 seconds and then switch off the pump



Capital letters of alphabet refer to Table 1
The figures are in mm. (Tolerances are ±1 mm unless otherwise stated.)

Fig. 1 Glass impinger

of the apparatus.

Dismantle the apparatus and wash the inner wall surface of the upper impingement chamber collecting the washings in a volumetric flask. Wash the inner wall surface of the lower impingement chamber collecting the washings in a second volumetric flask. Finally, wash the filter preceding the pump and its connections to the lower impingement chamber and combine the washings with those obtained from the lower impingement chamber. Determine the amount of active substance collected in each of the two flasks. Express the results for each of the two parts of the apparatus as a percentage of the total amount of active substance.

4.2. Procedure for metered-dose inhalers

Install a suitable mouthpiece adapter in position at the end of the throat. When the mouthpiece end of the inhaler is inserted in the mouthpiece adapter to a depth of about 10 mm, the mouthpiece end of the inhaler lines up along the horizontal axis of the throat. The open end of the inhaler, which accepts the pressurized container, is uppermost and in the same vertical plane as the rest of the apparatus.

Introduce 7 mL and 30 mL of a suitable solvent into the upper and lower impingement chambers, respectively.

Connect all the component parts. Ensure that the assembly is vertical and adequately supported and that the jet spacer peg of the lower jet assembly just touches the bottom of the lower impingement chamber. Connect a suitable pump to the outlet of the apparatus. Adjust the air flow through the apparatus, as measured at the inlet to the throat, to 60 ± 5 L per minute.

Unless otherwise prescribed in the patient instruction, shake for 5 seconds and discharge once to waste. After not less than 5 seconds, shake and discharge again to waste. Repeat the procedure a further three times.

Shake for about 5 seconds, switch on the pump to the apparatus and locate the mouthpiece end of the inhaler in the adapter, discharge once immediately in the apparatus.

Table 1 Component specification for apparatus shown in Fig. 1

Code	Item	Description	Dimensions*
A	Mouthpiece adapter	Moulded rubber adapter for actuator mouthpiece.	
B	Throat	Modified round-bottomed flask: —ground-glass inlet socket —ground-glass outlet cone	50 mL 29/32 24/29
C	Neck	Modified glass adapter: —ground-glass inlet socket —ground-glass outlet cone Lower outlet section of precision-bore glass tubing: —bore diameter Selected bore light-wall glass tubing: —external diameter	 24/29 24/29 14 17
D	Upper impingement chamber	Modified round-bottomed flask: —ground-glass inlet socket —ground-glass outlet cone	100 mL 24/29 14/23
E	Coupling tube	Medium-wall glass tubing: —ground-glass cone Bent section and upper vertical section: —external diameter Lower vertical section: —external diameter	 14/23 13 8
F	Screwthread, side-arm, adapter	Plastic screw cap Silicone rubber ring Polytetrafluoroethylene (PTFE) washer Glass screwthread: —thread size Side-arm outlet to vacuum pump: —minimum bore diameter	28/13 28/11 28/11 28 5
G	Lower jet assembly	Modified polypropylene filter holder connected to lower vertical section of coupling tube by PTFE tubing Acetal circular disc with the centres of four jets arranged on a projected circle of diameter 5.3 mm with an integral jet spacer peg: —peg diameter —peg protrusion	see Fig. 1 10 2 2
H	Lower impingement chamber	Conical flask —ground-glass inlet socket	250 mL 24/29

* Dimensions in mm, unless otherwise stated.

Remove the assembled inhaler from the adapter, shake for not less than 5 seconds, relocate the mouthpiece end of the inhaler in the adapter and discharge again. Repeat the discharge sequence. The number of discharges should be minimized and typically would not be greater than 10. After the final discharge wait for not less than 5 seconds and then switch off the pump.

Dismantle the apparatus. Wash the inner wall surface of the coupling tube to the lower impingement chamber and its outer wall surface that projects into the chamber with a suitable solvent, collecting the washings in the lower impingement chamber. Determine the content of active substance in this solution. Calculate the amount of active substance collected in the lower impingement chamber per discharge and express the results as a percentage of the active substance stated on the label.

4.3. Procedure for dry powder inhalers

Introduce 7 mL and 30 mL of a suitable solvent into the upper and lower impingement chambers, respectively.

Connect all the component parts. Ensure that the assembly is vertical and adequately supported and that the jet spacer peg of the lower jet assembly just touches the bottom of the lower impingement chamber. Connect a suitable pump to the outlet of the apparatus. Adjust the air flow through the apparatus, as measured at the inlet to the throat, to 60 ± 5 L per minute.

Prepare an inhaler and connect it to the throat using a suitable adapter. Switch on the pump of the apparatus, after 5 seconds switch off the pump of the apparatus, and repeat the discharge sequence. The number of discharges should be minimized and typically would not be greater than 10.

Dismantle the apparatus. Wash the inner wall surface of the coupling tube to the lower impingement chamber and its outer wall surface that projects into the chamber with a suitable solvent, collecting the washings in the lower impingement chamber. Determine the content of active substance in this solution. Calculate the amount of active substance collected in the lower impingement chamber per discharge and express the results as a percentage of the active substance stated on the label.

Tablet Hardness Determinations
<G6-4-180>

This General Information summarizes the principle, type, instrument configuration and points to be considered in the tablet hardness determination. Results, together with other information on physical integrity of tablets such as friability, are used to ensure their mechanical strength against stresses such as impact and pressure they experience from manufacturing process to usage.

The measurement is usually performed by placing a tablet between two platens and moving the one platen at a constant speed to obtain the force (N) just before the tablet is broken. The tablet hardness values reflect either the breaking force with which a compressive load generates breakage in a specific tablet plane, or the crushing strength with which substantial breaking cause the loss of structure, or both, depending on an apparatus used. In some cases, the tablet appearance may be lost after breakage. Criteria of the tablet hardness obtained by this measurement is different from the definition of hardness generally used in the field of materials science etc. (the resistance of a surface against penetration and pushing by a small probe).

Multiple hardness testers with different structure of the tablet-mounted part, the transfer mechanism of the platen, the measurement method of force, etc. are used. Manual or relatively simple-structured hardness testers include Monsanto (Stokes) tablet hardness tester which compresses a tablet with an indenter via a spring gauge and a screw, Pfizer tablet hardness tester which applies pressure to a tablet with a plier, Strong Cobb hardness tester which applies a load to a tablet by compressed air, and Erweka tablet hardness tester which applies a load to a tablet with an indenter using an electrically-powered weight load. Apparatuses which automate the process of hardness determination, correspond to various measurement modes and have a data correction function, etc. are also used.

The measurement of tablet hardness is affected by the shape, size, and orientation of a sample tablet, as well as the structure of an apparatus and the measurement conditions. Therefore, it is important to record the name of apparatus and conditions as well as results. The direct comparison of data requires measurements under the same conditions. The following points should be also considered in the measurement.

(i) Platen: Two platens with smooth area contacting a tablet should be used in parallel. The contact surface should be larger than the area of contact with the tablet. Make sure that tablets should be free of displacement by bending or twisting when the load is applied.

(ii) Loading rate: An apparatus having the mechanism which keeps the constant loading rate of compression force by platens should be used for the measurements. Or, the measurements should be performed by moving the platen at a low rate from a certain point immediately before contact with a tablet using an apparatus which keeps the moving speed of platens constant to suppress the variation of the loading rate. Faster movement of the platen would allow rapid measurement, while possible variation in the loading rate often leads to uncontrollable crushing and the rapid accumulation of compressive loads.

(iii) Measurement unit and calibration of apparatuses: An apparatus calibrated with accuracy of 1N or less should be used for the measurement.

(iv) Change of apparatus: It is desirable to change an apparatus to that having a similar mechanism because the mechanism such as the loading method of compression force and the measuring method force would vary between them. The risk due to the apparatus change should be controlled by considering their measurement mechanisms and by comparing the results obtained by multiple measurement parameters such as the loading rate, and the moving speed of platens, using the sample in a same lot.

(v) Tablet orientation: A round non-scored tablet is usually placed between two platens to allow the compression occurs across the tablet diameter. In the measurement of a scored tablet, the tablet is placed so that the score is perpendicular or parallel to the platens. In the case of tablets with unique or complicated shape, it is desirable to place them in the same orientation that can be easily reproduced. In general, a load is applied either across the diameter or parallel to the longest axis.

(vi) Unit: As a unit of tablet hardness, kgf, kp or Strong Cobb Unit (SCU), etc. as well as N is used.

(vii) Number of samples: In addition to the average of the measured values of tablet hardness, their variation should be also important. Therefore, the number of samples should be statistically appropriate for the purpose of measurement. Usually 6 or more samples, often 10 samples, are measured.

Tablet Friability Test <*G6-5-150*>

This test is harmonized with the European Pharmacopoeia and the U.S. Pharmacopeia.

Information on the harmonization with the European Pharmacopoeia and the U.S. Pharmacopeia is available on the website of the Pharmaceuticals and Medical Devices Agency.

The Tablet Friability Test is a method to determine the friability of compressed uncoated tablets. The test procedure presented in this chapter is generally applicable to most compressed tablets. Measurement of tablets friability supplements other physical strength measurement, such as tablet crushing strength.

Use a drum, with an internal diameter between 283 and 291 mm and a depth between 36 and 40 mm, of transparent synthetic polymer with polished internal surfaces, and subject to minimum static build-up (see figure for a typical apparatus). One side of the drum is removable. The tablets are tumbled at each turn of the drum by a curved projection with an inside radius between 75.5 and 85.5 mm that extends from the middle of the drum to the outer wall. The outer diameter of the central ring is between 24.5 and 25.5 mm. The drum is attached to the horizontal axis of a device that rotates at 25 ± 1 rpm. Thus, at each turn the tablets roll or slide and fall onto the drum wall or onto each other.

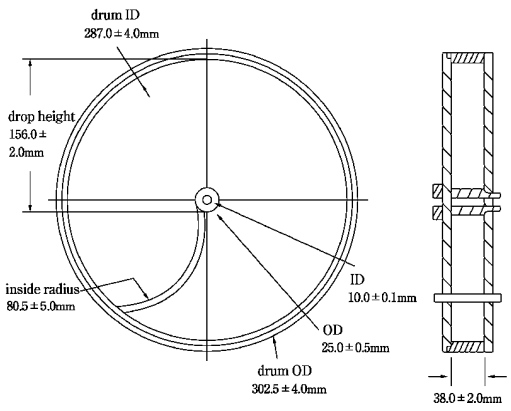
For tablets with a unit mass equal to or less than 650 mg, take a sample of whole tablets *n* corresponding as near as possible to 6.5 g. For tablets with a unit mass of more than 650 mg, take a sample of 10 whole tablets. The tablets should be carefully dedusted prior to testing. Accurately weigh the tablet sample, and place the tablets in the drum. Rotate the drum 100 times, and remove the tablets. Remove any loose dust from the tablets as before, and accurately weigh.

Generally, the test is run once. If obviously cracked, cleaved, or broken tablets are present in the tablet sample after tumbling, the sample fails the test. If the results are difficult to interpret or if the mass loss is greater than the targeted value, the test should be repeated twice and the mean of the three tests determined. A maximum mean mass loss from the three samples of not more than 1.0% is considered acceptable for most products.

If tablet size or shape causes irregular tumbling, adjust the drum base so that the base forms an angle of about 10° with the horizontal and the tablets no longer bind together when lying next to each other, which prevents them from falling freely.

Effervescent tablets and chewable tablets may have different specifications as far as friability is concerned. In the case of hygroscopic tablets, an appropriate humidity-controlled environment is required for testing.

Drums with dual scooping projections, or apparatus with more than one drum, for the running of multiple samples at one time, are also permitted.



pH Test for Gastrointestinal Medicine <*G6-6-131*>

In this test, medicine for the stomach and bowels, which is said to control stomach acid, is stirred in a fixed amount of the 0.1 mol/L hydrochloric acid for a fixed duration, and the pH value of this solution is measured. The pH value of a stomach medicine will be based on the dose and the dosage of the medicine (when the dosage varies, a minimum dosage is used) and expressed in the pH value obtained from the test performed by the following procedure.

1. Preparation of Sample

Solid medicine which conforms to the general regulations for medicine (the powdered medicine section) can be used as a sample. When the medicine is in separate packages, the content of 20 or more packages is accurately weighed to calculate the average mass for one dose and mixed evenly to make a sample. For granules and similar types in separate packages, among the solid medicine which does not conform to the general regulations for medicine (the powdered medicine section), the content of 20 or more packages is accurately weighed to calculate the average mass for one dose and is then powdered to make sample. For granules and similar types not in separate packages, among solid medicine which does not conform to the general regulations for medicine (the powdered medicine section), 20 doses or more are powdered to make a sample. For capsules and tablets, 20 doses or more are weighed accurately to calculate the average mass for one dose or average mass and then powdered to make a sample.

Liquid medicine is generously mixed to make a sample.

2. Procedure

Put 50 mL of the 0.1 mol/L hydrochloric acid with the molarity coefficient adjusted to 1.000, or equivalent 0.1 mol/L hydrochloric acid with its volume accurately measured in a 100-mL beaker. Stir this solution with a magnetic stirrer and a magnetic stirrer rotator (35 mm length, 8 mm diameter) at the speed of about 300 revolutions per minute. While stirring, add the accurately weighed one-dose sample. After 10 minutes, measure the pH value of the solution using the pH Determination. The solution temperature should be maintained at 37 ± 2°C throughout this operation.

Test for Trace Amounts of Aluminum in Total Parenteral Nutrition (TPN) Solutions <*G6-7-160*>

Total parenteral nutrition solutions (TPNs) are nutrient preparations for intravenous injection. Since toxic effects to the central nervous system, bone, etc. due to trace amounts of aluminum have recently been reported in several countries, testing methods for trace amounts of aluminum contaminating TPNs are required for the official standard. The

following three analytical methods are available: High-Performance Liquid Chromatography using a fluorescence photometric detector (HPLC with fluorescence detection), Inductivity Coupled Plasma-Atomic Emission Spectrometry (ICP-AES method), Inductivity Coupled Plasma-Mass Spectrometry (ICP-MS method). Detection sensitivity by HPLC with fluorescence detection is about 1 μg/L (ppb), while ICP-AES fitted with special apparatus and ICP-MS have higher sensitivity.

Since TPNs are nutrient preparations, they contain many nutrients such as sugars, amino acids, electrolytes, etc., in various compositions. Thus, care is needed in the selection of a suitable analytical method, because these coexisting components may affect the measurement of trace amounts of aluminum.

In view of the general availability of HPLC apparatus, the present general information describes procedures for the determination of trace levels of aluminum in TPNs by means of HPLC with a fluorescence photometric detector, using two kinds of fluorescent chelating agents, i.e., Quinolinol complexing method, Lumogallion complexing method.

1. Quinolinol complexing method

After forming a complex of aluminum ion in the sample solution with quinolinol, the assay for aluminum by HPLC fitted with a fluorescence photometer is performed.

1.1. Preparation of sample solution

Pipet 1 mL of the sample (TPNs) exactly, and after adding 10 μL of water for aluminum test, make up the sample solution to 10 mL exactly by adding the mobile phase.

1.2. Preparation of a series of standard solutions for calibration curve

Pipet 1 mL of water for aluminum test exactly, and after adding 10 μL each of standard solutions of aluminum (1)–(5), make up the standard solutions for calibration curve to 10 mL exactly by adding the mobile phase (Aluminum concentration: 0, 1.25, 2.5, 5.0, and 10.0 ppb).

1.3. Standard testing method

Pipet 0.1 mL each of the sample solution and standard solutions, and perform the test by HPLC under the following conditions. Calculate the aluminum cotent in the sample solution using a calibration curve method.

Operating conditions—

Detector: A fluorescence photometer (excitation wavelength: 380 nm, emission wavelength: 520 nm).

Column: A stainless steel column 4.6 mm in inside diameter and 15 cm in length, packed with phenylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 40°C.

Mobile phase: A mixture of 8-quinolinol in acetonitrile (3 in 100) and diluted 0.5 mol/L ammonium acetate TS (2 in 5) (1:1).

Flow rate: Adjust so that the retention time of aluminum/8-quinolinol complex is about 9 minutes.

System suitability—

The correlation coefficient of the calibration curve, which is prepared using a series of standard solutions, is not less than 0.99.

Furthermore there is an alternative method, in which the chelating agent 8-quinolinol is not included in the mobile phase.

In this method also, aluminum is detected as a complex with 8-quinolinol in the sample solution by using HPLC fitted with a fluorescence photometer. But it is necessary to form a more stable aluminum/8-quinolinol complex in the sample solution, because the chelating agent is not included in the mobile phase. Further, since the analytical wavelength for the fluorescence detection is different from that in the standard method, excitation wavelength: 370 nm, emission wavelength: 504 nm, the detection sensitivity is different. Thus, it is appropriate to obtain the calibration curve between 0 – 25 ppb of aluminum. Other than the above- mentioned differences, the size of column, column temperature, and the mobile phase are also different from those used in the standard method, so suitable analytical conditions should be established for performing precise and reproducible examinations of trace amounts of aluminum in the sample specimen.

2. Lumogallion complexing method

After forming a complex of aluminum ion in the sample specimen with the fluorescent reagent of lumogallion, the solution is examined by HPLC fitted with a fluorescence photometer.

