Guidelines for the Designation of Food Additives and Revision of Standards for Use of Food Additives

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This document is the English translation of "*食品添加物の指定及び使用基準改正に関する指針について*." The Ministry of Health, Labour and Welfare offers this translation as a service to a broad international audience/readers. While the ministry has attempted to obtain translation that is as faithful as possible to the Japanese version, we recognize that the translated version may not be as precise, clear, or complete as the original version. The official version of this document is the Japanese version.

Note: This document is the English translation of "食品添加物の指定及び使用基準改正に関する指針について." Since the Japanese original version was issued on March 22, 1996, some matters in the document have changed, including the names of the ministry and its divisions and article numbers of the Food Sanitation Act.

Attachment

Guidelines for the Designation of Food Additives and Revision of Standards for Use of Food Additives

I Purpose

The guidelines provide the procedures required to apply for the designation of chemically synthesized substances intended for use as food additives pursuant to Article 6 of the Food Sanitation Act and for the establishment of standards for use of food additives pursuant to Article 7(1) of the said Act, the scope of documents that should be submitted with the application, such as results of safety studies, and standard testing methods necessary to generate the documents.

II Basic policies on designation of food additives and revision of standards for use of food additives

Food additives should pose no risk to human health and provide consumers with some kind of benefit when used.

Thus, in designating food additives and in revising standards for use, it is important that the points shown below are scientifically assessed. For this reason, the Food Sanitation Investigation Council ("the Council") conducts scientific evaluations from the perspective of public health, in consideration of food consumptions in Japan as well as the overseas food additive standards, such as those of Joint FAO/WHO Codex Alimentarius Commission.

1. Safety

The safety of the food additive is proven or confirmed in the intended and applied use methods for which the application is made.

2. Effectiveness

It shall be proven or confirmed that the use of the food additive comes under one or more of the purposes specified below. However, if the manufacturing or processing methods/techniques for the target food can be improved or modified in relatively inexpensive ways and the improved or modified methods/techniques do not require the use of the additive, such a case is excluded from the necessity of the food additive/the use of the food additive is deemed unnecessary. (1) To preserve the nutritional quality of food

An intentional reduction of nutritional quality of the food may be justified, when section (2) below is applicable or the food is not important in ordinary diets.

(2) To provide ingredients or constituents necessary to manufacture food for certain groups of consumers who need specific diets

However, if the food additive is intended for the treatment of diseases or other medical purposes, such a case is excluded.

(3) To enhance the keeping quality or stability of food or to improve the organoleptic properties, such as taste and vision.

However, if its use may lead to any change in the nature, substance, or quality of the food, therefore deceiving the consumer, such a case is excluded

(4) To provide supplementary roles in the manufacture, processing, preparation, treatment, packing, transportation, or storage of food

However, if its use is intended to disguise the use of faulty ingredients or undesirable (including unhygienic) practices or techniques during any of the processes, such a case is excluded.

III Procedures for designation of food additives or revision of standards for use of food additives

1. Application

Those who apply for designation of a food additive or for revision of standards for use may submit an application to the Minister of Health and Welfare using Form No. 1 or Form No. 2 as described in the appendix. The application must be accompanied by documents, such as on draft specifications and draft standards for use of the food additive, as well as its safety.

Further, if the applicant resides overseas, a person who is able to respond responsibly in Japanese (Contact person in Japan) to matters concerning the application should be specified in the application. The application should be directly addressed to the Food Chemistry Division, the Environmental Health Bureau of the Ministry of Health and Welfare.

2. Submission of draft specifications and draft standards for use

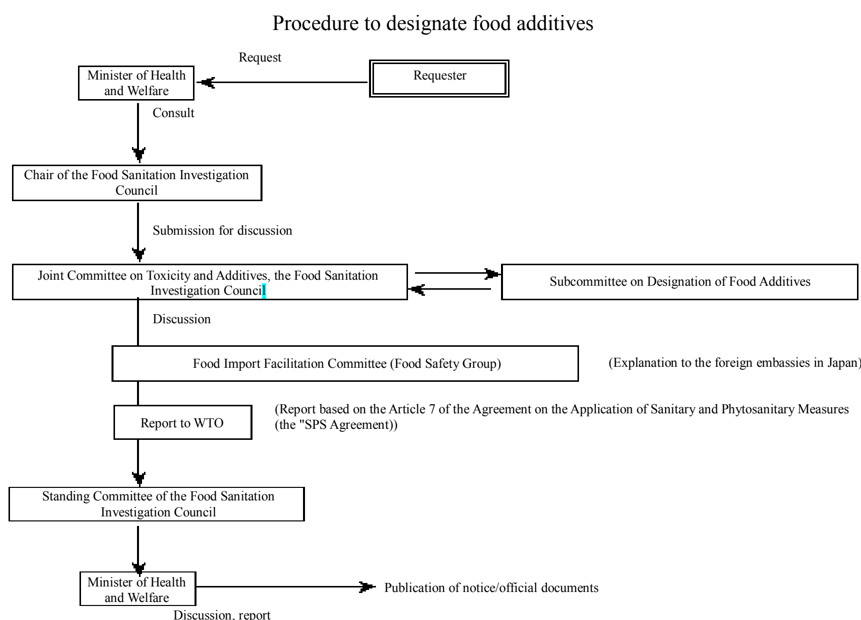
(1) For applying for the designation of a food additive, in principle, draft specifications for the food additive should accompany the application. Draft standards for use should be attached, if it is necessary to limit target foods in which the food additive is used and the amount and method of use of the food additive.

(2) For applying for revision of standards for use of a food additive, a list contrasting the existing standards and the proposed draft standards for the food additive should accompany the application.

3. Examination

The documents submitted for designation of food additives or revision of standards for use will first be reviewed by the Food Chemistry Division, the Environmental Health Bureau of the Ministry of Health and Welfare (the “MHW”). If it is deemed necessary to hear the opinion of the Council, administrative procedure to consult the Council will be initiated.

When the examination by the Council is completed, the Council will report the results for matters for which the council opinion has been sought to the Minister of Health and Welfare. Based on the report from the Council, the MHW will conduct necessary procedures, including revision of the Ordinance for Enforcement of the Food Sanitation Act (see the chart below). If necessary, the applicant may be requested to submit additional documents in the course of examination by the Council.



4. Processing period

The standard period of time required for processing, from the receipt of a filed application to the designation of the target food additive or the revision of existing standards for use of the target designated food additive, is one year. This period, however, does not include the time necessary for the applicant to revise the submitted documents and materials, if they are not sufficient, as well as the time necessary for the applicant to reply to inquiries by the Food Sanitary Investigation Council or other relevant parties.

IV Documents to accompany application for the designation of food additives and revision of standards for use of food additives

1. Scope of accompanying documents

(1) For application for designation of a food additive, in principle, the documents listed in Table 1 should be submitted.

If the food additive is a common food constituent or it is scientifically proven that the food additive will be decomposed in the food or in the human gastrointestinal tract into common food constituents, in principle, the documents concerning toxicity in Table 1 may be exempted from submission. However, it is preferable to submit documents on a 28-day repeated dose toxicity study and a genotoxicity study in rodents.

It should be determined whether the food additive meets the above-mentioned conditions for exemption from document submission after due examination/consideration of the items specified in Table 2.

(2) For application for revision of standards for use, the documents marked with ○ in Table 1 are necessary in principle. Documents marked with △ should be submitted, when new findings are obtained after the food additive was designated or deemed necessary.

(3) Notwithstanding the requirement of (1) and (2), the applicant may be exempted from the submission of some documents with the provision of the reason, when the food

additive is identical to an already designated food additive with exception in different bases, when the food additive is an isomer of an already designated food additive, or when any other legitimate reason exists.

(4) Documents that would raise doubts about the quality, safety, or effectiveness of the food additive for application should be submitted, without regard to the reliability of the submitted documents.