2.1. Preparation of sample solution

Pipet 70 μL of the sample specimen (TPN) exactly, add 0.15 mL of lumogallion hydrochloric acid TS and 0.6 mL of buffer solution for aluminum test (pH 7.2) exactly, then mix the solution. After this solution has been allowed to stand for 4 hours at 40°C, it can be used for the measurement as a sample solution.

2.2. Preparation of a series of standard solutions for calibration curve

Pipet 1 mL each of standard aluminum solutions (1) – (5) exactly, and add diluted nitric acid for aluminum test (1 in 100) to make exactly 100 mL. Pipet 70 μL each of these solutions exactly, and add exactly 0.15 mL of lumogallion hydrochloric acid TS and exactly 0.6 mL of buffer solution for aluminum test (pH 7.2) then allow to stand for 4 hours at 40°C to make a series of standard solutions for obtaining the calibration curve (Aluminum: 0, 1.07, 2.13, 4.27, and 8.54 ppb).

2.3. Standard examination method

Take 0.1 mL each of the sample solution and standard solutions for the calibration curve, and perform HPLC analysis under the following conditions. Calculate the aluminum content in the sample solution by using a calibration curve method.

Operating conditions—

Detector: A fluorescence photometer(excitation wavelength 505 nm, emission wavelength 574 nm).

Column: A stainless steel column 6.0 mm in inside diameter and 10 cm in length, packed with octylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 40°C.

Mobile phase: Take 100 mL of 2-propanol, and add a diluted 1 mol/L acetic acid-sodium acetate buffer solution of pH 5.0 (1 in 10) to make 1000 mL.

Flow rate: Adjust so that the retention time of aluminum/lumogallion complex is about 5 minutes.

System suitability—

The correlation coefficient of the calibration curve, which is prepared using a series of standard solutions for calibration curve, is not less than 0.99.

3. Notes

(i) Regarding water, solvents, reagents, vessels and other tools used for the examination, select those not contaminated with aluminum. Further, keep the testing environment clean and free from dust in the testing room.

(ii) Before the measurement, it is necessary to confirm that the characteristic properties of the sample do not affect the formation of the complex.

(iii) Reference substances of river water for analysis of trace elements, commercially distributed by the Japan Society for Analytical Chemistry, can be used to estimate the validity of test methods and results.

4. Standard Solutions, Reagents and Test Solutions

Other than the standard solutions, reagents and test solutions specified in the Japanese Pharmacopoeia, those described below can be used in this test.

(i) ***N*,*N*-Bis(2-hydroxyethyl)-2-aminoethane sulfonic acid** $C_6H_{15}NO_5S$ White crystals or powder.

(ii) **Hydrochloric acid for aluminum test** Same as the reagent *Hydrochloric acid*. Further, it contains not more than 1 ppb of aluminum.

(iii) **Lumogallion** $C_{12}H_9ClN_2O_6S$ Red-brown to dark brown powder. Further, it contains not more than 1 ppm of aluminum.

(iv) **Lumogallion hydrochloric acid TS** Dissolve 0.86 g of lumogallion in 300 mL of 2-propanol, and add 350 mL of diluted *Hydrochloric acid for aluminum test* (9 in 50) and *Water for aluminum test* to make 1000 mL exactly.

(v) **Nitric acid for aluminum test** Same as the reagent *Nitric acid*. Further, it contains not more than 1 ppb of aluminum.

(vi) **pH buffer solution for aluminum test (pH 7.2)** Dissolve 106.6 g of *N*,*N*-bis(2-hydroxyethyl)-2-aminoethane sulfonic acid in 800 mL of *Water for aluminum test*, adjust the pH 7.2 by using *Tetramethylammonium hydroxide aqueous solution*, and add *Water for aluminum test* to make 1000 mL.

(vii) **Standard aluminum solution** Pipet a constant volume each of *Water for aluminum test* or the *Standard aluminum stock solution*, dilute and adjust the aluminum concentration to 0, 1.25, 2.5, 5.0, and 10 ppm by using diluted *Nitric acid for aluminum test* (1 in 100), to make Standard aluminum solutions (1) – (5).

(viii) **Tetramethylammonium hydroxide TS** $[(CH_3)_4NOH]$ It is a 25% aqueous solution, prepared for aluminum test. Further, it contains not more than 1 ppb of aluminum.

(ix) **Water for aluminum test** It contains not more than 1 ppb of aluminum.

G7 Containers and Package

Glass Containers for Pharmaceutical Products <*G7-1-171*>

Glass containers for pharmaceutical products are widely used. Glass bottles are used for tight and well-closed containers for bulk packaging of solid preparations for oral administration such as tablets and capsules etc., and ampules, vials or glass syringes are for hermetic containers of injections etc.

Glass containers used as a primary packaging have characteristics of high chemical durability etc. in addition to high strength, high transparency, no air permeability and no moisture permeability. On the other hand, they are heavy, bulky, fragile and easy to be broken by a physical shock during manufacturing or transportation, so they require attention on handling.

This chapter provides basic information about glass containers, items to be confirmed for the selection of glass containers and for the proper performance of a quality evaluation that comes along with the selection, and information about the quality control at the manufacturing stage of preparations.

1. Basic information about glass containers for pharmaceutical products

Glass containers for pharmaceutical products do not interact physically or chemically with the contained medicaments to alter any property or quality. Glass containers for injections can protect the contained medicaments from the invasion of microbes by means of perfect sealing or other suitable process.

To ensure the quality of contained medicaments over the shelf life, it is necessary to select a suitable glass container. In the selection of container, it is necessary to consider the physicochemical condition of the contained medicaments, i.e., solid or liquid and the adoption of a well-closed container, a tight container, a hermetic container or a colored container to ensure the chemical stability of the contained medicaments. Furthermore, it is necessary to consider surface treatment on the inner surface of containers in the case where it is assumed that foreign substances occur by interactions with the preparation ingredients.

1.1 Glass composition and molding

Composition of the glass used for primary packaging of pharmaceutical products is either borosilicate glass or sodalime glass.

Borosilicate glass has a reticulated network made of silicon dioxide (silica: SiO_2) and diboron trioxide (B_2O_3). Borosilicate glass has a small coefficient of thermal expansion, relatively high hardness and high hydrolytic resistance[1]. Containers made of this chemical composition are classified as Type I glass in the USP and the EP.

Cylinder-shaped and long material glass tubes made of borosilicate glass are cut and undergo secondary processing to mold ampules, vials or syringes, which are mostly used for containers of small amount of injections or lyophilized preparations.

Sodalime glass is composed of silicon dioxide (silica: SiO_2), sodium oxide (Na_2O) and calcium oxide (CaO) as the principal components. It has low water resistance as a drawback, but it is easy to manufacture and process[1]. Containers made of this chemical composition are classified as Type II or III glass in the USP and the EP.

A glass container made of sodalime glass is called a blown bottle or a molded bottle because it is molded by pouring melted glass into a mold and blowing air. Also it is called a standard bottle or an automatic bottle because of its mass production at low cost. It is widely used not only for glass bottles of solid preparations for oral administration but also as containers for injections such as large volume vials of parenteral infusions or vials of powder injections for antibiotics etc.

1.2 Surface treatment of inner surface of glass containers for pharmaceutical products

Surface treatments are performed to modify the nature of the inner surface of glass containers. The treatments are such as dealkalization treatment and coating, etc.

The dealkalization treatment is a method to neutralize the surface layer of the glass by selectively extracting and removing alkali components using sulfur compounds at high temperature above the glass-transition, which results in exposure of the silica-rich surface. This treatment reduce the elution of alkali components. The coating includes methods using silica (SiO_2), silicon resin and fluorine resin, etc.

Silica processing is a method to form a thin film on an inner surface by melt coating of silica (SiO_2) on the inner surface of the glass at a high temperature. It is expected to suppress the elution of glass components and the occurrence of flakes, because the thin film is high purity silica with no water-soluble component such as alkali, weld to the inner surface of the glass container and the drug solution does not contact directly with the inner surface of the glass.

Silicone processing is a method to form a thin film of silicone resin on a glass surface by immersing the glass in dimethylpolysiloxane solution and baking. This treatment enhance water repellency and prevent a drug solution from remaining to the inner surface of the glass. Also it is expected to suppress the elution of glass components and the occurrence of flakes because the drug solution does not contact directly with the inner surface of the glass.

Fluorine resin processing is a method to form a thin film of fluorine resin on an inner surface by coating fluorine resin using coupling agents and baking. This treatment enhance water repellency and prevent a drug solution from remaining to the inner surface of the glass. Also it is expected to suppress the elution of glass components and the occurrence of flakes because the drug solution does not contact directly with the inner surface of the glass.

2. Quality evaluation of glass containers for pharmaceutical products at the design stage of preparations

At the design stage of preparations it is necessary to perform the quality evaluation of a glass container used and the compatibility of it with the contained medicaments.

Since each glass container for pharmaceutical products has characteristic properties and properties of pharmaceutical products packed in the glass containers are diverse, the compatibility of glass containers with pharmaceutical products should be judged by considering the combination of the both.

When evaluated, refer to General Rules for Preparations [2] General Notices for Packaging of Preparations, ''Basic Requirements and Terms for the Packaging of Pharmaceutical Products <*G0-5-170*>'' and ''Basic Requirements for Plastic Containers for Pharmaceutical Use and Rubber Closures for Containers for Aqueous Infusions <*G7-2-162*>'' under General Information, and verify that the glass container used for preparations conform to the basic requirements, i.e., the design specifications, based on tests and literatures.[2,3] The compatibility must be maintained based on an appropriate quality assurance plan.

2.1 Glass containers for pharmaceutical products equipped with closures

In the case of solid preparations for oral administration, glass containers with closures consist of a glass bottle and a resin cap with a packing or a metal cap with a compound, and in the case of lyophilized injections they consist of a vial and a rubber closure. In the case of syringe preparations they consist of a glass outer (barrel, some has a needle), a gasket and a top cap.

In the case of pharmaceutical products susceptible to be oxidized, it is unsuitable to select the closure material that permeate oxygen easily. In the case of aqueous pharmaceutical products and hygroscopic pharmaceutical products, it is unsuitable to select the closure material that permeate water vapor easily. Closures must not be deformed, deteriorated and degenerated by contained medicaments. Unacceptable loss of function of containers must not be caused by a possible high temperature or low temperature or their repetitions during storage and transportation and vibrations during transportation. Glass containers for multiple-dose pharmaceutical products equipped with closures are required to have an appropriate stability after opening.

The compatibility (fitting compatibility) of closures with glass containers for pharmaceutical products must be maintained based on an appropriate quality assurance plan.

2.2 Transparency of glass containers for pharmaceutical products and colored glass containers

In the case of pharmaceutical products such as injections where foreign matters and turbidity must be examined visually, glass containers for pharmaceutical products should have the required level of transparency that enables inspection.

On the other hand, the quality of contained medicaments unstable to light must not be lowered during storage because of a high transparency of glass containers for pharmaceutical products. A sufficient level of light shielding is required to ensure light stability, and the select of colored glass containers must be considered.

When colored glass containers are used for injections, they must meet the requirements of the light transmission test for light-resistant containers under Test for Glass Containers for Injections <*7.01*>.

2.3 Glass containers for pharmaceutical products required to be sterile

In selecting suitable glass containers (ampules) or glass containers with closures (vials, syringes) as a primary packaging for injections, it is desirable to obtain information on the manufacturing processes of the glass container including substances added.

For pharmaceutical products that require terminal sterilization, it is required for glass containers to satisfy the basic requirements even after the sterilization. There must be no residue or generation of new toxic substances of more than a certain quantity after the sterilization. Structures and materials of glass containers must cause no microbial contamination to contained medicaments during storage and transportation after the sterilization.

2.4 Foreign matters derived from glass containers for pharmaceutical products for injections

In the case of glass containers for injections, glass fragments generated at cutting ampules, flakes generated by peeling of inner surfaces of glass and insoluble foreign matters generated by elution of glass components or by stains on inner surfaces of glass should be examined.

Eluates and flakes etc. derived from glass containers must be sufficiently small from the viewpoint of safety. They must not damage the efficacy and safety of the contained medicaments.

Foreign matters derived from glass containers must be sufficiently evaluated at the design stage of preparations. It must be also evaluated, when the molding process or supplier is changed.

Scanning Electron Microscopy–Energy Dispersive X-ray Spectroscopy (SEM-EDX) is useful to analyze flakes derived from glass containers and inorganic foreign matters, for example aerosol of reaction products etc.

3. Test results to be recorded for each management unit

At the manufacturing stage of glass containers for pharmaceutical products the specification of the following test items should be set, and the test results should be recorded for each management unit of glass containers for pharmaceutical products.

1) Glass bottles used for solid preparations for oral administration etc.:
   (i) Appearance[4]: Shape and dimensions are correct, and there must not be failures of wall thickness,

failures of color tone, breakage, lacks, cracks, internal cracks, scratches, bubbles, foreign matters, striae, streaks, rough surfaces, burrs, stains and insoluble matters, which cause a hindrance in usage.

(ii) Quality tests: Soluble alkali test for a container, heat resistance and distortion.

(iii) Others: Items to be necessary.

2) Ampules or vials used for injections etc.:

(i) Appearance[4]: Shape and dimensions are correct, and there must not be failures of wall thickness, failures of color tone, breakage, lacks, cracks, internal cracks, scratches, foreign matters, striae, streaks, stains and insoluble matters, which cause a hindrance in usage.

(ii) Quality tests: Tests prescribed under Test for Glass Containers for Injections <*7.01*>, heat resistance (only for sodalime glass) and distortion.

(iii) Others: Items to be necessary.

4. References

1) Glossary of terms relating to fine ceramics, JIS R 1600: 2011, Japanese Industrial Standards.
2) US Pharmacopeia 40 (2017), <660> Containers-Glass.
3) US Pharmacopeia 40 (2017), <1660> Evaluation of the Inner Surface Durability of Glass Containers.
4) Glass bottles for drug, JIS R 3522: 1955, Japanese Industrial Standards.

Basic Requirements for Plastic Containers for Pharmaceutical Use and Rubber Closures for Containers for Aqueous Infusions <*G7-2-162*>

In this chapter, there describe basic requirements for plastic containers for pharmaceutical use and rubber closures for containers for aqueous infusions, and the methods to evaluate the toxicity of containers at design stage.

Containers for pharmaceutical use should not have the properties to deteriorate the efficacy, safety or stability of the pharmaceutical products to be packed in the container.

The compatibility of plastic containers with pharmaceutical products should be judged for combination of each material and the specific pharmaceutical product to be contained therein. Such judgment should be performed through verification that the container for the pharmaceutical preparation can comply with the essential requirements for the container, i.e., the design specifications, based on the data from the experiments on the prototype products of the container and/or information from scientific documentation, etc. In addition, such compatibility must be ensured based upon an appropriate quality assurance system.

1. Basic Requirements in Designing Containers for Pharmaceutical Use

Containers must not deteriorate the quality of the pharmaceutical products during storage. The concentration of the pharmaceuticals must not be decreased by more than a certain level due to the adsorption of the pharmaceuticals on the surface of the container or the migration of the pharmaceuticals into the inside of the material of the container. Also, the pharmaceutical products contained therein must not be degraded by an interaction with the material of the container.

The container should not be deformed, should not deteriorate and should not be degraded by the pharmaceutical products contained therein. Unacceptable loss of function of the container should not result from a possible high temperature or low temperature or their repetitions encountered during storage or transportation.

The leachable or migrants from the container should not deteriorate the efficacy or stability of the pharmaceutical products contained therein. Furthermore, the amounts of leachable or migratable chemical substances, such as monomers and additives, from the containers to the pharmaceutical products contained therein must be sufficiently small from the viewpoint of safety.

In the case of pharmaceutical products which must be sterilized, it is required to satisfy the above-mentioned essential requirements of the container after the sterilization, if there is a possibility that the quality of the container may change after the sterilization. There should not be any residue or generation of new toxic substances of more than certain risk level after the sterilization. In addition, the container should not have any inappropriate structure and/or material that might result in any bacterial contamination of the pharmaceutical products contained therein during storage and transportation after sterilization.

1.1. Plastic containers for pharmaceutical use

The plastic material used for the container should be of high quality. Therefore, recycled plastic materials, which are of unknown constitution, must not be used.

In the case of pharmaceutical products which are unstable to light, the container should provide a sufficient level of light shielding. In the case of pharmaceutical products which are easily oxidized, the container material should not allow the permeation of oxygen. In the case of aqueous pharmaceutical products and pharmaceutical products that must be kept dry, the container material should not allow the permeation of water vapor. In addition, it is necessary to pay attention to the permeability of solvents other than water through the container.

The container should have a certain level of physical properties such as hardness, flexibility, shock resistance, tensile strength, tear strength, bending strength, heat resistance and the like, in accordance with its intended usage. The container should be of a required level of transparency, when it is necessary to examine foreign insoluble matter and/or turbidity of the pharmaceutical products by visual observation.

Furthermore, in introducing a plastic container, it is desirable that proper disposal method after use is taken into consideration.

1.2. Rubber closures for containers for aqueous infusions

For the rubber closures for container, natural rubber, which has the possibility to cause an allergic response, or recycled rubber material that can not be guaranteed its material composition, should not be used. As the closure systems, the appropriate materials should be used to prevent the permeation of oxygen, water vapor and solvents.

Further, the rubber closure should have a certain level of physical properties such as air tightness, hermetic seal, penetrability of a needle, coring-resistance and self-sealing after penetration, in accordance with its intended usage.

2. Toxicity Evaluation of Container at Design Stage

For design verification, the toxicity of the container should be evaluated. For the toxicity evaluation, it is desirable to select appropriate test methods and acceptance criteria for the evaluation, and to explain the rationale for the selection clearly. The tests should be conducted using samples of the whole or a part of the prototype products of the container. If the container consists of plural parts of different materials, each part should be tested separately. Such

materials as laminates, composites, and the like are regarded as a single material. To test containers made of such materials, it is recommended to expose the inner surface of the container, which is in contact with the pharmaceutical products contained therein, to the extraction media used in the tests as far as possible.

It is recommended to select the test items and the test methods for the evaluation of the toxicity of the containers, depending on their application site, in accordance with the standard test methods on medical devices and materials published in Japan, a notice entitled Basic Principle of Biological Safety Evaluation Required for Application for Approval to Market Medical Devices (MHLW Notification by Director, OMDE Yakusyokuki 0301 No.20 on March 1, 2012).

3. Test Results to be recorded per Production Unit for Plastic containers for pharmaceutical use and Rubber closures for containers for aqueous Infusions

3.1. Plastic containers for pharmaceutical use

At the commercial production phase, it is required to establish acceptance criteria on at least the test items listed below and to record the test results of each production unit of plastic containers for pharmaceutical products. In addition, it is desirable to explain the rationale for setting the acceptance criteria clearly. However, these requirements should not be applied to orally administered preparations except for liquid preparations.

(i) Combustion Tests: Residue on ignition, heavy metals. If necessary, the amounts of specified metals (lead, cadmium, etc.)

(ii) Extraction Tests: pH, UV spectrum, potassium permanganate-reducing substances, foaming test, residue on evaporation

(iii) Cytotoxicity Test

(iv) Any other tests necessary for the specific container for aqueous infusions.

3.2. Rubber closures for containers for aqueous infusions

At the commercial production phase of rubber closures, it is required to establish acceptance criteria on the test items that should be controlled other than those specified in the general chapter of <*7.03*> Test for Rubber Closure for Aqueous Infusions. And the test results of each production unit of rubber closures for containers for aqueous infusions should be recorded. In addition, it is desirable to explain the rationale for setting the acceptance criteria.

Moisture Permeability Test for Blister Packaging of Solid Preparations <*G7-3-171*>

The test is the method to measure the moisture transmission rate of the blister packaging represented by PTP packaging. It can be used for the following studies to evaluate moisture transmission through packaging of drug preparation.

(i) Screening of the material and/or thickness for plastic sheets, forming conditions and/or size of pockets, etc. in the development phase.

(ii) Comparison of the moisture transmission rate of a plastic sheet before and after the change in material, thickness, forming conditions, and/or size of pockets, etc. in the development or production phase.

Note that when a sufficient amount of desiccant cannot be filled up in the pockets due to the minute pockets a reliable result might be not obtained. The test is intended to determine the moisture transmission rate of successfully prepared blister packaging, but not to detect the leakage due to pinholes and the like.

1. Terms

(i) Molding materials: Materials forming pockets and sealing areas. Usually, a single or double layer plastic sheet or that laminated with aluminum foil is used.

(ii) Sealing materials: Materials to seal tightly pockets packed with drug preparations. Usually, an aluminum foil is used.

(iii) Pockets: Places where the molding material is inflated in a convex shape to put drug preparations.

(iv) Moisture transmission rate: An amount of water transmitted into the pockets of blister packaging per unit time (mg/day/pocket).

2. Apparatus

(i) Constant temperature and humidity chamber: An apparatus which can maintain a temperature and humidity storage condition.

(ii) Balance: A chemical balance.

3. Desiccants

It may be chosen from the following.

(i) Calcium chloride for water determination

Pretreatment before use: Put the desiccant taking out of fine powder in a depthless vessel, dry at 110°C for 1 hour, then allow to cool in a desiccator [phosphorus (V) oxide].

(ii) Synthetic zeolite for drying

Moisture adsorption ability: Not less than 15%. Weigh accurately about 10 g of the desiccant, allow to stand at 40°C and 75% relative humidity for 24 hours, then weigh the mass, and calculate the gain in weight.

Pretreatment before use: Put the desiccant in a depthless vessel, dry at 350 − 600°C for 2 hours, then allow to cool in a desiccator [phosphorus (V) oxide].

4. Samples

4.1. Preparation of sample

Amount of the desiccant filled in a pocket is determined appropriately depending on the form or size of the pocket, however, it should be about 80% of the capacity of the pocket for avoiding the deforming or impairing of the covering material. For preparation of the sample, carefully prepare the sample avoiding moisture adsorption of the desiccant. Fill the desiccant in all the pockets as evenly as possible, seal with a sealing material, and cut out to a suitable size. Separately, prepare a control in the same manner by packing with the similar mass of glass beads. The form and size of the sample and the control should be as identical as possible.

Examine the appearances of the prepared sample and control with the naked eye or by using a stereomicroscope, and use them whose pockets maintain their shapes as prescribed, and without any forming faults, aberrant wrinkles on the sealing material, pinholes or any sealing faults.

4.2. Number of samples

Five to ten sheets are used for the sample with not less than 10 pockets per sheet. An appropriate number of sheets equivalent to 20 to 100 pockets (not less than 10 sheets), depending on the number of pockets per sheet are used for the sample with less than 10 pockets per sheet. The number of the control is at least 2 sheets, however, desirable to be the same as the sample number.

5. Method

5.1. Storage conditions

The following conditions are desirable, though other conditions may be used.

(i) 25 ± 2°C/60 ± 5%RH

(ii) 40 ± 2°C/75 ± 5%RH

5.2. Storage

Place the samples and controls in a constant temperature and humidity chamber without overlapping each other of the sheets, not in standing position, as the pocket facing upwards, as not intercepting the air circulation and avoiding exposure to the air flow from the outlet.

5.3. Mass measuring

Take out the samples and controls from the chamber, allow cooling to room temperature, measure the mass of each sheet quickly, and place them back to the chamber. Weigh exactly the masses of them to a degree of 0.1 mg.

5.4. Measuring intervals

Intervals of the measurement are adjusted depending on the moisture transmission rate and avoiding large change in the temperature and humidity inside of the chamber (for example, 0, 1, 3, 7, 14, 21 and 28 days).

5.5. Termination of measurements

Measure the mass of each sheet of the sample and control at each measuring point, and calculate the differences in their average values (the increasing amount of the sample mass). Prepare a linear regression equation by the least-squares method by plotting the increasing amount (mg) of the sample mass on the vertical axis against the time (day) on the horizontal axis. The measurement should be finished when the increase in the mass shows linearity in at least three sequenced points (expect for the starting point) and before the desiccant absorbs moisture of 10% amount of the mass of packed desiccant. The correlation coefficient of the linearity is desirable to be not less than 0.98.

5.6. Others

Data of samples showing extremely larger mass increase as compared to the others should be excluded since the package may have some leakage due to pinholes or the like. Appropriate statistical tests are performed, as needed.

6. Calculation of moisture transmission rate

The moisture transmission rate (mg/day/pocket) is calculated by dividing the slope, i.e. the mass increasing amount (mg/day), obtained by the least-squares method, by the number of pocket per sheet. Record the moisture transmission rate together with the storage conditions and the name of the desiccant used.

7. Information

7.1. Factors affecting the moisture transmission rate

There are as follows:

(i) Qualities (molecular structure, density, degree of crystallinity, etc.), composition and/or thickness of the molding materials

(ii) Methods and conditions to form the pocket

(iii) Size and/ or uniformity of wall thickness of the pocket

(iv) Storage conditions, water activity inside the pocket

7.2. Measurement of pocket wall thickness

Measure the wall thickness of at least one position of upper or side face or R part of not less than 10 pockets of the sampling sheet to a unit of 1 μm, using a micrometer or dial gage with an accuracy of better than 1 μm or an equivalent measuring instrument, as necessary. Measuring position is selected in consideration of the shape of the pocket or difficulty of the measurement. It is desirable to identify the site that may become thinner in the phase of study for pocket forming conditions, and to measure the thickness of the site while paying attention to the pressure.

8. Reference

1) T. Okubo, *et al.*: *PMDRS*, 45(2), 155 – 165, (2014).

Packaging Integrity Evaluation of Sterile Products <*G7-4-180*>

1. Introduction

Package integrity for sterile pharmaceutical products is ability to prevent microbial ingress and the entry or escape of substances, which is required for the packaging for sterile preparations to maintain their quality.

This General Information is used to evaluate the integrity of the primary packaging or the secondary packaging of sterile pharmaceutical products that are required to have a barrier function from microorganisms, reactive gases and other substances that affect quality, from the viewpoint of protection of products. A packaging defect is defined as the situation that unexpected leakage caused by incorrect design or by some abnormality occurred during manufacturing processes or storage up to the shelf life of preparations induces the loss of the intended barrier function of the packaging, resulting in the unsustainability of the quality of the preparation including sterility.

The package integrity testing is applied throughout the product life cycle from the development of preparations and in the product stability programs after the launch.

2. Package integrity and testing

2.1. Concept of package integrity

Package integrity for sterile pharmaceutical products is necessary to ensure the quality of products until use. Primary packaging for sterile pharmaceutical products should ensure no ingress of microorganisms from the outside. In addition, if a gas such as water vapor or oxygen affects the quality of products by its moving between in and out of the primary packaging, the quality should be maintained by controlling the amount of gas transfer in the primary packaging, or combining multiple packaging materials including secondary packaging.

It is necessary to recognize that most packaging have gas leakage and permeation depending on their type. In many cases, it is difficult to distinguish between leakage and permeation for qualified packaging. Therefore, complete packaging is to prevent the ingress of microorganisms and to prevent the quality deterioration of the product due to the ingress/transfer of gas/other substances by conforming to the maximum allowable leakage limit of individual preparation packaging, and the product should be ensured to meet physicochemical and microbiological specifications by data. The package integrity test methods include a physicochemical method to find leaks (leak test), a method to ensure that no leakage occurs by confirming the qualification of the sealed part of a package (seal quality test), and a method to confirm a barrier property against microorganisms by microbiological methods (microbial challenge test). The, package integrity of sterile pharmaceutical products is guaranteed by any one or more of these tests.

The application of the quantitative leak test requires optimization according to the characteristics of the package of each preparation. In addition, it is required to understand the detection limit, accuracy and precision of the test method to be set.

2.1.1. Leak test

The leak test guarantees ability to maintain the integrity of packaging by qualitatively detecting or quantitatively measuring holes or pathways, where leaks occur, by a physicochemical method. There are two types of leak tests; qualitative leak test and quantitative leak test.

Since the results of the qualitative leak test are accompanied by uncertainty, the test requires a large sample size and rigorous control of test conditions to obtain reliable results. The qualitative leak test is a useful mean to detect leaks, but is not suitable for the deterministic verification of package integrity. On the other hand, it is an effective test to locate leak positions correctly.

The quantitative leak test is a test to evaluate quantitatively the physicochemical changes accompanied with leaks and to obtain objective data to set a maximum allowable leakage limit and control.

Examples of qualitative and quantitative leak test methods are listed below. Other methods may be used according to a purpose.

<Qualitative leak test methods>
Liquid immersion test
Liquid leak test
Tracer liquid test (dye penetration test)
Sniffer method (helium leak test method 1)
<Quantitative leak test methods>
Sealed chamber method (pressure change leak test method 1)
Vacuum decay method (pressure change leak test method 2)
Pressure integration method (helium leak test method 2)
Vacuum chamber method (helium leak test method 3)
Immersion method (helium leak test method 4)
High-voltage leak test (pinhole test method)
Laser-based gas headspace analysis

2.1.2. Seal quality test

The seal quality test is used to indirectly ensure ability to maintain package integrity by confirming that parameters related to the container seal or fitting are valid. Conducting the seal quality test set based on evidence is useful for the continuous understanding of the characteristics required for closure and maintaining package integrity. In addition to examples shown as the seal quality test methods (Table 1), various methods are used.

2.1.3. Microbial challenge test

The microbial challenge test is a microbiological test to estimate qualitatively package integrity by using live microorganisms or microbial spores. The microbial challenge test is useful for acquiring the direct evidence of preventing microbial ingress. Microbial ingress evaluated in the test includes the passage through pathways by microbial growth or movement and the passive transport of microorganisms via liquid.

Conducting the microbiological test is effective when appropriate physicochemical leak test methods, which obtain the evidence of preventing microbial ingress, have not been established, or when the maximum allowable leakage limit depends on the possibility of microbial ingress.

The recommended general practices are as follows. For the test, strains of microorganisms that are appropriately maintained should be used. Other scientifically appropriate methods can also be used.

Put a fluid medium aseptically in the primary package of a preparation to be tested, and immerse the preparation in an appropriate bacterial solution of 10^5 CFU/ml or more for at least 30 minutes or more. Cultivate the preparation and confirm the presence or absence of turbidity in the medium.

2.2. Package integrity and testing in the development and manufacturing of preparations

Selection of test methods according to the stage of the product lifecycle is important to ensure package integrity for sterile pharmaceutical products.

2.2.1. Design of packaging

In the packaging design of the product development stage, the maximum allowable leakage limit is required to be set based on evaluations of not only the risk of sterility failure due to microbial ingress but also the effect of various gases passing through the primary packaging on the quality. For the evaluation, it is desirable to use the quantitative leak test method that has been verified to be able to detect leaks that affect product quality. Samples used for the evaluation should be prepared assuming the worst case in design.

If influence of other than microorganisms can be ignored, the allowable leak limit to be controlled is set by considering the risk of microbial ingress. This can be set by verifying by the microbial challenge test, or by proving that there is logically no ingress of microorganisms by leak tests. On the other hand, the allowable leakage limit should be set to control the passage of substances in addition to preventing microbial ingress for products that require to keep low headspace oxygen concentration to maintain the quality of preparations etc. Verification only by the qualitative microbial challenge test should not be sufficient. Other qualitative tests are also valuable to obtain information appropriate for the purposes.

2.2.2. Manufacturing of preparations

Package integrity testing in the manufacturing of content-filled products is important to prevent the release of incompletely packaged pharmaceuticals. Based on packaging defects recognized in the development stage and initial process validation, tests are established by leak tests, seal quality tests and appropriate combination of visual inspections during manufacturing to obtain supplemental information.

Examples of leak tests used for package integrity evaluation of preparations in manufacturing processes include liquid immersion test, liquid leak test, tracer liquid test (dye ingress method), sealed chamber method (pressure change leak test method 1), vacuum decay method (pressure change leak test method 2), high voltage leak test (pinhole test method), and laser-based gas headspace analysis. Moreover, examples of the seal quality test methods are shown in Table 1.

Sampling tests using a part of a production lot as a sample during the process provide means to verify package integrity of the lot by ensuring that the process is maintained in a validated state. For sampling frequency and the number of samples, it is required to set the necessary number of samples and demonstrate its validity based on the results of statistical process controls obtained in the process validation stage and the trend analysis of product quality after the start of production. In contrast, non-destructive leak tests for all products of a production lot provide continuous and all-product guarantees of package integrity.

If relevance between seal quality test results and package integrity is verified in advance, the conduction of the seal quality test can indirectly ensure the package integrity. For the glass or plastic ampoules that are sealed by sealing or welding the opening, nondestructive leak tests are usually performed with all samples.

The main purpose of package integrity testing in process validation is to obtain high quality product packaging in the process which is operated with no problem within operating parameters set and to keep the incidence of serious packaging defects low enough. Package integrity testing of in-process and final products complements complete packaging de-

Table 1 Examples of seal quality test methods

Name of seal quality test method	Packaging applied	Contents of test
Tensile strength test	Bag, blister pack, etc.	Measure force required to separate two bonded surfaces.
Closure (opening and closing) torque test	Packaging closed by screws	Measure torque required for opening or closing a plug.
Package burst test	Bag, blister pack, etc.	Apply pressure to a package seal to rupture and open, and measure the pressure or force at the rupture.
Residual seal force test	Vial, etc.	Push a cap downward at a constant speed from the top of a vial, and measure the repulsive force when the plot of the transfer distance–repulsive force reaches the inflection point. Non-destructive testing is possible.
Rubber closure depression test	Vial, etc.	Push a rubber closure downward at a constant rate from the top of a vial, and measure intensity to the depression.
Rotation resistance test of winding cap	Vial, etc.	Measure an initial resistance value when idling a cap. Similar to the residual seal strength test, it is possible to estimate the seal property due to the elastic force of rubber closures.
Airborne ultrasound method	Packaging joined by welding/crimping	Pass an ultrasonic signal through the seal area of a package or an article, and inspect the seal quality by measuring the signal strength. The ultrasonic energy of an area with a bad seal decreases compared with a suitable package seal. Non-destructive testing is possible.

sign, therefore cannot replace confirmation at initial design, even if performed.

2.2.3. Evaluation of package integrity in stability tests and stability monitoring

In order to assess the risk of new leaks generated during storage of pharmaceuticals, it is necessary to evaluate package integrity as a part of a stability test during the product development stage. It is also desirable to evaluate package integrity in the stability monitoring after the launch. It is recommended that test methods with detection ability as close as possible to the maximum allowable leakage limit are used based on the understanding of the mechanism and the rationale for ensuring no contamination.

The amount of sample required for the package integrity test in stability tests should be the amount that can achieve the purpose of the test in consideration of the past development and validation tests. If the test is a non-destructive test, the package integrity of a sample to be tested for preparation stability can be inspected prior to the stability test.