2. General considerations for preparation of accompanying documents

(1) Applicants should submit documents for application on their responsibility and assume full responsibility for the reliability of the information given in the documents.

(2) The main part of accompanying documents (e full responsibility for the reliability of full resp However, documents other than the overview documentation (quoted documents listed in categories 2 to 6 in Table 1) may be submitted in English.

(3) Studies necessary to prepare the required documents should be conducted at laboratories that have facilities, equipment, and personnel adequate to ensure the credibility of the study results, and that are recognized as appropriately managed.

3. Specific considerations on the preparation of accompanying documents

(1) Overview documentation

① The overview documentation should be described concisely, according to the document category. To identify the documents related, the number of the document being referenced should appear on the upper left or right corner of the corresponding page. In addition, sequential page numbers must be entered on all pages.

② If any quoted documents listed in the Table 1 are omitted from attachment, the reason for the omission should be stated.

(2) Documents on the origin or details of development and use status in other countries

① Origin or details of development
The background that led to application should be described, including information on when and in which country the target substance (hereinafter the target substance refers to “substance for which the designation is requested”) was developed and subsequently in which country it came to be used as a food additive.

② Use status in other countries
Overseas status of the target substance should be described, including authorization status, the names of the foods in which it is permitted for use, the standards for use, and specifications. Also, safety assessments, the standards for use, and specifications of international organizations should be described.

(3) Documents on the physiochemical properties and specifications

Documents should be prepared based on results of the tests appropriately conducted in accordance with “General Tests” and “General Notice” in the official compilation of food additives (Japan’s Specification and Standards for Food Additives, hereinafter the “official compilation of food additives” is referred to as the “compilation”).

① Name
The general name, chemical name (IUPAC name), and other appropriate name should be indicated.

② Structural or rational formula
This item should be described referring to the compilation.

③ Molecular formula and molecular weight
These items should follow "General Notice" of the compilation.

④ Assay
Assay should be established to guarantee constant quality in safety and effectiveness, based on the manufacturing processes, quantitative error, stability, and other conditions.
The content of an effective ingredient as a food additive should be described in percentage. When two or more effective ingredients exist, the content should be described for individual ingredients.

⑤ Manufacturing methods
Since different manufacturing processes could result in different type or amount of impurities, the manufacturing methods should be described concisely.

⑥ Description
Information on description should include items that are required for identification and handling at the time of use, such as the taste, odor, color, and form of the target substance.

⑦ Identification tests
Identification tests are conducted to confirm whether the substance concerned is the target food additive, based on its characteristics. The tests, therefore, should be specific to the characteristics of the substance's chemical structure.
If other tests than the identification tests can specify the food additive, the tests can be considered. For instance, when a highly specific chromatography is employed for quantification, the identification tests can be simplified and overlapping items can be exempted.
Ordinarily, conceivable test methods for identification are based on spectral analysis and chemical reaction. Methods based on chemical reaction should be set if they are appropriate for confirming the characteristics of the chemical structure.

⑧ Specific properties
Specific properties are expressed as numeral values measured by physical and chemical methods, such as absorbance, optical rotation, pH, and melting point. Parameters that are essential to ensure the quality shall be specified

⑨ Purity tests
Purity tests are conducted to determine impurities in the food additive. The purity tests as well as assay define the purity of the food additive. Tests should be set for necessary substances among those that may be present as contaminants in the food additive (raw materials, intermediates, by-products, decomposition products, reagents/catalysts, heavy metals/inorganic salts, and solvents).

⑩ Loss on drying, loss on ignition, or water content
A loss on drying test is conducted to measure the amount of the substances in the food additive that are lost by drying, including free water, all or part of the crystal water, and volatile substances. A loss on ignition test is conducted on the inorganic substances that lose a part of the structural components or inclusions by ignition. Water determination is conducted to identify the water content in the food additive.

⑪ Ignition residue (Residue on ignition)
A residue on ignition test is generally conducted to identify the content of the inorganic substances present as impurities in organic substances. In some cases, the test is conducted to measure the amount of the inorganic substances present in organic substances as the structural components, or the impurities in inorganic substances that volatilize when heated.

⑫ Methods of assay
Assay is a test to measure the content of effective components by physical, chemical, or biological methods. When a comparative test method is set, specifications should be established for the reference standard used in the assay.
Methods shall be established with particular attention on preciseness, reproducibility, and specificity.
However, even low in specificity to the food additive, if the level of substances mixed is controlled with a proper purity test, a method can be employed to ensure the measurement with reproducibility. In such cases, a method with high specificity to the food additive should compensate the lack of specificity to the food additive in assay method.
If constituents to be determined are more than one, they should be described in the order of importance.

⑬ Stability of food additives
The stability of food additives should be examined, including the retrieved information on decomposition products.

⑭ Analytical methods of food additives in foods
In principle, a method to be able to qualitatively and quantitatively identify the addition of the food additive should be established, for example, including chemical analysis, for foods in which the food additive is likely to be used. Examination should be made as to whether the food additive can be quantitatively separated from other food additives with similar purposes.
If establishment of standards for use is not necessary or the food additive does not remain in the food, establishment of quantitative methods may be exempted from the analytical methods of food additives in food.

⑮ Grounds for establishing draft specifications

a) The draft specifications should be established as necessary to ensure a certain level of quality concerning safety and effectiveness of the food additive, based on the information shown in ①-⑫, taking into consideration the specifications established by international organizations.

b) A table comparing the draft specifications with the specifications established by international organizations and foreign countries should be attached.

(4) Documents on effectiveness

①Studies on effectiveness should be conducted to demonstrate the expected effects of the food additives in each use.

For instance, for antioxidants, the tests should elucidate antioxidant effects to the foods in relation to its amount used and time elapsed. For preservatives, tests should elucidate the effectiveness of the food additive in improvement of the preserving property of the foods.

② If food additives with the same use have already been designated, it is preferable to make a comparison with those food additives in terms of effectiveness.

③ The stability of the food additive in food should be examined If it is not stable, the type and quantity of the primary decomposition products generated should be examined.

④ The effect of the food additive on the main nutritional components of the food should be also examined.

(5) Documents on safety

① Documents on toxicity

a) In order to ensure reliability of the toxicity test data, these studies should be conducted in accordance with appropriate GLP (Good Laboratory Practice), such as standards for the conduct of safety studies on drugs.

b) For each toxicity study, standard methods are described in Chapter V to facilitate proper evaluation of the safety of the food additive.
However, it is not reasonable to apply uniform methods to all food additives. New methods may be developed, keeping pace with the advance in scientific technology. If obtained findings can enable scientific safety evaluation of the food additive, examiners may not necessarily adhere to the methods specified in Chapter V.
For instance, studies complying with OECD (Organization for Economic Cooperation and Development) guidelines or USFDA (Food and Drug Administration in US) guidelines are basically acceptable.

c) If a 90-day repeated dose toxicity study is conducted on a rodent or non-rodent species, a 28-day repeated dose toxicity study on the corresponding species may be exempted.

d) If a one-year repeated dose toxicity study and a carcinogenicity study are both conducted on the animal species specified, the combined one-year repeated dose toxicity/ carcinogenicity study is not necessary.
If a combined one-year repeated dose toxicity/carcinogenicity study is conducted on a rodent, a one-year repeated dose toxicity study and a carcinogenicity study on rodent may be exempted.

e) Safety of decomposed product of the food additive and its impurities should be, as necessary, examined.

② Documents on metabolism and pharmacokinetic study

a) A metabolism and pharmacokinetic study should be conducted to estimate absorption, distribution, metabolism, and excretion of the food additive in the living body when consumed by human. Thus, the document should include not only summaries of results on animal studies, but also considerations for extrapolation to human on metabolism and pharmacokinetic and possible adverse effects.

b) General methods on metabolism and pharmacokinetic study are also described in Chapter V. The note on (1) b) above for toxicity also applies.