In the case of applying a physicochemical leak test method or any other test method that can appropriately evaluate the ingress of microorganisms with a certain level, it can be substituted for a sterility test in stability tests. On the contrary, for products for which influence of other than microorganisms on quality can be ignored, sterility tests can be substituted for package integrity evaluation of products in stability monitoring by considering the possibility of microbial ingress.

2.3. Criteria for the selection of test methods

The method of an individual leak test or seal quality test cannot cover all of the packaging of products. Depending on preparation packaging, multiple test methods may be required during the product life cycle. Therefore, for ensuring package integrity, it is necessary to select appropriate test methods, set parameters, and verify that the selected test methods can be applied to the product. The following product properties are taken into consideration for the choice of test methods.

Contents of package: Physical state (liquid, solid), electrical conductivity of liquid, presence or absence and type of headspace gas, and compatibility with test materials/test conditions.

Package structure and physicochemical properties: Package hardness, presence or absence of mobility, effect of volatiles added to polymers, electrical conductivity and capacitance of materials, and the amount of passed gas that is not a leak.

Impact on packaging and contents (destructive tests and non-destructive tests): For example, package integrity testing for ampoules, etc. requiring total inspection should be a nondestructive test that does not affect the quality of packaging and contents.

2.4. Setting and verification of test methods

The optimization of test conditions is required to ensure highly sensitive, accurate, robust, highly reproducible leak detection for individual product packaging systems to which leak or seal quality tests are applied. For the design and verification of test methods, the design of a package closure system, packaging materials, the nature of leaks to be predicted, and the effect of the contents of products on the test results should be taken into account, and positive controls (packages with intentional or known leaks) and negative controls (packages with no known leak) are used. For quantitative evaluation, it is necessary to make an opening with a certain diameter in consideration of the type and structure of materials that compose packages.

3. Glossary

The definitions of terms used in this General Information are as follows.

Package integrity: Package integrity is the ability of a package to prevent the loss of preparations, to prevent microorganism ingress, and to limit entry of detrimental gases or other substances, thus ensuring that the product meets all necessary safety and quality standards. ''Container closure system integrity'' and ''container integrity'' mean ''package

integrity''.

Quantitative leak test method: In the quantitative leak test method, the leak to be detected or measured is based on a phenomenon caused by a predictable series of events. Furthermore, the means of leak detection can be easily controlled and monitored, and is based on the physicochemical techniques that can obtain concrete quantitative data.

Qualitative leak test method: The qualitative leak test method is essentially probabilistic. Qualitative tests depend on a series of continuous or simultaneous events, each of which is accompanied by a random result represented by a probability distribution. Therefore, the results have uncertainty and require a large sample size and the rigorous control of test conditions to obtain meaningful results.

Leakage: The transfer of liquid or gas through a breach in a package material or through a gap between package materials. Leakage is expressed in the measure (in mass or volume units) of the flow rate of gas that pass through leakage pathways under specified temperature and pressure conditions. The leak rate has the dimension of pressure multiplied by volume, divided by time. For example, the international standard SI nomenclature is pascal cubic meter per second ($Pa \cdot m^3 \cdot s^{-1}$).

Leak: A leakage, or a hole, or a pathway where a leakage occurs.

Permeation: The passage of substances through a package material. Permeation of gases, including water vapor, can usually occurs in the packaging of sterile pharmaceuticals. The ''water vapor permeability test'' applies to the permeation of water vapor in plastic containers (mainly aqueous injection containers).

Maximum allowable leakage limit: A maximum leakage rate (or hole, or pathway size) allowable for a product package that can assure no risk to product safety and no or negligible impact on product stability.

Positive and negative controls: A package having different type and size of defects is used as a positive control. Generally, packages having large size defects are used as positive controls during the development of test methods, and packages having small size defects are used as positive controls for the development of test methods and for validation.

Leak Tests for Packaging of Sterile Products <*G7-5-180*>

This General Information describes test methods to measure the entry and escape of gases and liquids in packages and containers of sterile pharmaceuticals, and to recognize their unintentional fluid transfer due to leakage. The measured values may indicate the presence, location and size of leak channels as well as leak amount.

The leak tests are classified to qualitative leak tests and quantitative leak tests. This General Information describes liquid immersion test, liquid leak test, tracer liquid test (dye penetration test) and sniffing method (helium leak test method 1) as qualitative leak tests, and sealed chamber method (pressure change leak test method 1), vacuum decay method (pressure change leak test method 2), pressure integration method (helium leak test method 2), vacuum chamber method (helium leak test method 3), immersion method (helium leak test method 4), high-voltage leak test (pinhole test method) and laser-based gas headspace analysis as quantitative leak tests. Other methods that have been validated can also be used.

Test methods should be selected according to the characteristics of samples and the purpose of the test[1-6]. Samples, the configuration of test apparatus, and conditions such as temperature, pressure and time, are set appropriately based on various technical data, because they affect the validity of results and the safety of operations. It is desirable to use the apparatus that has been calibrated by a standard traceable to the national measurement standard as needed. The leak tests are applied to stoppered rigid or flexible packages and containers, which are empty or contain liquid or solid sterile pharmaceuticals. Specifically, ampoules, vials, syringes, containers for ophthalmic solutions, plastic bags, etc. are subject to the tests.

1. Qualitative leak tests

The qualitative leak tests are test methods to directly observe or measure the leak phenomena, and are used to confirm the presence, position(s) and conditions of leakage.

1.1. Liquid immersion test

The liquid immersion test method is used to detect the presence and locations of leaks by observation of gas emission occurred from defect regions as bubbles, when a sample containing gas in its inside is immersed in liquid and the headspace of the liquid tank is depressurized. In many cases, water is used as the liquid, and in that case, it is also called a water immersion test. It observes the generation of gas bubbles after the completion of reduced pressure until the prescribed time, and evaluate the location(s) of leak(s), the size and the occurrence frequency of the gas bubbles. In some cases, bubbles are observed in a liquid tank using a sample pressurized with gas. The amount of leakage can be quantified by collecting the generated gas bubbles in a liquid tank with a measuring cylinder etc. for defined time and measuring the amount. The amount of leakage can be expressed as a function of the sampling time for collecting gas bubbles and the collected quantity being corrected with a reference pressure or one atmospheric pressure. The test is performed at prescribed temperature as required, and the value of reduced pressure and measurement time of the headspace of a liquid tank are set according to the pressure resistance of a sample and assumed defects. It is applied to rigid or flexible packages and containers.

1.2. Liquid leak test

The liquid leak test methods are test methods to visualize and observe the transfer of liquid due to leakage using an additive or a developer. The liquid leak test methods include the method that adds liquid containing a fluorescent dye to a sample to detect the leaked liquid by irradiation with ultraviolet light, and the method that coats the surface of a sample with a developer to observe an indication pattern generated by a chemical reaction of the leaked liquid and the developer (Table 1).

In the fluorescent dye method, liquid containing fluorescent dye is injected inside a sample, or is dissolved in liquid inside a sample, and leakage is detected under ultraviolet light in a dark place. The inside of the sample is pressurized, as necessary, and change due to leakage is observed. In the test methods using a developer, the adequately stirred developer is applied evenly on the surface of a sample by a spray or a brush. After drying of the developed coating film, an indication pattern due to leakage is observed under white light in the cases of the white development method and the color development method. In the fluorescent development method, an indication pattern due to leakage is observed under ultraviolet light in a dark place. Record the indication pattern due to leakage as the description of position, size and number, etc. or as an image. These test methods are applied to rigid or flexible packages and containers.

Table 1 Types of liquid leak tests

	Methods	Excipients added to liquid	Developers	Observation	Indication pattern
Using an additive	Fluorescent dye method	Fluorescent dye	no	under ultraviolet light (in a dark place)	fluorescence
Using a developer	White development method	no	White developer	under white light	gray color
	Color forming development method	no	Color-forming developer	under white light	red color
	Fluorescence development method	no	Fluorescence developer	under ultraviolet light (in a dark place)	fluorescence

1.3. Tracer liquid test (dye penetration test)

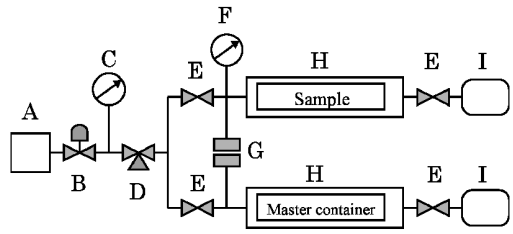
The tracer liquid test method is a method to observe the inflow or outflow of tracer liquid by immersing a sample in liquid. The test method is used to detect leak location and to evaluate the relative amount of leakage in non-porous rigid or flexible containers. A dye solution or a solution containing metal ions is used as the tracer liquid. The transfer of the dye is observed visually or measured using instruments. The test method is applied to clear, pressure-tight or other, stoppered rigid or flexible packages and containers which are empty or contain contents (liquid or solid).

a) In the test method where a tracer liquid is introduced, immerse a sample containing no tracer liquid in a chamber filled with the tracer liquid, cover the chamber, and pressurize or depressurize the chamber so that the head space part has a prescribed pressure, and hold. After prescribed time has elapsed, the head space part is opened to the atmosphere and left for prescribed time. Then the sample is taken out, and the surface is cleaned. The tracer liquid which has invaded into the sample is observed visually or measured by chemical analysis.

b) In the test method where a tracer liquid is flowed out, immerse a sample containing a tracer liquid in a chamber filled with a solution containing no tracer liquid. Then cover the chamber, pressurize or depressurize so that the head space part becomes a prescribed pressure, and hold for prescribed time to flow out the tracer liquid. After the head space part is left for a prescribed time under the atmospheric pressure, the sample is taken out, and the transfer of the tracer liquid is measured by observation of the liquid inside the chamber or by chemical analysis. The test method is applied to rigid containers.

1.4. Sniffer method (Helium leak test method 1)[7]

The helium leak test method by the sniffer method is also called the suction method. This test method is a method to detect leakage by filling helium gas in a sample under normal pressure or pressurized condition and sucking the helium gas that leaks into the outside by a suction probe. In addition, some methods that apply a suction probe to measurement regions or scan with the probe to detect the presence and the position of leakage are also used. The test method is applied to rigid or flexible packages and containers.



A : Pressurizing or depressurizing apparatus
B : Pressure regulating valve
C : Pressure gauge
D : Pressure and exhaust valve
E : Shut-off valve
F : Vacuum gauge
G : Differential pressure gauge
H : Chamber
I : Release container

Fig. 1 Example of apparatus configuration for the sealed chamber method (pressure method)

2. Quantitative leak tests

The quantitative leak test methods provide the amount of leakage in numerical values of physical quantities. Because measured values are affected by conditions (sample temperature, testing time, etc.) and environmental factors (air temperature, humidity, atmospheric pressure, etc.) of the test method, it is necessary to use them with sufficient consideration of these factors.

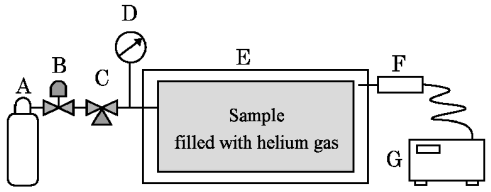
2.1. Sealed chamber method (Pressure change leak test 1)[8]

The sealed chamber pressure change leak test method is used to measure the leakage of a stoppered sample by pressure change after pressurizing or depressurizing the chambers which contain the sample and a master container (a leak-free container having same structure as the sample), respectively. The method may be destructive or non-destructive depending on the pressure resistance of the sample and the pressure setting of a chamber. It is applied to rigid or flexible packages and containers.

In this test method, use an apparatus shown in Fig. 1, place a sample and a master container in each chamber, close valves after pressurizing or depressurizing the chambers, and measure pressure difference between the chambers after prescribed time. To detect large leaks, measure pressure difference after releasing the pressure of the inside of both chambers into discharge containers. The amount of leakage is expressed as a function of the value of the pressure difference between the chambers, the space volume of the sample and the chamber, the volume ratio of the chamber and the discharge container, etc.

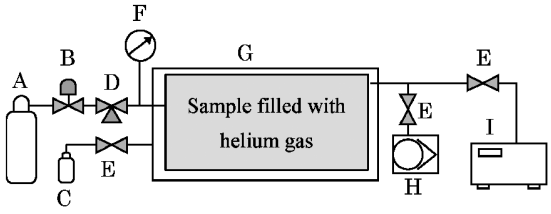
2.2. Vacuum decay method (Pressure change leak test method 2)

The vacuum decay pressure change leak test is applied to test samples containing liquids. An apparatus similar to that used in the sealed chamber method is used for this test. In the operation, a sample and a master container are placed in chambers, respectively, pressure in the chambers are depressurized below the vapor pressure of the liquid. Changes in the chamber pressure due to the evaporation of the leaked liquid is measured by a vacuum gauge or a differential pressure sensor. The degree of pressure rise is expressed as a function of gap volume between the sample and the chamber and the measurement time, and is affected by the amount of leaked liquid, vapor pressure, degree of vacuum and the temperature of the liquid. This method is applied to rigid or flexible packages and containers that contain liquid and have no head space.



A : Helium cylinder
B : Pressure regulating valve
C : Pressure and exhaust valve
D : Pressure gauge
E : Chamber or cover
F : Suction probe
G : Helium leak detector

Fig. 2 Example of apparatus configuration for pressure integration method



A : Helium cylinder
B : Pressure regulating valve
C : Calibration leak
D : Pressure and exhaust valve
E : Shut-off valve
F : Vacuum gauge
G : Vacuum chamber
H : Auxiliary exhaust apparatus
I : Helium leak detector

Fig. 3 Example of apparatus for the vacuum chamber method

2.3. Pressure integration method (Helium leak test method 2)

In the pressure integration method, the sample filled with helium gas under normal or pressurized pressure is hold in a chamber or a cover with hood (coating material) to collect helium gas leaked to space between the hood and the sample for a prescribed time. Measure the leakage by sucking the collected gas using a suction probe.

For this test method, an apparatus shown in Fig. 2 is used, and the amount of leakage is expressed as a function of the concentration of helium gas, the gap volume between the hood and the sample, the time for collecting helium gas, the amount of suction by a suction probe, etc. This method is capable of measuring leakage from a whole sample, and is less susceptible to the concentration of surrounding helium. This method is applied to non-stoppered rigid containers without contents.

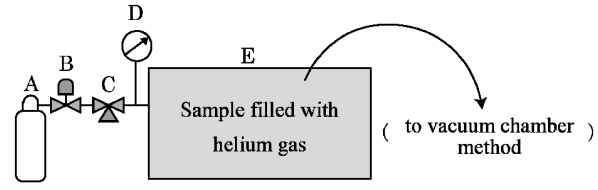
2.4. Vacuum chamber method (Helium leak test method 3),

The vacuum chamber method helium leak test is also called the vacuum container method. This test measures helium gas leaked from a sample filled with helium gas in the chamber maintained at high degree of vacuum by exhausting. High detection sensitivity is obtained compared to the pressure integration method. This method is applied to non-stoppered rigid containers without contents.

For this test method, use an apparatus shown in Fig. 3, set a sample filled with pressured helium gas in a vacuum chamber, close the chamber, and depressurize. The value of leakage is obtained from the difference between the amount of helium in the chamber, in the presence and absence of the sample, at the time when the inside of the chamber reaches prescribed vacuum.

2.5. Immersion method (Helium leak test method 4)

The immersion method helium leak test is also called the bombing method. This test measures outflow due to leakage by the vacuum chamber method after introducing helium gas into the space inside a sample through defects in a chamber filled with helium gas and then taking out the sample, using an apparatus shown in Fig. 4. A sample having space inside is placed in a chamber pressured with helium gas and the concentration of helium inside the sample is increased by immersing helium gas through defect holes from the outside. Then the leakage of the sample is measured by the vacuum chamber method. This method cannot be applied to samples having large leaks. The degree of leakage is expressed as a function of leaving-time in the air, inside volume of a sample, time for filling, pressure for filling, etc. This method is applied to stoppered rigid containers having space inside a sample.



A : Helium cylinder
B : Pressure regulating valve
C : Pressure and exhaust valve
D : Vacuum gage
E : Helium gas filling chamber

Fig. 4 Example of apparatus configuration for immersion method

2.6. High-voltage leak test (Pinhole test)

The high voltage leak test is used to detect pinholes which allow leakage, in the area between where electrodes are applied. The electric current that flows upon application of the high voltage to the sample is measured. Rapid non-destructive tests are possible in appropriate measurement conditions. This method is applied to rigid or flexible packages and containers with non-conductive packaging materials, containing conductive contents that are not affected by applied voltage.

2.7. Laser-based gas headspace analysis

The laser-based headspace gas analysis is a method that detect changes in the headspace gas of a sample due to leakage from the absorbance or frequency modulation of specific frequency bands in the transmission of a laser light. Information such as the concentration of oxygen, carbon dioxide or water vapor and an internal pressure are obtained by irradiating a sample held between a light source and a detector with a light having a wavelength suitable for the gas to be measured. The possibility of leakage is judged by comparing the measurement value with the value obtained for a reference sample having controlled defects. Non-destructive tests are possible in appropriate measurement conditions, however, it is necessary to perform the tests under environment suitable for the purpose because temperature and humidity affect the results. This method is applied to light-transmitting containers that have a gas part and to pharmaceutical preparations that have a head-space part.

3. References

1) JIS Z 2330: 2012, Non-destructive testing-Selection of leak testing method.
2) The Japanese Society for Non-Destructive Inspection, Leak testing I, (2012).
3) The Japanese Society for Non-Destructive Inspection,

Leak testing II, (2012).
4) The Japanese Society for Non-Destructive Inspection, Leak testing III, (2016).
5) US Pharmacopeia 40 (2017), <1207.1> Package Integrity Testing in The Product Life Cycle—Test Method Selection and Validation.
6) US Pharmacopeia 40 (2017), <1207.2> Package Integrity Leak Test Technologies.
7) JIS Z2331: 2006, Method for helium leak testing.
8) JIS Z2332: 2012, Leak testing method using pressure change.

G8 Reference Standards

Reference Standards and Reference Materials Specified in the Japanese Pharmacopoeia <*G8-1-170*>

Reference materials are materials that are used as standards in quantitative and qualitative measurement of drug products, calibration and accuracy confirmation of apparatus, and suitability tests of analytical systems. Reference standards specified in the Japanese Pharmacopoeia (JP) as described in Reference Standards <*9.01*> are reference materials prepared to the specified quality necessary with regard to their intended use such as quality evaluation tests of drugs, and they are provided with public assurance that the substances have suitable quality for the specified use.

This chapter includes the definitions and explanations of basic terms regarding reference standards, and the particulars of the JP reference standards mainly used for chemical drugs, such as classification by use, requirements for establishment, required quality evaluation items, distribution and precautions for use. The descriptions show only basic policies, and therefore flexible management is required for practical application for the time being.

1. Basic terms of reference standards

- Reference materials: ''The materials used as the standards'' for determination of characteristic values of materials and substances. Reference materials are sufficiently homogeneous and stable with respect to one or more specified properties and are produced so that they are suitable for their intended use in measuring processes [JIS Q 0035:2008]. Reference materials for drug products are materials used as the standards in quantitative and qualitative measurements, calibration and accuracy confirmation of apparatus, and they are prepared so that they are suitable for their intended use.
- Reference standards: The reference materials prepared to a specified quality necessary with regard to their intended use, such as quality evaluation tests of drugs, and they are provided with public assurance that the substances have suitable quality for the specified use.
- JP reference standard: The reference standards specified in the Monographs or General Tests in the JP.
- Certified reference materials: The reference materials with one or more specified characteristic values determined by appropriate metrological procedure. Their qualities are guaranteed by a certificate that describes their characteristic values, uncertainties of the values, and metrological traceability [JIS Q 0035:2008].

2. Classification of the JP reference standards by use

JP reference standards include various types and are used for assays, identification, purity, calibration of apparatus, suitability tests of analytical systems, etc., and they are broadly classified by their use such as quantitative tests, qualitative tests, and calibration of apparatus. These reference standards are further subclassified according to their specific use, such as assays of active ingredients and indicator ingredients, assays of related substances, identification using spectral measurement or chromatography, calibration of apparatus used in tests, determinations and assays that are specified in the General Tests, and suitability tests for analytical systems.

2.1. Reference standards used for quantitative tests

2.1.1. Reference standards for assays of active ingredients etc.: Reference standards used for quantitative assays of products specified in the Monographs, such as chemical agents, antibiotics, additives, and biotechnological/biological products.

2.1.2. Reference standards for assays of indicator ingredients:
Reference standards used for quantitative assays of indicator ingredients of crude drugs specified in the Monographs.

2.1.3. Reference standards for assays of related substances: Reference standards used for quantitative assays of specific related substances in purity for products specified in the Monographs, such as chemical agents, antibiotics, additives, and biotechnological/biological products.

2.1.4. Other reference standards for assays:
Reference standards needed for quantitative assays specified in the General Tests.

2.2. Reference standards used for qualitative tests

2.2.1. Reference standards for identification:
Reference standards used for identification tests of products specified in the Monographs, such as chemical agents, antibiotics, additives, biotechnological/biological products, and crude drugs. These identification include the comparison of ultraviolet-visible absorption spectra, infrared absorption spectra, nuclear magnetic resonance spectra, retention time or *R*f values in chromatography, and mobility in electrophoresis.

2.2.2. Reference standards for purity:
Reference standards used for identification of peaks or spots, or for limit tests of related substances, in purity tests of products specified in the Monographs, such as chemical agents, antibiotics, additives, biotechnological/biological products, and crude drugs.

2.3. Reference standards used for system suitability

2.3.1. Reference standards for system suitability:
Reference standards used for system suitability of products specified in the Monographs, such as chemical agents, antibiotics, additives, biotechnological/biological products, and crude drugs.

2.4. Reference standards used for calibration and suitability confirmation of apparatus

2.4.1. Reference standards for calibration of apparatus:
Reference standards used for secondary calibration of apparatus that are specified in the General Tests.

2.4.2. Reference standards for suitability confirmation of apparatus:
Reference standards used in order to confirm that the measured values gained from an apparatus specified in the General Tests are within the defined range.

3. Names and uses of JP reference standards

JP reference standards are used for tests specified in the

Monographs and General Tests, such as Assays, Identification, Purity, calibration of apparatus, and suitability tests of analytical systems. These reference standards include materials with only one specific use and materials with multiple uses. JP reference standards used for assays and quantitative determinations of active ingredients in uniformity of dosage units and dissolution are named in principle by attachment of the phrase ''reference standard'' to the material name. Reference standards used for quantitative tests can be used for other tests such as identification as the reference standards, if possible. Reference standards only used for uses other than quantitative tests are named by attachment of the name of their use. Some possible uses are shown below with examples in brackets.

- For identification (Montelukast Sodium for Identification RS)
- For purity (○○○ for Purity RS)
- For calibration of apparatus (Calcium Oxalate Monohydrate for Calibration of Apparatus RS)
- For system suitability (Montelukast for System Suitability RS)

4. Requirements for establishment of JP reference standards

The JP reference standards have been mainly used as standard substances for quantitative assays of active ingredients so far. On the other hand, the European Pharmacopoeia (Ph. Eur.) and the U.S. Pharmacopeia (USP) have been actively establishing reference standards or reference materials that have specific uses other than the use for quantitative assays of active ingredients. Such specified reference standards include standards for contaminants in purity, for suitability of analytical systems, and for identification. This situation requires JP to change its policy on the reference standards along with the international trends, however, careful deliberation is needed to establish new JP reference standards in consideration of the points shown below.

(1) To adopt a relative determination such as chromatography for a quantitative test, in principle, establish the reference standard for the assay or establish the reference materials, of which purity with metrological traceability is indicated, as a reagent.

(2) To appoint a comparison method for an identification, such as comparison of ultraviolet-visible absorption spectra, infrared absorption spectra, or nuclear magnetic resonance spectra, comparison of retention times or *R*f values in chromatography, or comparison of electrophoretic mobility, in principle, it is desirable to establish the reference standard for the identification unless the use of some reference standard for assays is applicable.

(3) In a purity test where a specific related substance or contaminant is analyzed, it is recommended to establish the reference standard dedicated to the purity if the specific related substance or contaminant cannot be identified from the relative retention time of its peak on the chromatogram, and/or the limit cannot be specified by area percentage method or by comparison of the peaks with the sample solution and with the standard solution derived from the sample solution.

(4) Establish a reference standard for suitability of an analytical system, if the system suitability cannot be adequately evaluated by conventional JP methods (determination of the number of theoretical plates and the symmetry factor, etc.)

(5) If the continuous supply of the raw materials for the reference standard is uncertain, a reference standard for a related substance for purity or a reference standard for system suitability should not be established.

(6) When a material utilized as standard has the uses other than for quantitative assays and can be obtained as a certified reference material or as a reference material for other tests, that can be specified in the Reagents, Test Solutions <*9.41*> without establishing the materials as a JP reference standard.

(7) When a material utilized as standard has the uses other than for quantitative assay and can be obtained as a reagent, that can be specify the material in the Reagents, Test Solutions <*9.41*> establishing specifications and test methods according to its use, without establishing the material as a JP reference standard.

5. Quality evaluation items required for JP reference standards

Quality evaluation items required to establish a JP reference standard are shown below. The items are chosen mainly on the assumption that the materials are used as reference standards for chemical agents.

5.1. Quality evaluation items for reference standards used for quantitative assays

(i) Description: color and shape

(ii) Identification: establish a test method to identify or confirm the chemical structure.

i) Ultraviolet-visible absorption spectrum
ii) Infrared absorption spectrum
iii) Nuclear magnetic resonance spectrum (^{1}H)
iv) X-ray powder diffraction image※(when the crystal form is specified)
v) Retention time or *R*f value in chromatography※ (when chromatography is applicable to the identification)
vi) Counter ion※

(iii) Purity

i) Related substances (total amount)
ii) Residual solvents
iii) Other contaminants※

(iv) Characteristic values※

i) Specific rotation
ii) Melting point

(v) Water/Loss on drying

(vi) Residue on ignition※

(vii) Purity determination by a mass balance method: Regarding purity evaluation in mass balance method, calculate the purity (%) on the dried or anhydrous basis setting related substances, residue on ignition, residual solvent, and other contaminants as deductions.

(viii) Assay (if possible, establish an absolute quantification method such as titration, etc.)

[Note] Quality evaluation items attached with※ marks are items whose adoption should be considered taking into account the material's use as a reference standard.

5.2. Quality evaluation items for reference standards used for other than quantitative assays

Shown below are examples of quality evaluation items whose adoption should be considered taking into account the material's use as a reference standard.

(i) Description: color and shape

(ii) Identification: establish a test method to identify or confirm the chemical structure.

i) Ultraviolet-visible absorption spectrum
ii) Infrared absorption spectrum
iii) Nuclear magnetic resonance spectrum (^{1}H)
iv) Mass spectrum
v) X-ray powder diffraction image (when the crystal form is specified)
vi) Retention time or *R*f value in chromatography

(iii) Purity

i) Related substances (total amount)
ii) Residual solvents
iii) Other contaminants
(iv) Water/Loss on drying
(v) Purity determination by mass balance method
(vi) Tests related to the uses of the materials

i) A reference standard for system suitability used for peak identification needs identification of relative retention time of the peak under the same conditions to the test method that is specified in the Monographs.

ii) A reference standard for system suitability needs identification of the resolution under the same conditions to the test method that is specified in the Monographs.

6. Reference materials specified in the JP

Materials that correspond to reference materials are described in the Reagents, Test Solutions <*9.41*> in the JP. Such materials are shown as follows:

- Materials described as reagents for assays
- Materials used for identification that described as reagents for thin-layer chromatography (some materials do not correspond to reference materials)
- Materials specified as reagents for purity
- Materials described as specific related substances in the item of Related substances in Purity in the Monographs
- Reference materials specified by JIS

In the JP (except ''Crude Drugs''), ''reagents for assays'' is specified as reference materials for assays of active ingredients in drug products, and some active ingredients with quality above a certain level are specified as reagents that are used as reference materials for identification of the active ingredients in drug products by thin-layer chromatography. However, these reference materials specified as reagents are not officially provided. Assays should be performed using reference standards, and it is considered appropriate that the ''reagents for assays'' described as reagents in the Reagents, Test Solutions <*9.41*> should be established as reference standards. Regarding new assay reagents that will be established for drug products except ''Crude Drugs,'' there is a need to consider gradual establishment of these ''reagents for assays'' as reference standards.

On the other hand, it is difficult to establish reference standards for indicator ingredients of ''Crude Drugs''; therefore, reference materials for assays of indicator ingredients are specified as reagents and quantitative NMR is included in specifications of the reagents so that a specification for assay with metrological traceability is established.

7. Precautions for the use of JP reference standards

7.1. JP reference standards are reference standards whose uses are specified in the Monographs and General Tests in the JP. Their detailed uses are described in the Monographs and their adequate qualities as reference standards are guaranteed when they are used for the described uses. Accordingly, their qualities for other uses are not guaranteed.

7.2. When a JP reference standard is used for a quantitative test specified in the Monographs, if a correction coefficient is indicated on documents such as package insert, multiply the standard amount by the correction coefficient to calculate the corrected amount for the use. If a correction coefficient is not indicated, do not correct the amount for use assuming that the purity of the reference standard is 100.0%.

7.3. If there is a description of ''amount of the reference standard, calculated on the dried basis'' or ''amount of the reference standard, calculated on the anhydrous basis'' in a calculation formula for an assay method in the Monographs, extra measurement of the loss on drying or the water content of the reference standard is required. If, however, the value of loss on drying or water content is indicated on the document of the reference standard such as package insert, use of the indicated value is permitted.

7.4. JP reference standards have no established expiration date, therefore, obtain needed amount of a reference standard when it is needed, and then store the reference standard under specified conditions and use up it as quick as possible.

7.5. The quality of reference standards stored after unsealing of the packages are not guaranteed.

7.6. Normally, one packing unit of a reference standard contains the amount that enables several times of repeated test. However, some packages of reference standards whose raw material supply is scarce contain the amounts that enable only one-time test.

7.7. Information of JP reference standards necessary for the uses specified in the Monographs or General Tests is described on documents such as package inserts. However, their test results are not disclosed and certificates of analysis are not issued.

GZ Others

Water to be used in the Tests of Drugs <*GZ-1-161*>

The water to be used in the tests of drugs is defined as ''the water suitable for performing the relevant test'' in the paragraph 21 under General Notices of the JP. Therefore, it is necessary to confirm that the water to be used in a test of a drug is suitable for the purpose of the relevant test before its use.

Unless otherwise specified in the individual test method, Purified Water, Purified Water in Containers or the water produced by an appropriate process, such as ion exchange or ultrafiltration, may be used for these purposes. Water produced for these purposes at other individual facilities may also be used.

Examples of the water for tests specified in General Tests in the JP are as follows:

- Water for ammonium limit test: <*1.02*> Ammonium Limit Test (Standard Ammonium Solution)
- Water used for measuring organic carbon (water for measurement): <*2.59*> Test for Total Organic Carbon
- Water for ICP analysis: <*2.63*> Inductively Coupled Plasma-Atomic Emission Spectrometry and Inductively Coupled Plasma-Mass Spectrometry
- Water for bacterial endotoxins test: <*4.01*> Bacterial Endotoxins Test
- Water for particulate matter test (for injections): <*6.07*> Insoluble Particulate Matter Test for Injections
- Water for particulate matter test (for ophthalmic solutions): <*6.08*> Insoluble Particulate Matter Test for Ophthalmic Solutions
- Water for particulate matter test (for plastic containers): <*7.02*> Test Methods for Plastic Containers

The water for tests specified in General Information in the JP is as follows:

- Water for aluminum test: Test for Trace Amounts of Aluminum in Total Parenteral Nutrition (TPN) Solu-

tions

The term ''water'' described in the text concerning tests of drugs means ''the water to be used in the tests of drugs'' as defined in the paragraph 21 under General Notices.

Quality Control of Water for Pharmaceutical Use <*GZ-2-172*>

Water used for manufacturing pharmaceutical products and for cleaning their containers and equipments used in the manufacture of the products is referred to as ''pharmaceutical water.'' For assuring the quality of pharmaceutical water consistently, it is important to verify through appropriate process validation of water processing system that water with the quality suitable for its intended use is produced and supplied, and to keep the quality of produced water through routine works for controlling the water processing system.

1. Types of Pharmaceutical Water

1.1. *Water*

The specification for ''*Water*'' is prescribed in the Japanese Pharmacopoeia (JP) monograph. It is required for *Water* to meet the Quality Standards for Drinking Water provided under the Article 4 of the Japanese Water Supply Law. In the case that *Water* is produced at individual facilities using well water or industrial water as source water, it is necessary for produced water to meet the Quality Standards for Drinking Water and an additional requirement for ammonium of ''not more than 0.05 mg/L.'' Furthermore, when *Water* is to be used after storing for a period of time, it is necessary to prevent microbial proliferation.

Water is used as source water for *Purified Water* and *Water for Injection*. It is also used for manufacturing intermediates of active pharmaceutical ingredients (APIs), and for pre-washing of the equipment used in the manufacture of pharmaceutical products.

1.2. *Purified Water*

The specifications for ''*Purified Water*'' and ''*Purified Water in Containers*'' are prescribed in the JP monographs. *Purified Water* is prepared by distillation, ion-exchange, reverse osmosis (RO), ultrafiltration (UF) capable of removing microorganisms and substances with molecular masses of not less than approximately 6000, or a combination of these processes from *Water*, after applying some adequate pretreatments if necessary. For the production of *Purified Water*, appropriate control of microorganisms is required. Particularly, in the case that *Purified Water* is prepared by ion-exchange, RO or UF, it is necessary to apply the treatments adequate for preventing microbial proliferation, or to sanitize the system periodically.

When *Purified Water* is treated with chemical agents for sterilizing, preventing microbial proliferation, or maintaining the endotoxin level within an appropriate control range, a specification suitable for the intended use of treated water should be established individually, and a process control for keeping the quality of treated water in compliance with the specification thus established should be performed.

''*Purified Water in Containers*'' is prepared from *Purified Water* by introducing it in a tight container.

1.3. *Sterile Purified Water*

The specification for ''*Sterile Purified Water in Containers*'' (its alternative name is *Sterile Purified Water*) is prescribed in the JP monograph.

Sterile Purified Water in Container is prepared from *Purified Water* by 1) introducing it into a hermetic container, sealing up the container, then sterilizing the product, or 2) making it sterile by using a suitable method, introducing the sterilized water into a sterile hermetic container by applying aseptic manipulation, then sealing up the container.