③ Documents on daily intake of the food additive

a) Daily intake of the food additive should be estimated by multiplying the

daily intake of target foods by the amount of the food additives used in each food. The daily intake of target foods in Japan should be properly estimated using data on the food intake from the National Nutrition Survey or other related sources.

b) Safety of the food additive should be examined by comparing the daily intake with the acceptable daily intake determined by toxicity studies. Safety of the food additive should be also examined, when similar food additives are simultaneously consumed.

Attention should, if necessary, be given to overintake of specific nutrients and effect on the balance of electrolytes in the living body, due to the intake of the food additive, in the light of conditions of Japanese food intake.

(6) Documents on draft standards for use

① When, as a result of comprehensive review of safety and effectiveness of the food additive, it is determined necessary to set standards for use to limit target foods and the amount of food additive used in the target foods, the grounds for the setting of the standards for use should be stated based on documents shown in (2) to (5) above. Draft standards for use should be set by referring to the existing standards for use for other food additives.

② If it is determined unnecessary to set standards for use, the grounds should be stated based on documents shown in (2) to (5) above.

4. Specific considerations on the preparation of documents required to apply for revision of standards for use

"Specific considerations on the preparation of accompanying documents" in Section 3 should be followed. The documents for setting of standards for use should specify the grounds for the revision of the existing standards, including the addition of target foods and the change of the amount of food additive used.

Table 1. Documents required to apply for designation of food additives or revision of standards for use

Document category	Designation	Revision
1. Overview documentation	○	○
2. Documents on the origin or details of development and use status in other countries		
(1) Origin or details of development	○	△
(2) Use status in other countries	○	○
3. Documents on physiochemical properties and specifications		
(1) Name	○	△
(2) Structural or rational formula	○	△
(3) Molecular formula and molecular weight	○	△
(4) Assay	○	△
(5) Manufacturing methods	○	△
(6) Description	○	△
(7) Identification tests	○	△
(8) Specific properties	○	△
(9) Purity tests	○	△
(10) Loss on drying, loss on ignition, or water content	○	△
(11) Ignition residue (Residue on ignition)	○	△
(12) Methods of assay	○	△
(13) Stability of food additives	○	△
(14) Analytical methods of food additives in food	○	△
(15) Grounds for establishing draft specifications	○	△
4. Documents on effectiveness		
(1) Effectiveness and comparison in effects with other similar food additives	○	○
(2) Stability in food	○	△
(3) Effects on nutritional components in food	○	△
5. Documents on safety		
(1) Documents on toxicity	○	△
① 28-day toxicity study	○	△
② 90-day toxicity study	○	△
③ One-year toxicity study	○	△
④ Reproduction study	○	△
⑤ Teratogenicity study	○	△
⑥ Carcinogenicity study	○	△
⑦ Combined chronical toxicity/carcinogenicity study	○	△
⑧ Antigenicity study	○	△
⑨ Mutagenicity study	○	△
⑩ General pharmacological study	○	△
(2) Metabolism and pharmacokinetic studies	○	△
(3) The daily intake of the food additive	○	○
6. Standards for use	○	○

Note:○: Documents necessary to be attached. △: Document should be submitted when deemed necessary (e.g., when a new finding is shown).

Table 2. Consideration to confirm that the food additive be decomposed into common food constituents either in the foods or gastrointestinal tract

1. Under the ordinary conditions to use the food additive, the substance is readily decomposed either in the foods or in the gastrointestinal tract into common food components.

2. Major factors (e.g., pH and enzymes) associated with the decomposition in the foods or gastrointestinal tract are identified.

3. When proper amounts is used under proper usage conditions of the food additive, the substance is absorbed into the body at similar extent as the food components and the absorption of other food components is not interfered.

4. Unhydrolyzed or partially hydrolyzed substances ingested as a food additive are not secreted in stool in large amounts. In addition, unhydrolyzed or partially hydrolyzed substances do not accumulate in body tissues.

5. When foods with the food additive used are consumed, overloading of the major components of the foods does not occur.

Form 1

Date

Minister of the Health and Welfare

Address of applicant
(For a corporation, principal place of business)
Name of applicant
(For a corporation, its name and the representative's name)

Seal

I/We hereby apply for the designation of the substance below as a food additive unlikely to cause damage to human health, pursuant to Article 6 of the Food Sanitation Act.

(Name of the substance)

(Note)
1. Use JIS A4-size paper.
2. Use black ink. Type in clear block letters, if in Japanese.
3. If the applicant resides outside Japan, specify the contact in Japan. The seal may be replaced by the applicant's signature.

Form 2

Date

Minister of the Health and Welfare

Address of applicant
(For a corporation, principal place of business)
Name of applicant
(For a corporation, its name and the representative's name)

Seal

I/We hereby apply for the revision of standards for use of the substance below as a food additive unlikely to cause damage to human health, pursuant to Paragraph 1, Article 7 of the Food Sanitation Act.

(Name of the food additive
Proposed standards for use)

(Notes)
1. Use JIS A4-size paper.
2. Use black ink. Type in clear block letters, if in Japanese.
3. If the applicant resides outside Japan, specify the contact in Japan. The seal may be replaced by the applicant's signature.

V Recommended methods for safety studies

[1] 28-day toxicity study

This study is designed to provide information concerning toxic effects induced when a test substance is administered repeatedly for 28 days to rodents and non-rodents, and to provide dose-setting information for longer term toxicity studies (one year).

1. Animal species and sex

One rodent species (usually the rat) and one non-rodent species (usually the dog) are used. Equal numbers of males and females should be used.

Dosing of rodents (the rat or mouse) should begin as soon as possible after weaning and acclimation, usually before the animals are 5 to 6 weeks of age. Dosing of non-rodents (the dog) should begin between 4 and 6 months of age.

2. Number of animals

Each group should consist of at least 5 animals per sex for rodents, and 4 animals per sex for non-rodents. Animals should be allocated into each group by an appropriate stratified randomization, such as on the basis of body weight.

The number of animals should be sufficient to ensure adequate number of survivors at termination to permit proper evaluation of toxicity.

3. Route of administration

Administration should be oral, usually by incorporating the test substance into the diet or into the drinking water. Gavage administration may be adopted in the following cases where administration in diet or water is difficult: (1) the test substance is unstable in the diet or in water, (2) there is an analytical problem with the substance in the diet or in water, or (3) test animals reject the test diet or the water (unpalatability). Administration of the microcapsulated test substance may be applied under certain experimental conditions.

4. Duration of administration

The test substance should be administered daily for 28 consecutive days.

5. Dose levels and control groups

(1) Dose levels

At least three different dose levels and appropriate controls should be used. In order to establish a comprehensive profile of toxicity of the test substance and to estimate no-observed-adverse-effect level (NOAEL), the dose levels should include the highest level which induces any significant toxicity and the lowest level which does not induce toxicity. Also, the doses used should permit the demonstration of a dose-response relationship. The basis for dose selection must be specified.

Adverse nutritional effects should be avoided when the test substance is administered as a dietary admixture. The concentration of the test substance in the diet does not usually exceed 5% (w/w). For gavage administration, when the technically maximum feasible dose level (but not higher than 1000 mg/kg) fails to elicit any signs of toxicity, that level is to be taken as the highest dose level.

(2) Control groups

All test conditions of the control groups should be the same as for the dosed groups, except that the test substance is not administered.

When a vehicle is used to prepare the test diets or the drinking water solutions, there should be a vehicle control group that receives the same vehicle level as the highest dosed group. If there is insufficient information about the toxic properties of the vehicle used, an additional control group not exposed to the vehicle should be included.

6. Observation and examination

(1) General conditions, body weight, and diet (and/or water) consumption

All test animals should be observed daily.

Cage-side observation of general conditions should be made with special attention to the following;

general appearances including changes in skin, fur, eyes, ears, nose, mouth, perianal area and mucous membranes; exhaustion; obesity/emaciation, or abdominal swelling; body position/posture including prone, crawling, hunchback;

consciousness/attitude including excitement, aggression, inanimation, sedation, lethargy;

behavior including changes in exploration, grooming, motor activity, and abnormal gait;

nervous system (somatomotor activity) including tremors, convulsions, twitch, and reflex functions;

body temperature;

respiration;

excretion.

Body weights and diet and/or water consumption (when the test substance is administered in drinking water) should be measured at least once weekly.

(2) Hematological and blood biochemical examinations

Examinations should be performed on samples taken at necropsy for rodents, and before the start of administration and prior to or at necropsy for non-rodents.

The following parameters are evaluated. Additional tests may be performed as deemed appropriate. Only internationally accepted procedures should be used.

Hematological examination includes erythrocyte count, total and differential leukocyte counts, platelet count, hemoglobin concentration, hematocrit, and if necessary, reticulocyte count, and clotting potential such as prothrombin tine and activated partial thromboplastin time.

Blood biochemical determinations include serum (plasma) total protein, albumin, A/G ratio, protein fraction*, fasting glucose, cholesterol, triglyceride, bilirubin, urea nitrogen, creatinine, transaminases [AST(GOT), ALT(GPT)], ɤ-glutamyl transpeptidase, alkaline phosphatase, and electrolytes including Na, K, Cl, Ca, inorganic P.

*If some changes are found in protein faction, immunoglobulin fraction (IgG, IgM, IgE, and IgA) should be determined.

(3) Urinalyses

Urinalyses should be performed on all animals before the start of administration of the test substance and before necropsy (at the end of the administration period). The following are usually analyzed: urinary volume, pH, protein, glucose, ketones, bilirubin, urobilinogen, occult blood, sediments, specific gravity or osmotic pressure, and electrolytes such as Na and K.

(4) Ophthalmological examination

Ophthalmological examination should be performed on all rodents in the highest-dose group and the appropriate control group before necropsy, and on all non-rodents before the start of administration of the test substance and before necropsy. When changes are observed in rodents receiving the highest dose, then all remaining dosed groups are to be examined.

The examination is performed both macroscopically and ophthalmoscopically on the anterior portion of the eye, optic media, and ocular fundus.

(5) Examinations for other functions

Electorcardiography, examinations of renal function, sensory function, motor activity, measurements of more integrated behavior, and other functional tests should be performed, when deemed appropriate or necessary.

(6) Necropsy and histopathological examination

① Animals found dead during the administration period should be necropsied as soon as possible. All organs and tissues should be examined macroscopically. When necessary, selected organs should be weighed and selected organs/tissues should be examined histopathologically to identify the cause(s) of death and the nature of toxic at the time of death.

② Since animals which become moribund during the administration period provide more meaningful information when sacrificed than when allowed to die, moribund animals should be sacrificed as soon as possible and necropsied. Observation and examination should be performed in the manner described above to identify the cause(s) inducing moribundity and the nature of toxic changes at the time of sacrifice.

③ At the end of the administration period, blood and urine should be collected from all survivors, and the survivors sacrificed and necropsied. Organs and/or tissues should be examined macroscopically and selected organs are weighed.
The following organs are weighed and preserved for histopathological examination: heart, liver, spleen, kidneys, adrenals, prostate, testes, seminal vesicles, ovaries, uterus, brain, hypophysis, salivary glands, thymus, lungs, and thyroids with parathyroids,

Histopathological examination should be performed on all non-rodent test animals, and at least on the animals of both highest-dose group and appropriate control group for rodents.
Examinations should be performed on the following organs/tissues and any others deemed necessary based on macroscopic findings: skin, mammary gland, lymph nodes including cervical and mesenteric lymph nodes), aorta, salivary glands, bone and bone marrow (sternum and femur), thymus, trachea, lungs and bronchi, heart, thyroids with parathyroids, tongue, esophagus, stomach, duodenum, small, intestine (jejunum and

ileum), large intestine (cecum, colon, and rectum), liver and gall bladder, pancreas, spleen, kidneys, adrenals, urinary bladder, seminal vesicles, prostate, testes, epididymides, ovaries and oviducts, uterus, vagina, brain, pituitary, sciatic nerve, skeletal muscle, spinal cord, nasal cavity (turbinate), eyeballs and their accessory organs, Zymbal gland, and any other organs and/or tissues which exhibited macroscopic changes.

Particular attention should be paid to effects on the immune system, nervous system, and gonad.

Immune system: Immunohistochemical examination should be performed on the thymus, spleen, and lymph nodes/lymphoid tissues when deemed necessary. When the test substance is deemed immunotoxic, additional examinations should performed on bone marrow cellularity, spleen lymphocyte subpopulations, and NK cell activity.

Nervous system: When animals have exhibited neurotoxic effects attributable to administration of the test substance, nervous tissues from some dosed animals should be fixed *in situ* using a generally recognized perfusion technique. The issues to be examined neurohistopathologically include forebrain, center of cerebrum, midbrain, cerebellum, pons, medulla oblongata, spinal cord (cervical, thoracic, lumbar parts), spinal dorsal root ganglia, spinal dorsal and ventral roots, proximal sciatic nerve, sural nerve, and musculus gastrocnemius.

Gonad: Testes are normally fixed in Bouin's solution.

When any tissue/organ has exhibited macroscopical changes in other dosed groups of rodents, or any organ and/or tissues is deemed to require histopathological examination, judging from the findings on high-dose groups, examinations of such organs and tissues should be performed on all animals of the corresponding dosed group. Histopathological examinations of tissues from all rodents would further improve evaluation of the data.

[2] 90-day toxicity study

This study is designed to provide information concerning toxic effects induced when a test substance is administered repeatedly for 90 consecutive days to rodents and non-rodents, and to provide dose-setting information for longer term toxicity studies (e.g. one year, carcinogenicity, and combined chronic toxicity/carcinogenicity).

1. Animal species and sex

One rodent species (usually the rat) and one non-rodent species (usually the dog) are used. Equal numbers of males and females should be used.

Dosing of rodents (the rat or mouse) should begin as soon as possible after weaning and acclimation, usually before the animals are 5 to 6 weeks old. Dosing of non-rodents (the dog) should begin between 4 and 6 months of ages.

2. Number of animals

Each group should consist of at least 10 animals per sex for rodents and at least 4 animals per sex for non-rodents. Animals should be allocated into each group by appropriate stratified

randomization, such as on the basis of the body weight.

The number of animals should be sufficient to ensure an adequate number of survivors at termination to permit proper evaluation of toxicity. If interim sacrifices are planned, the initial number of animals should be increased by the number of animals required for interim sacrifices.

3. Route of administration

Administration should be oral, usually by incorporating the test substance into the diet or into the drinking water. Gavage administration may be adopted in the following cases where administration of the test substance in diet or in water is not appropriate: (1) the test substance is unstable in the diet or in water, (2) there is analytical problem with the substance in the diet or in water, or (3) test animals reject the test diet or the water (unpalatability). Administration of the microcapsulated test substance may be applied under certain experimental conditions.

4. Duration of administration

The test substance should be administered daily for 90 consecutive days.

5. Dose levels and control groups

(1) Dose levels

At least three different dose levels and appropriate control(s) should be used. In order to establish a comprehensive profile of toxicity of the test substance and to estimate no-observed-adverse-effect level (NOAEL), the dose levels should include: the highest level which induces any significant toxicity but without inducing excessive mortality (which could preclude proper evaluation) and the lowest level which does not induce toxicity.

The doses used should permit the demonstration of a dose-response relationship. The basis for dose selection must be specified.

Adverse nutritional effects should be avoided when the test substance is administered as a dietary admixture. The concentration of the test substance in the diet does not usually exceed 5% (w/w). For gavage administration, when the technically maximum feasible dose level (but not higher than 1000 mg/kg) fails to elicit any toxic signs, that level is to be taken as the highest dose level.