Plastic containers for aqueous injections may be used in place of hermetic containers.

1.4. *Water for Injection*

The specifications for ''*Water for Injection*'' and ''*Sterile Water for Injection in Containers*'' are prescribed in the JP monographs.

Water for Injection is prepared by distillation or reverse osmosis and/or ultrafiltration (RO/UF), either: from the water which is obtained by appropriate pretreatments such as ion exchange, RO, etc. on *Water*; or from *Purified Water*. In the case of water processing systems based on distillation, it is necessary to take care for avoiding contamination of produced water by the impurities accompanied with the entrain. In the case of water processing system based on RO/UF, it is required to provide water with equivalent quality to that prepared by distillation consistently, based on substantial process validation through long-term operation and elaborate routine control of the system. It is essential to ensure consistent production of water suitable for *Water for Injection* by the entire water processing system including pretreatment facilities, in any systems based on RO/UF. For the water supplied to the system, it is also required to keep the quality suitable as source water through adequate validation and routine control on the water. For the water processing system based on RO/UF, routine control should be performed by analyzing water specimens, monitoring some quality attributes using in-line apparatus and checking the volume of water passed through the system. In addition, it is recommended to carry out periodical appearance observation and air-leak test on the membranes being currently used. It is also recommended to establish protocols for keeping the performance of membrane modules within appropriate control ranges and for estimating the timing to exchange the modules, through diagnosis on the degree of deterioration based on the results of tensile strength test on the used membrane modules, and visual observation on those modules whether any leakages of membranes have occurred or not, and to what extent they have occurred. Furthermore, it is desirable to establish the frequency of membrane exchange considering with its actual condition of use.

In the case that *Water for Injection* is stored in the water processing system temporarily, a stringent control for microorganisms and endotoxins should be taken. An acceptable criterion of lower than 0.25 EU/mL for endotoxins is specified in the JP monograph of *Water for Injection*.

''*Sterile Water for Injection in Container*'' is prepared from *Water for Injection* by 1) introducing it into a hermetic container, sealing up the container, then sterilizing the product, or 2) making it sterile by using a suitable method, introducing the sterilized water into a sterile hermetic container by applying aseptic manipulation, then sealing up the container. Plastic containers for aqueous injections may be used in place of hermetic containers.

2. Reverse Osmosis and/or Ultrafiltration (RO/UF)

RO/UF are the methods for refining water by using membrane modules based on either reverse osmosis or ultrafiltration, or the modules combining them, and used as the alternative methods for distillation in the production of *Purified Water* or *Water for Injection*.

When *Water for Injection* is produced by RO/UF, a water processing system equipped with pretreatment facilities,

facilities for producing *Water for Injection* and facilities for supplying *Water for Injection* is usually used. The pretreatment facilities are used to remove solid particles, dissolved salts and colloids in source water, and placed before the facilities for producing *Water for Injection* so as to reduce the load on the facilities for producing *Water for Injection*. They consist of apparatus properly selected from aggregation apparatus, precipitation-separation apparatus, filtration apparatus, chorine sterilization apparatus, oxidation-reduction apparatus, residual chlorine-removing apparatus, precise filtration apparatus, reverse osmosis apparatus, ultrafiltration apparatus, ion exchange apparatus, etc., depending on the quality of source water. The facilities for producing *Water for Injection* consist of apparatus for supplying pretreated water, sterilization apparatus with ultraviolet rays, heat exchange apparatus, membrane modules, apparatus for cleaning and sterilizing the facilities, etc. The facilities for supplying *Water for Injection* consist of a reservoir tank for storing *Water for Injection* in the facilities temporarily, pipe lines, heat exchange apparatus, a pump for circulating *Water for Injection* in the facilities, pressure control apparatus, etc. When *Purified Water* is produced by RO/UF, basic composition of water processing system is almost the same as that for *Water for Injection* described above.

In the case that *Water for Injection* is stored in the water processing system temporarily, it should usually be circulated in a loop consisting of a reservoir tank and pipe line at a temperature not lower than 80°C for preventing microbial proliferation.

When RO/UF is utilized for preparing pharmaceutical water, it is necessary to select the most appropriate combination of membrane modules in consideration of the quality of source water and the quality of produced water required for its intended use. When the ultrafiltration membrane is used to prepare *Purified Water* or *Water for Injection*, membrane modules capable of removing microorganisms and substances with molecular masses not less than approximately 6000 should be used.

3. Selection of Pharmaceutical Water

Depending on the intended use of pharmaceutical water, the water suitable for assuring the quality of final products without causing any trouble during their manufacturing processes, should be selected from the above 4 types (1.1 – 1.4) of pharmaceutical water specified in the JP. Table 1 exemplifies a protocol for such selection.

Sterile Purified Water in Containers or *Water for Injection* (or *Sterile Water for Injection in Containers*) may be used in place of *Purified Water* (or *Purified Water in Containers*).

3.1. Drug Products

For the manufacture of sterile drug products such as Injections, for which endotoxins together with microorganisms should be severely controlled, *Water for Injection* (or *Sterile Water for Injection in Containers*) should be used. For the manufacture of sterile drug products such as Ophthalmic Preparations and Ophthalmic Ointments, for which contamination with microorganisms should be paid attention, *Purified Water* (or *Purified Water in Containers*), which viable count level is specified at low, can also be used.

For the manufacture of non-sterile drug products, water with a quality not lower than that of *Purified Water* (or *Purified Water in Containers*) should be used. For the Inhalations, Ear Preparations and Nasal Preparations, appropriately controlled *Purified Water* (or *Purified Water in Containers*) in vial count level should be used, and for Liquids and Solutions among Inhalations, strictly controlled *Purified Water* (or *Purified Water in Containers*) in vial count level should be used. For the Liquids and Solutions for Oral Administration, Syrups, Suppositories for Vaginal Use, Ointments and Creams, which require care against microbiological contamination, *Purified Water* (or *Purified Water in Containers*) adequately controlled from microbiological viewpoints should be used in consideration of the possible impacts of preservatives formulated in the drug products.

For the manufacture of drug products containing crude drugs, it is recommended to select adequate type of water considering viable counts of the crude drugs used for manufacturing the product and microbial limit required for the product.

Water used for pre-washing of containers or equipment surfaces that comes in direct contact with the drug products should have the quality not lower than that of *Water*. Water used for final rinsing should have an equivalent quality to that of water used for manufacturing drug products.

3.2. Drug Substances

Water used for manufacturing a drug substance should be selected in consideration of the characteristics of the drug product for which the drug substance is to be used, and its manufacturing process, so that the quality of the final drug product is assured.

Water used for manufacturing a drug substance or for cleaning containers or equipment surfaces that come in direct contact with the raw materials or drug substance intermediates, should have the quality not lower than that of *Water* adequately controlled from the chemical and microbiological viewpoints, even if the water is used at an earlier stage of synthetic or extraction process in the manufacture of drug substances. Water used in the final purification process should have the quality equal to or higher than that of *Purified Water* (or *Purified Water in Containers*). Water used for final rinsing of containers or equipment surfaces that comes in direct contact with the drug substances should have an equivalent quality to that of water used for manufacturing the drug substances.

For manufacturing sterile drug substances, *Sterile Purified Water in Containers* or *Water for Injection* (or *Sterile Water for Injection in Containers*) should be used. Similarly, for manufacturing drug substances used for drug products where endotoxin control is required and there are no subsequent processes capable of removing endotoxins, *Water for Injection* (or *Sterile Water for Injection in Containers*), or *Purified Water* (or *Purified Water in Containers*) for which endotoxins are controlled at a low level, should be used.

4. Quality Control of Pharmaceutical Water

4.1. Outline

Verification that water with the quality required for its intended use has been produced by the pharmaceutical water processing system through substantial validation studies at an earlier stage of its operation, is the prerequisite for conducting quality control on pharmaceutical water in a routine and periodical manner. If this prerequisite is fulfilled, the following methods are applicable for quality control of pharmaceutical water.

For routine control, it is very useful to control quality of produced water based on the monitoring of electrical conductivity (conductivity) and total organic carbon (TOC). In addition, items to be monitored periodically, such as some specified impurities, viable counts, endotoxins, insoluble particulate matters, etc., should be determined according to the intended use of pharmaceutical water. The frequency of measurement should be determined considering with the

Table 1 An Exemplified Protocol for Selecting Pharmaceutical Water
(Water Used in the Manufacture of Drug Products or Drug Substances)

Classification	Class of Pharmaceutical Water	Application	Remarks
Drug Product	*Water for Injection* or *Sterile Water for Injection in Containers*	Injections, Dialysis Agents (Peritoneal Dialysis Agents and Hemodialysis Agents)	For Hemodialysis Agents, unless otherwise specified, *Water for Injection*, *Water for Injection in Containers*, or water suitable for the dialysis.
	Purified Water or *Purified Water in Containers*	Ophthalmic Preparations, Ophthalmic Ointments, Inhalations, Ear Preparations, Nasal Preparations	For sterile drug products, such as Ophthalmic Preparations and Ophthalmic Ointments, for which precautions should be taken against microbial contamination, *Purified Water* (or *Purified Water in Containers*) kept its viable counts at low levels may be used. For Inhalations, Ear Preparations and Nasal Preparations, *Purified Water* (or *Purified Water in Containers*) of which viable counts are controlled at an appropriate level should be used. However, for the Inhalation Liquid Preparations among Inhalations, *Purified Water* (or *Purified Water in Containers*) that vial count is strictly controlled should be used.
		Preparations for Oral Administration, Preparations for Oro-mucosal Application, Preparations for Rectal Application, Preparations for Vaginal Application, Preparations for Cutaneous Application, and Tinctures and Aromatic Waters among Preparations Related to Crude Drugs.	For Liquids and Solutions for Oral Administration, Syrups, Suppositories for Vaginal Use, Ointments, Creams and so on for which precautions should be taken against microbial contamination, *Purified Water* (or *Purified Water in Containers*) adequately controlled from microbiological viewpoints should be used, taking in mind the affection of containing preservatives.
	Water	Among Preparations Related to Crude Drugs: Extracts, Pills, Infusions and Decoctions, Teabags, Fluidextracts	The viable counts in crude drugs and the objective microbial limits of product should be considered in selecting water to be used.
Drug Substances	*Water for Injection* or *Sterile Water for Injection in Containers*	Sterile Drug Substances	
	Purified Water or *Purified Water in Containers*	Drug Substances	In the manufacture of drug substances used for products to be rendered sterile in the formulation process, if there is no subsequent processes capable of removing endotoxins, *Purified Water* (or *Purified Water in Containers*) controlled endotoxins in an appropriate level should be used.
	Water	Drug Substances Intermediates	

variation in the quality of water to be monitored.

The following are points to consider in controlling the quality of produced water from microbiological and physicochemical (conductivity and TOC) viewpoints. It is necessary to monitor other items if necessary, and to confirm that they meet the specifications established individually.

4.2. Sampling

Monitoring should be conducted at an adequate frequency to ensure that the pharmaceutical water processing system is well-controlled and that water with acceptable quality is continuously produced and supplied. Specimens should be collected at the representative locations in the facilities for producing and supplying water, with particular care so that collected specimens reflect the operating condition of the pharmaceutical water processing system. An adequate protocol for the control of microorganisms at the sampling site should be established considering with the situation around the site.

Sampling frequency should be adequately established based on the data from validation studies on the system. For microbiological monitoring, it is adequate to use the water specimens for the test within 2 hours after sampling. In the case that it is not possible to test within 2 hours, the specimens should be kept at 2 – 8°C and be used for the test within 12 hours.

4.3. Alert and Action Levels

In producing pharmaceutical water using a water processing system, microbiological and physicochemical monitoring is usually carried out to assure that water with required quality is being continuously produced when the system is operating as it designed. The operating condition of the system can be estimated by the comparison of

Table 2 Methods for Assessment of Viable Counts in Pharmaceutical Water

Method	Pharmaceutical Water		
	Water	*Purified Water*	*Water for Injection*
Measurement Method	Pour Plate Method or Membrane Filtration	Pour Plate Method or Membrane Filtration	Membrane Filtration
Minimum Sample Size	1.0 mL	1.0 mL	100 mL
Media	R2A Agar Medium Standard Agar Medium	R2A Agar Medium	R2A Agar Medium
Incubation Period	R2A Agar Medium: 4 – 7 days (or longer) Standard Agar Medium: 48 – 72 hours (or longer)	4 – 7 days (or longer)	4 – 7 days (or longer)
Incubation Temperature	R2A Agar Medium: 20 – 25°C or 30 – 35°C Standard Agar Medium: 30 – 35°C	20 – 25°C or 30 – 35°C	20 – 25°C or 30 – 35°C

monitoring data thus obtained against the alert level, action level, other levels for controlling the system, and acceptance criteria specified for the water required for its intended use, and also by the trend analysis of monitoring data through plotting them in a control chart.

In this manner, the alert level and action level are used for controlling the process of water production, and not used for judging pass/fail of produced water.

4.3.1. Definition of Alert Level

''Alert level'' indicates that, when exceeded it, the system is threatening to deviate from its normal operating range. Alert levels are used for giving a warning, and exceeding them does not necessarily require a corrective action. Alert levels are generally established either at a mean + 2σ on the basis of past trend analysis, or at a level of 70% (50% for viable counts) of the action level, whichever is lower.

4.3.2. Definition of Action Level

''Action level'' indicates that, when exceeded it, the system has deviated from its normal operating range. Exceeding it indicates that corrective action must be taken to bring the system back within its normal operating range.

Alert and action levels should be established within the processes and the quality specifications of products in consideration of available technologies and the quality required for the products. Consequently, exceeding an alert or action level does not necessarily indicate that the quality of produced water has become inadequate for its intended use.

4.4. Microbiological Monitoring

The main purpose of a microbiological monitoring program for a pharmaceutical water processing system is to foresee any microbiological quality deterioration of the produced water, and to prevent any adverse effects on the quality of pharmaceutical products. Consequently, detecting all of the microorganisms present in the water to be monitored may not be necessary. However it is required to adopt a monitoring technique able to detect a wide range of microorganisms, including slow growing microorganisms.

The following indicate incubation-based microbiological monitoring techniques for pharmaceutical water processing systems. To adopt a rapid microorganism detection technique, it is necessary to confirm in advance that the microbial counts obtained by such techniques are equivalent to those obtained by the incubation-based monitoring techniques.

4.4.1. Media and Incubation Conditions

There are many mesophilic bacteria of heterotrophic type that are adapted to poor nutrient water environments. Heterotrophic bacteria may form bio-films in many pharmaceutical water processing systems, and to cause quality deterioration of the produced water. Therefore, it is useful to monitor microbiological quality of water by using the R2A Agar Medium, which is excellent for growing bacteria of oligotrophic type.

Table 2 shows examples of measurement methods, minimum sample sizes, media, and incubation periods for estimating viable counts.

The media shown in Table 2 are as follows.

(i) Standard Agar Medium

Casein peptone	5.0 g
Yeast extract	2.5 g
Glucose	1.0 g
Agar	15.0 g
Water	1000 mL

Mix all the ingredients, and sterilize by heating in an autoclave at 121°C for 15 – 20 minutes. pH after sterilization: 6.9 – 7.1.

(ii) R2A Agar Medium

Peptone (casein and animal tissue)	0.5 g
Casamino acid	0.5 g
Yeast extract	0.5 g
Sodium pyruvate	0.3 g
Glucose	0.5 g
Magnesium sulfate heptahydrate	50 mg
Soluble starch	0.5 g
Dipotassium hydrogen phosphate	0.3 g
Agar	15.0 g
Water	1000 mL

Mix all the ingredients, and sterilize by heating in an autoclave at 121°C for 15 – 20 minutes. pH after sterilization: 7.1 – 7.3.

The following reagents should be used for preparing the R2A Agar Medium.

(i) Casamino acid Prepared for microbial test, by the acid hydrolysis of casein.

Loss on drying ⟨*2.41*⟩: Not more than 8% (0.5 g, 105°C, constant mass).

Residue on ignition ⟨*2.44*⟩: Not more than 55% (0.5 g).

Nitrogen content ⟨*1.08*⟩: Not less than 7% (105°C, constant mass, after drying).

4.4.2. Media Growth Promotion Test

In the media growth promotion test with the R2A Agar Medium, use test strains listed below or other test strains

considered equivalent to these test strains.

Methylobacterium extorquens: NBRC 15911

Pseudomonas fluorescens: NBRC 15842, ATCC 17386, etc.

Prior to the test, inoculate these test strains into sterile purified water and starve them at 20 – 25°C for 3 days. Dilute the fluid containing the test strain starved with sterile purified water to prepare microbial suspensions. When inoculating the R2A Agar Medium with the micro-organisms ($5 \times 10^1 - 2 \times 10^2$ CFU) and incubating at 20 – 25°C for 4 – 7 days, sufficient proliferation of the inoculated strains must be observed.

In the media growth promotion test with the Standard Agar Medium, use test strains listed below or other test strains considered equivalent to these test strains.

Staphylococcus aureus: ATCC 6538, NCIMB 9518, CIP 4.83 or NBRC 13276

Pseudomonas aeruginosa: ATCC 9027, NCIMB 8626, CIP 82.118 or NBRC 13275

Escherichia coli: ATCC 8739, NCIMB 8545, CIP 53.126 or NBRC 3972

Prepare microbial suspensions containing the test strains according to the procedure prescribed in Microbiological Examination of Non-sterile Products <*4.05*>. When inoculating the Standard Agar Medium with a small number (not more than 100 CFU) of the micro-organisms and incubating at 30 – 35°C for 48 hours, sufficient proliferation of the inoculated strains must be observed.