(2) Control groups

All test conditions of the control groups should be the same as for the dosed groups, except that the test substance is not administered.

When a vehicle is used to prepare the test diets or the drinking water solutions, there should be a vehicle control group that receives the same vehicle level as the highest dosed group. If there is insufficient information about the toxic properties of the vehicle used, an additional control group not exposed to the vehicle should be included.

6. Observation and examination

(1) General conditions, body weight, and diet (and/or water) consumption

All test animals should be observed daily.

Cage-side observations of general conditions should be made with special attention to the

following:

general appearances including, changes in skin, fur, eyes, ears, nose, mouth, perianal area and mucous membranes; exhaustion; obesity/emaciation, or abdominal swelling; body position/posture including prone, crawling, hunchback;

consciousness/attitude including excitement, aggression, inanimation, sedation, lethargy;

behavior including changes in exploration, grooming, motor activity, and abnormal gait;

nervous system (somatomotor activity) including tremors, convulsions, twitch, and reflex functions;

body temperature;

respiration;

excretion.

Body weights and diet and/or water consumption (when the test substance is administered in drinking water) should be measured periodically before the start of the administration period and at least once weekly thereafter.

(2) Hematological and blood biochemical examinations

Examinations should be performed on samples taken from all test animals at least once before necropsy for rodents, and before the start of administration and before necropsy for non-rodents. Examinations on rodents may be performed on animals selected from each group for technical reasons.

The following parameters are evaluated. Additional tests may be performed as deemed appropriate. Only internationally accepted procedures should be used.

Hematological exanimation includes erythrocyte count, total and differential leukocyte counts, platelet count, hemoglobin concentration, hematocrit, and if necessary, reticulocyte count, and clotting potential such as prothrombin time and activated partial thromboplastin time.

Blood biochemical determinations include serum(plasma) total protein, albumin, A/G ratio, protein fraction *, fasting glucose, cholesterol, triglyceride, bilirubin, urea nitrogen, creatinine, transaminases [AST(GOT), ALT(GPT)], ɤ-glutamyl transpeptidase, alkaline phosphatase, and electrolytes including Na, K, Cl, Ca, inorganic P.

*If some changes are found in protein fraction, immunoglobulin fraction (IgG, IgM, IgE, and IgA) should be determined.

(3) Urinalyses

Urinalyses should be performed at the same time as analyses on blood. All non-rodents and constant number of rodents, at least five per sex per group, are examined.

The following are usually analyzed urinary volume, pH, protein, glucose, ketones, bilirubin, urobilinogen, occult blood, sediments, specific gravity or osmotic pressure, and electrolytes such as Na and K.

(4) Ophthalmological examination

Ophthalmological examination should be performed for rodents on a constant number of randomly selected animals, at least five per sex per group, at least once during the administration period, and for all non-rodents before the start of the administration of the test substance, at least once during the administration period, and before necropsy.

The examination is performed both macroscopically and ophthalmoscopically on the anterior portion of the eye, optic media, and ocular fundus.

(5) Examinations for other functions

Electrocardiography, examinations of renal function, sensory function motor activity, measurements of more integrated behavior, and other functional tests should be performed when deemed appropriate or necessary.

(6) Necropsy and histopathological examination

① Animals found dead during the administration period should be necropsied as soon as possible. All organs and tissues should be examined macroscopically. When possible, selected organs should be weighed and selected organs/tissues should be examined histopathologically to identify the cause(s) of death and the nature of toxic changes at the time of death.

② Since animals which become moribund during the administration period provide more meaningful information when sacrificed than when allowed to die, moribund animals should be sacrificed as soon as possible and necropsied. Observation and examination should be performed in the manner described above to identify the cause(s) inducing moribundity and the nature of toxic changes at the time of sacrifice.

③ At the end of the administration period, blood and urine should be collected from all survivors, and the survivors sacrificed and necropsied, Organs and/or tissues should be macroscopically examined and selected organs are weighed.
The following organs are weighed and preserved for histopathological examination: heart, liver, spleen, kidneys, adrenals, prostate, testes, seminal vesicles, ovaries, uterus, brain, hypophysis, salivary glands, thymus, lungs, and thyroids with parathyroids.

Histopathological examination should be performed on all test animals for non-rodent, and at least on the animals of both highest-dose group and appropriate control group for rodents.
Examination should be performed on the following organs/tissues and any others deemed necessary based on macroscopic findings: skin, mammary gland, lymph nodes (including cervical and mesenteric lymph nodes), aorta, salivary glands, bone and bone marrow (sternum and femur), thymus, trachea, lungs and bronchi, heart, thyroids with parathyroids, tongue, esophagus, stomach, duodenum, small intestine (jejunum and ileum), large intestine (cecum, colon, and rectum), liver and gall bladder, pancreas, spleen, kidneys, adrenals, urinary bladder, seminal vesicles, prostate, testes, epididymides, ovaries and oviducts, uterus, vagina, brain, pituitary, sciatic nerve, skeletal muscle, spinal cord, nasal cavity (turbinate), eyeballs and their accessory organs, Zymbal gland, and any other organs and/or tissues which exhibited macroscopic changes.

Particular attention should be paid to effects on the immune system, nervous system, and gonad.

Immune system: Immunohistochemical examination should be performed on the thymus, spleen, and lymph nodes/lymphoid issues when deemed necessary. When the test substance is deemed immunotoxic, additional examinations should be performed on bone marrow cellularity, spleen lymphocyte subpopulations, and NK cell activity.

Nervous system: When animals have exhibited neurotoxic effects attributable to administration of the test substance, nervous tissues from some dosed animals should be fixed *in situ* using a generally recognized perfusion technique. The issues to be examined neurohistopathologically include forebrain, center of cerebrum, midbrain,

cerebellum, pons, medulla oblongata, spinal cord (cervical, thoracic, lumbar parts), spinal dorsal root ganglia, spinal dorsal and ventral roots, proximal sciatic nerve, sural nerve, and musculus gastrocnemius.

Gonad: Testes are normally fixed in Bouin's solution

When any tissue/organ has exhibited macroscopical changes in other dosed groups of rodents or any tissue/organ is deemed to require histopathological examination judging from the findings on high-dose groups, examinations of such organs and/or tissues should be performed on all animals in the corresponding dosed group. Aside from the above descriptions, conducting histopathological examinations on all rodents would further improve evaluation of the data.

[3] one-year toxicity study

This study is designed to provide information concerning toxic effects induced when a test substance is administered repeatedly for one year to rodents and non-rodents, to establish dose-response relationships, to define the mechanisms of toxicity, to identify a no observed adverse effect level (NOAEL).

1. Animal species and sex

One rodent species (usually the rat) and one non-rodent species (usually the dog) are used. Equal numbers of young healthy males and females should be used.

Dosing of rodents (the rat or mouse) should begin as soon as possible after weaning and acclimation, usually before the animals are 5 to 6 weeks of age. Dosing of non-rodents (the dog) should begin between 4 and 6 months of age.

2. Number of animals

Each group should consist of at least 20 animals per sex for rodents and at least 4 animals per sex for non-rodents. Animals should be allocated into each group by an appropriate stratified randomization such as on the basis of the body weight.

The number of animals should be sufficient to ensure an adequate number of survivors at termination to permit proper evaluation of toxicity. If interim sacrifices are planned, the initial number of animals should be increased by the number of animals required for the proper conduct of these additional studies.

3. Route of administration

Administration should be oral, usually by incorporating the test substance into the diet or into the drinking water. Gavage administration may be adopted in the following cases where administration in the diet or water is difficult (1) the test substance is unstable in the diet or in water, (2) there is analytical problem with the substance in the diet or in water, or (3) test animals reject the test diet or the water (unpalatability). Administration of the microcapsulated test substance may be applied under certain experimental conditions.

4. Duration of administration

The test substance should be administered daily for one year.

5. Dose levels and control group

(1) Dose levels

At least three different dose levels and appropriate control(s) should be used. In order to establish a comprehensive profile of toxicity of the test substance and to estimate no-observed-adverse-effect level (NOAEL), the dose levels should include: the highest level which induces any significant toxicity but without inducing excessive mortality (which could preclude proper evaluation) and the lowest level which does not induce toxicity. The doses used should permit the demonstration of a dose-response relationship. The basis for dose selection must be specified. In selecting dose, the results of the 28-day and 90-day studies should be consulted.

Adverse nutritional effects should be avoided when the test material is administered as a dietary admixture. The concentration of the test substance in the diet dose not usually exceed 5% (w/w). For gavage administration, when the technically maximum feasible dose level (but not higher than 1000 mg/kg) fails to elicit any toxic signs, that level is to be taken as the highest dose level.

(2) Control groups

All test conditions of the control groups should be the same as for the dosed groups, except that the test substance is not administered.

When a vehicle is used to prepare the test diets or the drinking water solutions, there should be a vehicle control group that receives the same vehicle level as the highest dosed group. If there is insufficient information about the toxic properties of the vehicle used, an additional control group not exposed to the vehicle should be included.

6. Observation and examination

(1) General conditions, body weight, and diet (and/or water) consumption

All test animal should be observed daily.

Cate-side observations of general conditions should be made with special attention to the following:

general appearances including morbidity and mortality, changes in skin, fur, eyes, ears, nose, mouth, perianal area and mucous membranes; exhaustion; obesity/emaciation, abdominal swelling or cramping;

body position/posture including prone, crawling, hunchback;

consciousness/attitude including excitement, aggression, inanimation, sedation, lethargy;

behavior including changes in exploration, grooming, motor activity, and abnormal gait;

nervous system (somatomotor activity) including tremors, convulsions, twitch and reflex functions;

body temperature;

respiration;

excretion.

Body weights and diet and/or water consumption (when the test substance is administered in drinking water) should be measured before the start of the administration period, at least once weekly during the first 3 months of the administration period, and at least once every 4 weeks thereafter.

Individual measurements of diet and/or water consumption are preferred, but cage measurements may be allowed.

(2) Hematological and blood biochemical examinations

Examinations should be performed on samples taken at least once before necropsy for rodents, and before the start of administration, every three months during the administration period, and before necropsy (at the end of the administration period) for non-rodents. The most meaningful data are derived from examinations on all animals and this is encouraged.

The following parameters are evaluated. Additional test may be performed as deemed appropriate. Only internationally accepted procedures should be used.

Hematological examination includes erythrocyte count, total and differential leukocyte counts, platelet count, hemoglobin concentration, hematocrit, and if necessary, reticulocyte count, and clotting potential such as prothrombin time and activated partial thromboplastin time.

Blood biochemical determinations include serum (plasma) total protein, albumin, A/G ratio, protein fraction, fasting glucose, cholesterol, triglyceride, bilirubin, urea nitrogen, creatinine, transaminases [AST(GOT), ALT(GPT)], ɤ-glutamyl transpeptidase, alkaline phosphatase, and electrolytes including Na, K, Cl, Ca, inorganic P.

(3) Urinalyses

Urinalyses should be performed at the same time as analyses on blood. All non-rodents and a constant number of rodents, at least 10 per sex per group, are examined. The following are usually analyzed: urinary volume, pH, protein, glucose, ketones, bilirubin, urobilinogen, occult blood, sediments, specific gravity or osmotic pressure, and electrolytes such as Na and K.

(4) Ophthalmological examination

Ophthalmological examination should be performed for rodents on a constant number of randomly selected animals per sex per group, at least 10, at least once during the administration period (at the end of the exposure period), and for all non-rodents before the start of the administration of the test substance, at least once during the administration period, and before necropsy.

The examination is performed both macroscopically and ophthalmoscopically on the anterior portion of the eye, optic media, and ocular fundus.

(5) Examinations for other functions

Electrocardiography, examinations of renal function, optic function and auditory function, and other functional tests should be performed when deemed appropriate or necessary.

(6) Necropsy and histopathological examination

① Animals found dead during the administration period should be necropsied as soon as possible. All organs and tissues should be examined macroscopically. When possible, selected organs should be weighed and selected organs/tissues should be examined histopathologically to identify the cause(s) of death and the nature of toxic changes at the time of death.

② Since animals which become moribund during the administration period provide more meaningful information when sacrificed than when allowed to die, moribund animals should be sacrificed as soon as possible and necropsied. Observation and

examination should be performed in the manner described above to identify the cause(s) inducing moribundity and the nature of toxic changes at the time of sacrifice.

③ At the end of the administration period, blood and urine should be collected from all survivors, and the survivors sacrificed and necropsied. Organs and/or tissues should be macroscopically examined and selected organs are weighed.
The following organs are weighed and preserved for histopathological examination: heart, liver, spleen, kidneys, adrenals, prostate, testes, seminal vesicles, ovaries, uterus, brain, hypophysis, salivary glands, thymus, lungs, and thyroids with parathyroids.

Histopathological examination should be performed on all non-rodent test animals and at least on the animals of both highest-dose group and appropriate control group for rodents.
Examination should be performed on the following organs/tissues and any others deemed necessary based on macroscopic findings: skin, mammary gland, lymph nodes (including cervical and mesenteric lymph nodes), aorta, salivary glands, bone and bone marrow (sternum and femur), thymus, trachea, lungs and bronchi, heart, thyroids with parathyroids, tongue, esophagus, stomach, duodenum, small intestine (jejunum and ileum), large intestine (cecum, colon, and rectum), live and gall bladder, pancreas, spleen, kidneys, adrenals, urinary bladder, seminal vesicles, prostate, testes, epididymides, ovaries and oviducts, uterus, vagina, brain, pituitary, sciatic nerve, skeletal muscle, spinal cord, nasal cavity (turbinate), eyeballs and their accessory organs, Zymbal gland, and any other organs and/or tissues which exhibited macroscopic changes.

When any tissue/organ has exhibited macroscopical changes in other dosed groups of rodents or any tissue/organ is deemed to require histopathological examination, judging from the findings on high-dose groups, examinations of such organs and/or tissues should be performed on all animals in the corresponding dosed group. Conducting histopathological examinations on all rodents would further improve evaluation of the data.

[4] Reproduction study

This study is designed to provide information concerning the effects of a test substance on male and female reproductive functions (including estrous cycles, copulation or mating behavior, conception, gestation, delivery, neonatal morbidity and mortality, lactation), weaning, and post-natal growth and development, and reproductive capacity of the offspring when the test substance is administered over two generations (first generation: F0, second generation, F1). This study can provide supplementary information concerning fetal mortality and teratogenicity, which will serve as a reference for related toxicological studies. The data provided can provide the basis for further testing.

1. Animal species and sex

One or more rodent species (usually the rat) should be used. Equal numbers of males and females should be used. All females used should be virgin.

Species and strains should be selected from among those usually used for general toxicity or reproduction studies, avoiding those strains with low fecundity.

Dosing of rats should begin after the acclimation of not less than one week, usually when males are 5 to 7 weeks of age and the females are 8 to 10 weeks of age.

2. Number of animals

Each group should contain sufficient animals able to obtain at least 20 pairs of animals at the time of mating. Animals should be allocated into each group by an appropriate stratified randomization, such as on the basis of body weight.

When species other than rats are employed, sufficient number of animals for proper evaluation of the results should be employed.

3. Route of administration

Administration should be oral, usually by incorporating the test substance into the diet or into the drinking water. Gavage administration may be adopted in the following cases where administration in the diet or in water is difficult (1) the test substance is unstable in the diet or in water, (2) there is an analytical problem with the substance in the diet or in water, or (3) test animals reject the test diet or the water (unpalatability). The dosage for each animal should be based on the body weight of the individual animal and should be adjusted weekly. For pregnant females, dosage may be based on the body weight on days 0 and 6 of pregnancy.