4.4.3. Action Levels for Microorganisms for Pharmaceutical Water Processing System

The following action levels are considered appropriate and generally applicable to pharmaceutical water processing systems.

Action Levels for viable counts in various types of pharmaceutical water

Water: 10^2 CFU/mL* (Acceptance criterion prescribed in the Quality Standards for Drinking Water provided under the Article 4 of the Water Supply Law)

Purified Water: 10^2 CFU/mL**

Water for Injection: 10^1 CFU/100 mL**

(*Viable counts obtained using the Standard Agar Medium, ** Viable counts obtained using the R2A Agar Medium)

Although the action level for *Purified Water* shown above is set at the same level as that for *Water*, it is recommended for each facility to perform a higher level of microbiological control of water processing system based on the action level established individually.

When actual counts in validation studies or routine control exceed the above action levels, it is necessary to isolate and identify the microorganisms present in the water, and to sanitize or disinfect the affected system.

4.5. Physicochemical Monitoring

Physicochemical monitoring of a pharmaceutical water processing system is usually performed using conductivity and TOC as the indicators for water quality. By monitoring conductivity, total amounts of inorganic salts present in the water can be estimated, and by monitoring TOC, total amount of organic compounds present in the water can be estimated. Normally, Conductivity Measurements <*2.51*> and Test for Total Organic Carbon <*2.59*> specified in the General Tests, Processes and Apparatus of the JP should be applied to these physicochemical monitoring. However, since tests for monitoring are performed in the situations different from those for judging pass/fail to the acceptance criteria prescribed in the monographs, supplements necessary to cover the situations to which the JP general tests cannot be applied, are described below.

To adopt the monitoring using conductivity and TOC as the indicators for inorganic and organic impurities at individual facility, appropriate alert and action levels for each indicator, and countermeasures against unexpected apparatus failures should be established.

4.5.1. Monitoring of Conductivity as the Indicator for Inorganic Impurities

Measurement of conductivity for monitoring is usually conducted continuously using an in-line apparatus with a flow-through type or pipe-insertion type cell. Alternatively, offline batch testing may be performed using a dip type cell with water specimens taken at appropriate locations of the pharmaceutical water processing system.

For the operation control of a pharmaceutical water processing system, guides for judging whether it is adequate to continue the operation of the system or not based on the results from monitoring of conductivity, are shown below, both for the cases of monitoring at the standard temperature (20°C) by applying Conductivity Measurements <*2.51*> of the JP and monitoring at temperatures other than 20°C by applying <645> *WATER CONDUCTIVITY* of the United States Pharmacopeia (USP) with some modifications.

4.5.1.1. Monitoring of Conductivity by applying Conductivity Measurements <*2.51*> of the JP

When the monitoring of the conductivity of *Purified Water* and *Water for Injection* is performed at the standard temperature (20°C), measure the conductivity after confirming that the measure temperature is within a range of 20 ± 1°C. In this case, the recommended allowable conductivity (action level) for *Purified Water* and *Water for Injection* is as follows.

- Action Level $1.1\ \mu S \cdot cm^{-1}$ (20°C)

Since the above allowable conductivity is established for in-line monitoring, an alternative action level may be used for the monitoring based on offline batch testing.

4.5.1.2. Monitoring of Conductivity by applying <645> *WATER CONDUCTIVITY* of the USP with some modification

Usually, it is somewhat difficult to control the temperature exactly in in-line conductivity monitoring. Therefore, the following approach can be applied for the monitoring at temperatures other than the standard temperature (20°C) of the JP. This approach is based on the Stages 1 and 2 of the three-stage approach described in ''<645> *WATER CONDUCTIVITY*'' of the USP and in the monographs being associated with water for pharmaceutical use (''Purified Water'', ''Highly Purified Water'' and ''Water for Injections'') of the European Pharmacopoeia (EP).

Stage 1 (In-line Measurement)

(i) Determine the temperature and the conductivity of the water specimens using a non-temperature-compensated conductivity reading.

(ii) From the Table 3, find the temperature value equal to or just lower than the measured temperature. Adopt the corresponding conductivity value on this table as the allowable conductivity at the measured temperature.

(iii) If the observed conductivity is not greater than the allowable conductivity adopted above, the water tested meets the requirement for monitoring conductivity. If the observed conductivity exceeds the allowable conductivity, proceed with Stage 2.

Stage 2 (Off-line Measurement)

(i) Measure the conductivity of the water specimen, by transferring it into a container and agitating it vigorous-

Table 3 Stage 1 Allowable Conductivity for Different Temperatures*

Temperature (°C)	Allowable Conductivity ($\mu S \cdot cm^{-1}$)	Temperature (°C)	Allowable Conductivity ($\mu S \cdot cm^{-1}$)
0	0.6		
5	0.8	55	2.1
10	0.9	60	2.2
15	1.0	65	2.4
20	1.1	70	2.5
25	1.3	75	2.7
30	1.4	80	2.7
35	1.5	85	2.7
40	1.7	90	2.7
45	1.8	95	2.9
50	1.9	100	3.1

* Applicable only to non-temperature-compensated conductivity measurements.

ly in order to attain equilibrium between the water specimen and the atmosphere on absorbing/desorbing carbon dioxide.

(ii) Transfer a sufficient amount of water to be tested into a suitable container, and stir the water specimen. Adjust the temperature to 25 ± 1°C, and begin agitating the water specimen vigorously, while observing the conductivity periodically. When the change in conductivity becomes not greater than 0.1 $\mu S \cdot cm^{-1}$ per 5 minutes, adopt the observed value as the conductivity (25°C) of the water specimen.

(iii) If the conductivity of the water specimen at 25°C obtained above is not greater than 2.1 $\mu S \cdot cm^{-1}$, the water tested meets the requirement for monitoring conductivity. If the observed value exceeds 2.1 $\mu S \cdot cm^{-1}$, it should be judged that the water tested does not meet the requirement for monitoring conductivity.

4.5.2. Monitoring of TOC as the Indicator for Organic Impurities

The acceptance criterion of TOC is specified as ''not greater than 0.50 mg/L (500 ppb)'' in the monographs of *Purified Water* and *Water for Injection*. However it is recommended for each facility preparing pharmaceutical water to conduct operation control of pharmaceutical water processing system through TOC monitoring on produced water based on its own alert and action levels for TOC determined individually. The following are the recommended action levels for TOC.

- Action Level: ≦300 ppb (in-line)
  ≦400 ppb (off-line)

The Quality Standards for Drinking Water provided under the Article 4 of the Japanese Water Supply Law require that TOC of tap water (*Water*) should be ''not greater than 3 mg/L (3 ppm)''. Taking the above recommended action levels into consideration, it is also recommended for each facility to conduct quality control of source water through TOC monitoring based on its own alert and action levels for TOC determined individually.

The JP specifies *Test for Total Organic Carbon* <*2.59*>, and normally, TOC measurement should be conducted using an apparatus which meets the requirements described in the JP method. However, if a TOC apparatus conforms to the apparatus suitability test requirements described in ''<643> *TOTAL ORGANIC CARBON*'' of the USP, or those described in the ''*Methods of Analysis 2.2.44. TOTAL ORGANIC CARBON IN WATER FOR PHARMACEUTICAL USE*'' of the EP, the apparatus can be used for the monitoring of pharmaceutical water processing system, if sufficiently pure water not contaminated with ionic organic substances, or organic substances having nitrogen, sulfur, phosphorus or halogen atoms in their chemical structures, is used as the source water supplied to the system.

A TOC apparatus, characterized by calculating the amount of organic carbon from the difference in conductivity before and after the decomposition of organic substances without separating carbon dioxide from the sample solution, may be influenced negatively or positively, when applied to the water specimens containing ionic organic substances, or organic substances having nitrogen, sulfur, phosphorus or halogen atoms in their chemical structures. Therefore, the apparatus used for TOC monitoring should be selected appropriately in consideration of the purity of the water to be monitored and the contamination risk in the case of apparatus failure.

4.6. Storage of *Water for Injection*

In storing *Water for Injection* temporarily, adequate measures able to prevent microbial proliferation stringently, such as circulating it in a loop at a high temperature must be taken, and an appropriate storage time should also be established based on the validation studies, in consideration of the risks of contamination and quality deterioration.

5. Points to Consider for Assuring the Quality of Pharmaceutical Water in Containers

There are some specific points to consider for assuring the quality of pharmaceutical water in containers (*Purified Water in Containers*, *Sterile Purified Water in Containers* and *Sterile Water for Injection in Containers*), which are available as commercially products.

5.1. Methods for Preparing Sterile Pharmaceutical Water in Containers

The following two different preparation methods are described in the monographs of *Sterile Purified Water in Containers* and *Sterile Water for Injection in Containers*.

(i) Introduce *Purified Water* or *Water for Injection* into a hermetic container, seal up the container, then sterilize the product.

(ii) Make *Purified Water* or *Water for Injection* sterile by using a suitable method, introduce the sterilized water into a sterile hermetic container by applying aseptic manipulation, then seal up the container.

For assuring the sterility of pharmaceutical water products, only the validation of final sterilization process is required in the case of preparation method (i), whereas validations of all the processes are indispensable in the case of preparation method (ii), since the latter is based on the idea to assure the sterility of pharmaceutical water products by ''aseptically'' introducing *Purified Water* (or *Water for Injection*) treated in advance with filtration sterilization, etc. into a sterile hermetic container, and sealing it up.

5.2. Deterioration of Water Quality during the Storage in Containers

5.2.1. Inorganic impurities (Conductivity as the indicator)

The conductivity of pharmaceutical water in containers may increase to some higher levels due to the absorption of carbon dioxide from the atmosphere at the time of its preparation and that passed through plastic layer of the containers during storage, and also due to ionic substances released from the containers, even if the conductivity of *Purified Water* or *Water for Injection* used for its production is maintained at the level not more than 1.0 $\mu S \cdot cm^{-1}$. Particularly in the cases of pharmaceutical water products packed in

small scale glass containers, it is necessary to pay attention to the change of conductivity during storage.

5.2.2. Organic impurities (Potassium Permanganate-reducing Substances or Total Organic Carbon (TOC) as the indicator)

The JP specifies the classical test of potassium permanganate-reducing substances in the monographs of *Purified Water in Containers*, *Sterile Purified Water in Containers* and *Sterile Water for Injection in Containers* for controlling organic impurities in pharmaceutical water in containers. It forms a remarkable contrast to the specifications of *Purified Water* and *Water for Injection*, in which the JP requires to control organic impurities in pharmaceutical water in bulk based on the test of TOC (acceptance criterion: not more than 0.5 mg/L (500 ppb)). This is because that it is considered difficult to establish the specification of pharmaceutical water in containers for organic impurities based on the test of TOC from the facts that there were many cases of remarkable increases in TOC values after storage of water in containers. Particularly in the cases of pharmaceutical water products packed in small scale plastic containers, it is necessary to pay attention to the increase of materials released from containers during storage.

The test of potassium permanganate-reducing substances is retained in the specifications of pharmaceutical water in containers, not as the most suitable method for the test of organic impurities present in the water in containers, but as a counter measure for performing the test of the water in containers with the same test method despite of the material (glass, polyethylene, polypropylene, etc.) and the size (0.5 – 2000 mL) of the containers, and the duration of storage. Therefore, it is recommended to adopt the test of TOC as the alternative for the test of potassium permanganate-reducing substances, and to perform quality control of pharmaceutical water in containers based on TOC measurements under the responsibility of each manufacturer, if possible.

In such cases, it is recommended to adopt the following values as the levels preferable to attain.

For products containing not more than 10 mL of water:
TOC not greater than 1500 ppb
For products containing more than 10 mL of water:
TOC not greater than 1000 ppb

As for the pharmaceutical water packed in the plastic containers made of polyethylene, polypropylene, etc., in addition to the concern for the release of materials such as monomer, oligomers, plasticizers, etc. from plastics, it is necessary to pay attention to the storage environment of the products to avoid the contaminations with low molecular volatile organics such as ethanol, or low molecular air pollutants such as nitrogen oxides, since these plastics have the properties of permeating various gases and water.

5.2.3. Microbial Limit (Total Aerobic Viable Counts)

For *Purified Water in Containers*, it is not required to assure the sterility, but it is necessary to produce it by using sanitary or aseptic processes in order to meet the acceptance criterion of ''10^2 CFU/mL'' for total aerobic viable counts throughout the period of their storages. It is also necessary to take special care against microbial contamination during its circulation. In addition, it is recommended to use them as soon as possible after opening their seals.

The acceptance criterion of ''10^2 CFU/mL'' for total aerobic viable counts of *Purified Water in Container* is at the same level as the action level for viable counts in the production of *Purified Water* (in bulk). However, different from the case of microbiological monitoring of *Purified Water*, Soybean-Casein Digest Agar Medium is used for the test of total aerobic viable counts of *Purified Water in Containers* to detect microorganisms contaminated from the surroundings during its storage.

5.3. Points to consider in the case that commercially available products of pharmaceutical water in containers are used for the manufacture of pharmaceutical products

It is allowable to use commercially available products of pharmaceutical water in containers (*Purified Water in Containers*, *Sterile Purified Water in Containers* and *Sterile Water for Injection in Containers*) for the manufacture of pharmaceutical products and products for clinical trial, and for the tests of pharmaceutical products. In such cases, it is necessary to consider the following points.

(i) Use them soon after confirming their compliances to the requirements of the JP monograph from the test results at the time of its receipt or those offered from the supplier of the products.

(ii) In the case that such products are used for manufacturing pharmaceutical products, it is necessary to validate the process in which the water was used as a part of process validation of pharmaceutical products. In the case that they are used for manufacturing products for clinical trial, it is necessary to confirm that the water doesn't give any adverse effects on the quality of the products.

(iii) The products of sterile pharmaceutical water in containers should be used only once after opening their seals, and it must be avoided to use them again after storage.

(iv) It is recommended to prepare a standard operation practice (SOP) adequate for its intended use, considering that the contamination and quality deterioration of the water due to human and laboratory environmental origins might go on rapidly immediately after opening the product seal.

International Harmonization Implemented in the Japanese Pharmacopoeia Eighteenth Edition

〈GZ-3-180〉

Information on items for which harmonization has been agreed among the European Pharmacopoeia, the United States Pharmacopeia and the Japanese Pharmacopoeia is available on the following websites of Pharmaceuticals and Medical Devices Agency:

General chapters:
https://www.pmda.go.jp/rs-std-jp/standards-development/jp/0021.html
Monographs:
https://www.pmda.go.jp/rs-std-jp/standards-development/jp/0020.html

Appendix

Atomic Weight Table (2017)

Atomic Weights Subcommittee of the Chemical Society of Japan

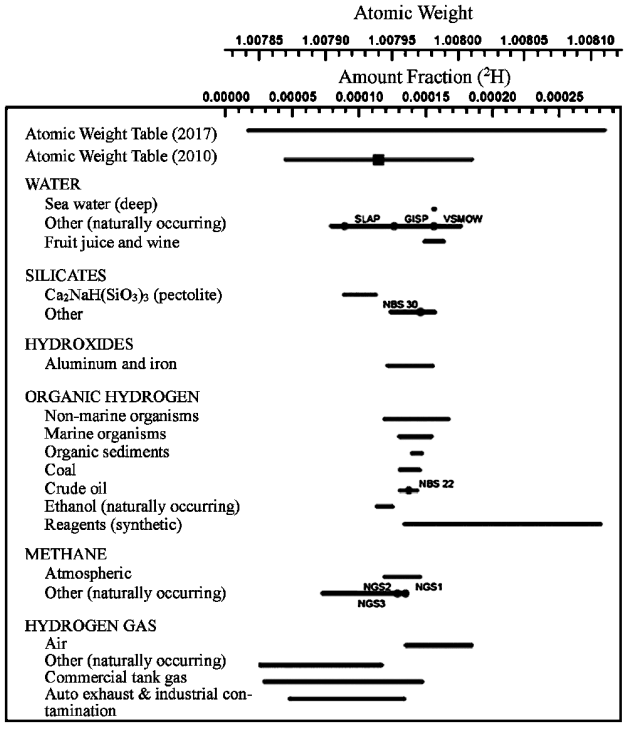
In 1961 it was decided that the atomic weights of the elements would be based on values relative to the mass number of 12 (no fractions) for carbon (^{12}C). Ever since, there has been a marked improvement in the quality and quantity of data on the nuclide masses and isotope ratios of the elements using physical methods such as mass spectrometry. The Commission on Isotope Abundances and Atomic Weights (CIAAW) of the International Union of Pure and Applied Chemistry (IUPAC) collected and examined newly measured data and revises the atomic weight table every two years (in the odd years). Based on this table, in April of each year the Atomic Weights Subcommittee of the Chemical Society of Japan also publishes an atomic weight table. The numbers of the following Atomic Weight Table (2017) is based on the numbers approved by the IUPAC in 2015[1]. For a more detailed explanation, the user is referred to a report[2] and a review[3] published by the CIAAW.