4. Duration of administration

(1) Females of the first generation (F0)

Administration of the test substance should be started at 8-10 weeks of age. Females should be subjected to mating after 2 or more weeks of administration.

Administration of the test substance should be continued throughout the period of mating, pregnancy, and lactation until the offspring (F1) are weaned.

(2) Males of the first generation (F0)

Administration of the test material should be started at 5-7 weeks of age. Males should be subjected to mating after 8 or more weeks of administration. Administration of the test substance should be continued throughout the mating period.

(3) The second generation (F1)

Administration of the test substance should be started at weaning, and continued until the offspring of the second generation (F2) are weaned for females and until the end of mating for males.

5. Dose levels and control groups

(1) Dose levels

At least three different dose levels and appropriate controls should be employed. In order to establish a comprehensive profile of reproductive toxicity and to estimate no-observed-adverse-effect level (NOAEL), the dose levels should include: the highest level which induces any significant toxicity in dams such as the reduction of body weight gain without excess mortality and the lowest level which does not induce toxicity in both parent animals and their offspring. The doses selected should permit the demonstration of a dose-response relationship. The basis for dose selection must be specified.

Adverse nutritional effects should be avoided when the test material is administered as

a dietary admixture. The concentration of test substance in the diet does not usually exceed 5% (w/w). For gavage administration, when the technically maximum feasible dose level (but not higher than 1000 mg/g body weight) fails to elicit any toxic signs, that level is to be taken as the highest dose level.

(2) Control groups

All test conditions of the control groups should be the same as the dosed groups, except that the test substance is not administered.

When a vehicle is used to prepare the test diets or the drinking water solutions, there should be a vehicle control group that receives the same vehicle level as the highest dosed group. If there is insufficient information about the toxic properties of the vehicle used, an additional control group not exposed to the vehicle should be included.

6. Mating and selection of the second generation (F1)

(1) The first generation (F0)

Females and males in the same group should be caged in pairs (1:1 mating) and mated until copulation is confirmed. The mating period should be up to three weeks. Each female should be examined every morning for presence of sperm in the vaginal smear or for the presence of vaginal (mucus) plugs. The day on which sperm or a vaginal plug is first observed is designated as day 0 of pregnancy.

(2) The second generation (F1)

① Standardization of litter size

On post-natal day 4, litters should be culled randomly to 4 pups per sex per litter. When this standardization of 4 pups per sex per litter cannot be accomplished, the total number of animals culled should be 8; for example, 5 males and 3 females. If the number of pups is 8 or less, culling should not be conducted.

② Selection of F1 for mating

At weaning of the F1 offspring, 20 to 25 pups per sex should be selected randomly as mating animals to produce the F2 generation. Pups from as many different litters as possible should be selected, usually 1 to 2 pups per sex per dam.

③ Mating

The F1 offspring should be mated when they are 10 to 13 weeks of age. Sibling matings are to be avoided.

7. Observation and examination

The following should be observed or examined.
If necessary, effects on the immune and nervous systems of parents and their offspring should also be examined.

(1) General conditions

All test animals (F0, F1, F2) should be observed at least once daily for general conditions, pregnancy, and delivery.

F0 and F1 animals after weaning

Parameters to be observed include morbidity and mortality, external appearance,

neurobehavioral and somatomotor effects including excitement, seizures, sedation, and abnormal gait, abortion, premature birth, and delayed delivery.

Neonates

Parameters to be observed include physical development (for example, pinna unfolding, incisor eruption, coat growth, eye opening, the development of genital organs) and functional development (for example, surface righting, motor activity, auditory startle).

(2) Body weight and diet (or water) consumption

Body weights and diet and/or water consumption (if the test substance is added to drinking water) should be measured periodically for F0 animals and F1 animals retained for mating. Periodic measurement of body weights and diet and/or water consumption should be conducted before the start of administration of the test substance and at least once weekly during the administration period.

(3) Pregnancy and delivery

The following parameters should be calculated.

$$\text{Copulation index} = \frac{\text{Number of animals with successful copulation}}{\text{Number of mated aminals}} \times 100$$

$$\text{Fertility index} = \frac{\text{Number of pregnant females}}{\text{Number of females with successful copulation}} \times 100$$

$$\frac{\text{Birth index}}{(\text{Gestation Index})} = \frac{\text{Number of females with live pups}}{\text{Number of pregnant females}} \times 100$$

$$\text{Viability index at weaning} = \frac{\text{Number of live pups at weanig}}{\text{Number of pups alive immediately after culling on post-natal day 4}} \times 100$$

Males and females not having successfully copulated with more than one mate should be examined to determine the basis for this. The estrous cycle and spermatogenesis should be evaluated; histopathological examination of genital organs may be necessary. The examination for spermatogenesis should include the number, motility, and morphology of sperm at the time of sacrifice, in cases where pregnancy does not take place.

(4) Neonates

Litter size, viability, sex and external changes of neonates of each dam should be examined at the earliest possible time after birth.

Dead pups and F1 pups sacrificed on day 4 after birth should be necropsied. The live pups should be weighed at birth or at the earliest possible time after birth, and on post-natal days 4, 7, 14(optional), and 21.

(5) Necropsy

① F0 animals and F1 animals used for mating should be sacrificed and necropsied immediately after weaning of their respective offspring. F1 animals not used for mating and F2 animals should be necropsied immediately after weaning. Observation should be performed macroscopically with special attention to genital organs. Animals found to be moribund during the examination period should be immediately sacrificed and necropsied in the appropriate manner. All animals found dead during the

examination period should be appropriately necropsied.

② Organs or tissues having exhibited macroscopical which may be attributable to the administration of the test substance should be examined histopathologically. In evaluating these data, results of repeated-administration toxicity studies should be consulted.

8. Analysis of data

(1) Data obtained should be presented in tables or figures with appropriate statistical and biological evaluation of results. Summary tables which present an overview of the results from all groups should be prepared. Data for individual animals should be tabulated for reference purposes.

(2) For statistical analysis of data obtained before weaning, it is desirable that the litter, instead of the individual pups, serve as the unit for analysis.

(3) The evaluation must include a no-observed-adverse-effect level (NOAEL) of the test substance on the reproductive performance of parent animals and on the development of offspring.

[5] Teratogenicity study

This study (*1) is designed to provide information concerning the structural and functional effects on the developments and growth of embryos and fetuses, especially teratogenicity, of a test substance administered to pregnant females during the period of intrauterine development.

1. Animal species

At least one rodent species (usually the rat) and at least one non-rodent species (usually the rabbit) should be used.

2. Number of animals

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[1] The teratogenicity study may be combined with the reproduction study using fetuses of the 2nd litter of the 2nd generation (F2 b) obtained from the continuously administered and repeatedly mated F1 pups after F1's offspring (F2 a) are weaned. When the combined study is conducted, the following conditions must be met:

① Toxic effects in parent animals and their offspring by an expected highest dose for teratogenicity study can be regarded as being not significantly different from those by the highest dose set for the reproduction study, based on knowledge of the general toxicity and/or biokinetics of the test substance.

② The test substance is believed not to have the capability to alter significantly the toxic effects by the long-period administration of females in the study (for example, through induction of drug metabolizing enzymes or as a result of liver damage).

The number of animals per sex group should be sufficient to allow meaningful interpretation of the data (*2).

3. Route of administration

Administration of the test substance should be by gavage and should be made at approximately the same time each day. Divided administrations may be acceptable, provided that all the administrations are made within 6 hours.

If it has been established from appropriate biodispositional and kinetic studies including measuring concentrations of the test substance or its metabolites in blood or diet consumption that a constant amount of the test substance is absorbed from the gastrointestinal tract, then administration in the diet or drinking water is acceptable.

4. Duration of administration

The test substance should be administered every day throughout the period of major organogenesis.