The atomic weight values of each of the elements shown in the atomic weight tables can, with the exception of single nuclide elements (elements consisting of one stable nuclide), change depending on a variety of factors, such as the method of treatment or the origin of the substance containing that element. This is because the atomic weight is dependent on the relative frequency (isotope ratio of an element) of the stable nuclides comprising each of the respective elements. Due to advancements in measurement techniques, the isotopic frequencies of each of the elements are not necessarily constant, and fluctuate due to a variety of processes that occur on the Earth. We have come to learn that this is reflected in the atomic weights. Based on such background, in 2009, the IUPAC decided to indicate the atomic weights for 10 elements not as single values but as ranges[4]. The Atomic Weights Subcommittee of the Chemical Society of Japan discussed the change and decided to reflect the IUPAC's guidelines to the Atomic Weight Table (2011) and subsequent atomic weight tables and to indicate the atomic weights of such elements as ranges and, as before, those of other elements as single values with uncertainty.

Expression of Atomic Weight as Range

At present, the range is used for indicating the atomic weight for 12 elements: hydrogen, lithium, boron, carbon, nitrogen, oxygen, magnesium, silicon, sulfur, chlorine, bromine, and thallium. The isotope composition of these elements in samples collected on Earth or reagents is known to vary greatly. An atomic weight value and its uncertainty were previously defined so as to the range would be included, and an element having geological samples of which the range was not included was indicated by a ''g'' and an element possibly used as isotopes that had undergone artificial fractionation as a reagent was indicated by an ''m''. Furthermore, an ''r'' is attached to an element for which a precise atomic weight cannot be given due to such great range, no matter how much progress is made in techniques for measuring. The figure below shows the isotope compositions of hydrogen in various samples and corresponding atomic weights. The top line indicates the atomic weight range (1.00784–1.00811), and the second line indicates the atomic weight in the Atomic Weight Table (2010) (1.00794 ± 0.00007), followed by values measured in various samples. Black dots indicate values of typical isotope reference materials; the precision of measurement of the isotope composition of hydrogen is ± 0.000 000 05 according to ''best measurement''[5], which is not more than 1/1000 of the uncertainty shown in the Atomic Weight Table (2010). Under such situation, expressing atomic weights as single values with uncertainty included the following problems:

• uncertainty of the atomic weight might be misunderstood as the precision of measurement;



• distribution of atomic weight values is not always a Gaussian distribution and depends on elements;

• a new measured value exceeding the current atomic weight range might cause modification of not only the uncertainty but also the atomic weight so as to include the new value; and

• discovering of a real substance having a defined atomic weight value is often difficult or rather impossible.

An atomic weight of such elements is represented not as a single value but as a range so as to include atomic weights in all known samples in this revision, which clearly shows that the atomic weight is not constant. Furthermore, the distribution within the range is not shown in the Atomic Weight Table and is various depending on elements[4]. The range should, therefore, be used with attention to the following points:

• the intermediate value of the range shall not be expressed as the atomic weight value and the half width of the range shall not be expressed as the uncertainty;

• the upper and lower limits themselves have no uncertainty although they are determined on the basis of values measured in ordinary substances on Earth in addition to measurement errors; and

• the atomic weight values are expressed in possible digits and should be expressed fully even if the last digit is zero.

1) IUPAC Inorganic Chemistry Division, CIAAW: Standard Atomic Weight of Ytterbium Revised, *Chem. Int.*, **37** (5-6), 26 (2015).
2) J. Meija *et al.*: Atomic Weights of the Elements 2015 (IUPAC Technical Report), *Pure Appl. Chem.*, to be published. J. Meija *et al.*: Atomic Weights of the Elements 2013 (IUPAC Technical Report), *Pure Appl. Chem.*, **88**, 265 (2016).
3) J. R. De Laeter *et al.*: Atomic Weights of the Elements: Review 2000 (IUPAC Technical Report), *Pure Appl. Chem.*, **75**, 683 (2003).
4) M. E. Wieser and T. B. Coplen: Atomic Weights of the Elements 2009 (IUPAC Technical Report), *Pure Appl. Chem.*, **83**, 359 (2011).
5) M. Berglund and M. E. Wieser: Isotopic Compositions of the Elements 2009 (IUPAC Technical Report), *Pure Appl. Chem.*, **83**, 397 (2011).

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Standard Atomic Weights 2017

(Scaled to $A_r(^{12}C) = 12$, where ^{12}C is a neutral atom in its nuclear and electronic ground state.)

The atomic weights, $A_r(E)$, of many elements vary because of variations in the abundances of their isotopes in normal materials. For 12 such elements, an atomic-weight interval is given with the symbol [*a*, *b*] to denote the set of atomic-weight values in normal materials; thus, $a \leq A_r(E) \leq b$ for the element E. The symbols *a* and *b* denote the bounds of the interval [*a*, *b*]. If a more accurate $A_r(E)$ value for a specific material is required, it should be determined. For 72 elements, $A_r(E)$ values and their evaluated uncertainties (in parentheses, following the last significant digit to which they are attributed) are given.

Name	Symbol	Atomic Number	Atomic Weight	Footnotes
Hydrogen	H	1	[1.00784, 1.00811]	m
Helium	He	2	4.002602(2)	g r
Lithium	Li	3	[6.938, 6.997]	m
Beryllium	Be	4	9.0121831(5)	
Boron	B	5	[10.806, 10.821]	m
Carbon	C	6	[12.0096, 12.0116]	
Nitrogen	N	7	[14.00643, 14.00728]	m
Oxygen	O	8	[15.99903, 15.99977]	m
Fluorine	F	9	18.998403163(6)	
Neon	Ne	10	20.1797(6)	g m
Sodium	Na	11	22.98976928(2)	
Magnesium	Mg	12	[24.304, 24.307]	
Aluminium (Aluminum)	Al	13	26.9815385(7)	
Silicon	Si	14	[28.084, 28.086]	
Phosphorus	P	15	30.973761998(5)	
Sulfur	S	16	[32.059, 32.076]	
Chlorine	Cl	17	[35.446, 35.457]	m
Argon	Ar	18	39.948(1)	g r
Potassium	K	19	39.0983(1)	
Calcium	Ca	20	40.078(4)	g
Scandium	Sc	21	44.955908(5)	
Titanium	Ti	22	47.867(1)	
Vanadium	V	23	50.9415(1)	
Chromium	Cr	24	51.9961(6)	
Manganese	Mn	25	54.938044(3)	
Iron	Fe	26	55.845(2)	
Cobalt	Co	27	58.933194(4)	
Nickel	Ni	28	58.6934(4)	r
Copper	Cu	29	63.546(3)	r
Zinc	Zn	30	65.38(2)	r
Gallium	Ga	31	69.723(1)	
Germanium	Ge	32	72.630(8)	
Arsenic	As	33	74.921595(6)	
Selenium	Se	34	78.971(8)	r
Bromine	Br	35	[79.901, 79.907]	
Krypton	Kr	36	83.798(2)	g m
Rubidium	Rb	37	85.4678(3)	g
Strontium	Sr	38	87.62(1)	g r
Yttrium	Y	39	88.90584(2)	
Zirconium	Zr	40	91.224(2)	g
Niobium	Nb	41	92.90637(2)	
Molybdenum	Mo	42	95.95(1)	g
Technetium*	Tc	43		
Ruthenium	Ru	44	101.07(2)	g
Rhodium	Rh	45	102.90550(2)	
Palladium	Pd	46	106.42(1)	g
Silver	Ag	47	107.8682(2)	g
Cadmium	Cd	48	112.414(4)	g
Indium	In	49	114.818(1)	
Tin	Sn	50	118.710(7)	g
Antimony	Sb	51	121.760(1)	g
Tellurium	Te	52	127.60(3)	g
Iodine	I	53	126.90447(3)	
Xenon	Xe	54	131.293(6)	g m
Caesium (Cesium)	Cs	55	132.9054519(2)	
Barium	Ba	56	137.327(7)	
lanthanum	La	57	138.90547(7)	g
Cerium	Ce	58	140.116(1)	g
Praseodymium	Pr	59	140.90766(2)	
Neodymium	Nd	60	144.242(3)	g
Promethium*	Pm	61		
Samarium	Sm	62	150.36(2)	g
Europium	Eu	63	151.964(1)	g
Gadolinium	Gd	64	157.25(3)	g
Terbium	Tb	65	158.92535(2)	
Dysprosium	Dy	66	162.500(1)	g
Holmium	Ho	67	164.93033(2)	
Erbium	Er	68	167.259(3)	g
Thulium	Tm	69	168.93422(2)	
Ytterbium	Yb	70	173.045(10)	g
Lutetium	Lu	71	174.9668(1)	g
Hafnium	Hf	72	178.49(2)	
Tantalum	Ta	73	180.94788(2)	
Tungsten	W	74	183.84(1)	
Rhenium	Re	75	186.207(1)	
Osmium	Os	76	190.23(3)	g
Iridium	Ir	77	192.217(3)	
Platinum	Pt	78	195.084(9)	
Gold	Au	79	196.966569(5)	
Mercury	Hg	80	200.592(3)	
Thallium	Tl	81	[204.382, 204.385]	
Lead	Pb	82	207.2(1)	g r
Bismuth*	Bi	83	208.98040(1)	
Polonium*	Po	84		
Astatine*	At	85		
Radon*	Rn	86		
Francium*	Fr	87		
Radium*	Ra	88		
Actinium*	Ac	89		
Thorium*	Th	90	232.0377(4)	g
Protactinium*	Pa	91	231.03588(2)	
Uranium*	U	92	238.02891(3)	g m
Neptunium*	Np	93		
Plutonium*	Pu	94		
Americium*	Am	95		
Curium*	Cm	96		
Berkelium*	Bk	97		
Californium*	Cf	98		
Einsteinium*	Es	99		
Fermium*	Fm	100		
Mendelevium*	Md	101		
Nobelium*	No	102		
Lawrencium*	Lr	103		
Rutherfordium*	Rf	104		

Name	Symbol	Atomic Number	Atomic Weight	Footnotes
Dubnium*	Db	105		
Seaborgium*	Sg	106		
Bohrium*	Bh	107		
Hassium*	Hs	108		
Meitnerium*	Mt	109		
Darmstadtium*	Ds	110		
Roentgenium*	Rg	111		
Copernicium*	Cn	112		
Nihonium*	Nh	113		
Flerovium*	Fl	114		
Moscovium*	Mc	115		
Livermorium*	Lv	116		
Tennessine*	Ts	117		
Oganesson*	Og	118		

*: Element has no stable isotopes. However, four elements (Bi, Th, Pa, and U) do have a characteristic isotopic composition, and for these elements, standard atomic weights are tabulated.

g: Geological specimens are known in which the element has an isotopic composition outside the limits for normal material. The difference between the atomic weight of the element in such specimens and that given in the table may exceed the stated uncertainty.

m: Modified isotopic compositions may be found in com-mercially available material because the material has been subjected to an undisclosed or inadvertent isotopic fractionation. Substantial deviations in atomic weight of the element from that given in the table can occur.

r: Range in isotopic composition of normal terrestrial material prevents a more precise A_r(E) being given: the tabulated A_r(E) value and uncertainty should be appli-cable to normal material.

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Standard Atomic Weights 2010

(Scaled to $A_r(^{12}C) = 12$, where ^{12}C is a neutral atom in its nuclear and electronic ground state)

The atomic weights of many elements are not invariant but depend on the origin and treatment of the material. The standard values of $A_r(E)$ and the uncertainties (in parentheses, following the last significant figure to which they are attributed) apply to elements of natural terrestrial origin. The footnotes to this table elaborate the types of variation which may occur for individual elements and which may be larger than the listed uncertainties of values of $A_r(E)$. Names of elements with atomic number 112 to 118 are provisional.

Name	Symbol	Atomic Number	Atomic Weight	Footnotes
Hydrogen	H	1	1.00794(7)	g m r
Helium	He	2	4.002602(2)	g r
Lithium	Li	3	[6.941(2)]†	g m r
Beryllium	Be	4	9.012182(3)	
Boron	B	5	10.811(7)	g m r
Carbon	C	6	12.0107(8)	g r
Nitrogen	N	7	14.0067(2)	g r
Oxygen	O	8	15.9994(3)	g r
Fluorine	F	9	18.9984032(5)	
Neon	Ne	10	20.1797(6)	g m
Sodium	Na	11	22.98976928(2)	
Magnesium	Mg	12	24.3050(6)	
Aluminium	Al	13	26.9815386(8)	
Silicon	Si	14	28.0855(3)	r
Phosphorus	P	15	30.973762(2)	
Sulfur	S	16	32.065(5)	g r
Chlorine	Cl	17	35.453(2)	g m r
Argon	Ar	18	39.948(1)	g r
Potassium	K	19	39.0983(1)	
Calcium	Ca	20	40.078(4)	g
Scandium	Sc	21	44.955912(6)	
Titanium	Ti	22	47.867(1)	
Vanadium	V	23	50.9415(1)	
Chromium	Cr	24	51.9961(6)	
Manganese	Mn	25	54.938045(5)	
Iron	Fe	26	55.845(2)	
Cobalt	Co	27	58.933195(5)	
Nickel	Ni	28	58.6934(4)	r
Copper	Cu	29	63.546(3)	r
Zinc	Zn	30	65.38(2)	r
Gallium	Ga	31	69.723(1)	
Germanium	Ge	32	72.64(1)	
Arsenic	As	33	74.92160(2)	
Selenium	Se	34	78.96(3)	r
Bromine	Br	35	79.904(1)	
Krypton	Kr	36	83.798(2)	g m
Rubidium	Rb	37	85.4678(3)	g
Strontium	Sr	38	87.62(1)	g r
Yttrium	Y	39	88.90585(2)	
Zirconium	Zr	40	91.224(2)	g
Niobium	Nb	41	92.90638(2)	
Molybdenum	Mo	42	95.96(2)	g r
Technetium*	Tc	43		
Ruthenium	Ru	44	101.07(2)	g
Rhodium	Rh	45	102.90550(2)	
Palladium	Pd	46	106.42(1)	g
Silver	Ag	47	107.8682(2)	g
Cadmium	Cd	48	112.411(8)	g
Indium	In	49	114.818(3)	
Tin	Sn	50	118.710(7)	g
Antimony	Sb	51	121.760(1)	g
Tellurium	Te	52	127.60(3)	g
Iodine	I	53	126.90447(3)	
Xenon	Xe	54	131.293(6)	g m
Caesium (Cesium)	Cs	55	132.9054519(2)	
Barium	Ba	56	137.327(7)	
Lanthanum	La	57	138.90547(7)	g
Cerium	Ce	58	140.116(1)	g
Praseodymium	Pr	59	140.90765(2)	
Neodymium	Nd	60	144.242(3)	g
Promethium*	Pm	61		
Samarium	Sm	62	150.36(2)	g
Europium	Eu	63	151.964(1)	g
Gadolinium	Gd	64	157.25(3)	g
Terbium	Tb	65	158.92535(2)	
Dysprosium	Dy	66	162.500(1)	g
Holmium	Ho	67	164.93032(2)	
Erbium	Er	68	167.259(3)	g
Thulium	Tm	69	168.93421(2)	
Ytterbium	Yb	70	173.054(5)	g
Lutetium	Lu	71	174.9668(1)	g
Hafnium	Hf	72	178.49(2)	
Tantalum	Ta	73	180.94788(2)	
Tungsten	W	74	183.84(1)	
Rhenium	Re	75	186.207(1)	
Osmium	Os	76	190.23(3)	g
Iridium	Ir	77	192.217(3)	
Platinum	Pt	78	195.084(9)	
Gold	Au	79	196.966569(4)	
Mercury	Hg	80	200.59(2)	
Thallium	Tl	81	204.3833(2)	
Lead	Pb	82	207.2(1)	g r
Bismuth*	Bi	83	208.98040(1)	
Polonium*	Po	84		
Astatine*	At	85		
Radon*	Rn	86		
Francium*	Fr	87		
Radium*	Ra	88		
Actinium*	Ac	89		
Thorium*	Th	90	232.03806(2)	g
Protactinium*	Pa	91	231.03588(2)	
Uranium*	U	92	238.02891(3)	g m
Neptunium*	Np	93		
Plutonium*	Pu	94		
Americium*	Am	95		
Curium*	Cm	96		
Berkelium*	Bk	97		
Californium*	Cf	98		
Einsteinium*	Es	99		
Fermium*	Fm	100		
Mendelevium*	Md	101		
Nobelium*	No	102		
Lawrencium*	Lr	103		
Rutherfordium*	Rf	104		
Dubnium*	Db	105		
Seaborgium*	Sg	106		
Bohrium*	Bh	107		
Hassium*	Hs	108		

Name	Symbol	Atomic Number	Atomic Weight	Footnotes
Meitnerium*	Mt	109		
Darmstadtium*	Ds	110		
Roentgenium	Rg	111		
Copernicium*	Cn	112		
Ununtrium*	Uut	113		
Ununquadium*	Uuq	114		
Ununpentium*	Uup	115		
Ununhexium*	Uuh	116		
Ununoctium*	Uuo	118		

*: Element has no stable isotopes.

†: Commercially available Li materials have atomic weights that range between 6.939 and 6.996; if a more accurate value is required, it must be determined for the specific material.

g: Geological specimens are known in which the element has an isotopic composition outside the limits for normal material. The difference between the atomic weight of the element in such specimens and that given in the table may exceed the stated uncertainty.

m: Modified isotopic compositions may be found in commercially available material because it has been subjected to an undisclosed or inadvertent isotopic fractionation. Substantial deviations in atomic weight of the element from that given in the table can occur.

r: Range in isotopic composition of normal terrestrial material prevents a more precise A_r(E) being given: the tabulated A_r(E) value should be applicable to any normal material.

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