5. Dose levels and control groups

(1) Dose levels

At least three different dose levels and appropriate controls should be employed. In order to establish a comprehensive profile of developmental toxicity of the test substance and to estimate no-observed-adverse-effect level (NOAEL), the dose levels should include: the highest level which induces toxicity in the dams and the lowest level which does not induce toxicity in the dams or in the fetuses. Toxic effects in the dam include lower body weight gain or reduced dietary consumption. The doses selected should permit the demonstration of a dose-response relationship. The basis for dose level selection must be specified.

Adverse nutritional effects should be avoided when the test substance is administered as a dietary admixture. The concentration of the test substance in the diet does not usually exceed 5% (w/w).

For gavage administration, when the technically maximum feasible dose level (but not higher than 1000 mg/kg body weight) fails to elicit any signs of toxicity, that level is to be taken as the highest dose level.

(2) Control groups

All test conditions of the control groups should be the same as for the dosed groups, except that the test substance is not administered.

When a vehicle is used to administer the test substance, there should be a vehicle control group that receives the same level of vehicle as the highest dosed group. If there is insufficient information about the toxic properties of the vehicle used, an additional control group not exposed to the vehicle should be included.

6. Observation and examination

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[2] For all but the rarest events, evaluation of between16 to 20 litters for rodents and rabbits tends to provide a degree of consistency between studies. Below 16 litters per evaluation, between study results become inconsistent, above 20-24 litters per group consistency and precision is not greatly enhanced.

(1) General conditions, body weight, and diet (or water) consumption.

All dams should be observed at least once daily. Cage-side observations, should include morbidity and mortality, changes in appearance, neurobehavioral and somatomotor activity, excitement, seizures, sedation and abnormal gait. Abortions, premature births, and delayed delivery should also be noted.

Body weight should be determined on day 0 of pregnancy and once daily thereafter and at sacrifice.

Diet and/or water consumption should be measured at least once prior to the start of administration of the test substance, at least twice during the period of administration, and at least once after termination of administration.

(2) Necropsy

① Dams

All dams having exhibited signs of abortion or premature delivery should be sacrificed immediately, the uterus removed and examined, and all other organs and tissues observed macroscopically. Selected organs should be weighed and examined histopathologically in order to identify changes related to abnormal pregnancy. Dead or moribund dams should also be examined. The removed uterus should be examined as described below.

On the day before expected delivery, all dams should be sacrificed, the uterus removed, and all organs and tissues should be observed macroscopically. Selected organs should be weighed and examined histopathologically. The removed uterus should be examined as follows.

② Fetuses

The removed uterus should be examined for evidence of embryonic deaths, fetal deaths, and the number of live fetuses. The times of intrauterine deaths should be estimated. For rats and rabbits, the number of corpora lutea should be counted. Following removal, all fetuses should be sexed and weighed individually. The body weights should be recorded and the mean fetal body weight should be calculated or each sex.

All fetuses should be examined externally for external abnormalities. A third to a half of each rat (or mouse or hamster) litter should be prepared and examined for skeletal anomalies and the remaining fetuses should be prepared and examined for internal anomalies using appropriate methods. All rabbit fetuses should be examined by careful dissection for internal anomalies and then examined for skeletal anomalies using appropriate methods.

7. Analysis of data

(1) Data obtained should be presented in tables or figures with appropriate statistical and biological evaluation of the results. Summary tables which present an overview of the results from all groups should be prepared. Tables giving data for individual animals in each group should be prepared for reference.

(2) The litter is the unit used for statistical analysis

(3) The evaluation must include the no-observed-adverse-effect level (NOAEL) of the test substance on dams and fetuses.

[6] Carcinogenicity study

This study is designed to provide information concerning the carcinogenic potential of a test substance when administered orally to rodents.

1. Animal species and sex

At least two rodent species (usually the rat, mouse, or hamster) are used. Equal numbers of males and females should be used.
Dosing of animals should begin as soon as possible after weaning and acclimation, usually before the animals are 5 to 6 weeks of age.

The nature of species and strains to be selected, i.e. their particular sensitivities or susceptibilities, should be thoroughly known, including resistance to infectious diseases, life span, and susceptibilities to known carcinogenic substance. Also, the species and strains selected should be widely used as experimental animals. Preference in selection should be given to strains with accumulated data concerning the incidence of spontaneous tumors (tumor profile).

2. Number of animals

Each group should consist of at least 50 animals per sex. Animals should be allocated into each group by appropriate stratified randomization such as on the basis of body weight.

It is desirable that mortality due to causes other than tumors be within 50% at 24 months after the start of administration for rats, and at 18 months for mice and hamster.

More than 10% of any group should not be lost due to cannibalism or problems of animal husbandry management.

If interim sacrifices are planned, the initial number of animals should be increased by the number of animals required for interim sacrifices.

3. Route of Administration

Administration should be made orally, usually by incorporation the test substance into the diet or drinking water. Gavage administration may be adopted in the following cases where administration in the diet or in water is difficult: (1) the test substance is unstable in the diet or in water, (2) there is an analytical problem with the substance in the diet or in water, or (3) test animals reject the test diet or the water. Administration of the microcapsulated test substance may be applied under certain experimental conditions.

4. Duration of administration

The administration period should be not less than 24 months and not more than 30 months for rats, and not less than 18 months and not more than 24 months for mice and hamsters. The test substance should be administered daily, i.e. seven days per week.

Observation/examination should be terminated at the end of the administration period, or in one to 3 months after the end of administration period. The maximum duration of observation/examination should be 30 months for rats and 24 months for mice and hamsters.

If cumulative mortality reaches 75% for either sex of the animals in the lowest-dose group or control group, then the test should be terminated with sacrifices of the surviving

animals of that sex.

5. Dose levels and control groups

(1) Dose levels

At least three different dose levels and appropriate controls should be used. The doses selected should permit the demonstration of a dose-response relationship. In selecting dose for carcinogenicity studies, results of the 90-day study should be consulted. The basis for dose selection must be specified.

① Highest dose level

The highest dose level should induce minimum toxic effects without inducing excess mortality due to any cause other than tumors, when compared with the control groups. Adverse nutritional effects should be avoided when the test substance as a dietary admixture is administered. The concentration of the test substance in the diet does not usually exceed 5% (w/w). For gavage administration, when the technically maximum feasible dose level (but not higher than 1000 mg/kg) fails to elicit any toxic sighs, that level is to be taken as the highest dose level.

② Lowest dose level

The lowest dose level should be not lower than 10% of the highest dose level. However, selection of the lowest level should be done, considering the amount of the test substance expected to be added to food. If the lowest level normally set is extremely different from the estimated level of anticipated human exposure, an additional dose level may be set at less than 10% of the highest level.

③ Intermediate dose level

The intermediate dose level is usually the geometric mean between the highest level and the lowest level. Usually, the common ratio between the two groups should be 2 or 3.

(2) Control groups

All test conditions of the control groups should be the same as for the dosed groups, except that the test substance is not administered.

When a vehicle is used to administer the test substance, the vehicle to be used for the vehicle control group should be the same level as the maximum vehicle level used for the highest dosed groups. If there is insufficient information about the toxic properties of the vehicle used, and additional control group not exposed to the vehicle should be included.

6. Observation and examination

(1) General conditions, body weight, and diet (and/or water) consumption

All test animals should be observed at least once daily.

Body weights and diet and/or water consumption (when the test substance is administered in drinking water) should be measured periodically before the start of administration of the test substance, at least once weekly during the first 3 months of the administration period, and at least once every 4 weeks thereafter. Individual measurements of diet and/or water consumption are preferred, but cage measurements may be allowed.

(2) Examinations should be performed on blood samples taken from all surviving animals

before necropsy. The parameters evaluated include erythrocyte count, total leukocyte counts.

At sacrifice, blood smears should be prepared and examined for cases suggestive of hematopoietic organ tumors including anemia and swelling of lymph nodes, liver, and spleen.

(3) Necropsy and histopathological examination

① Animals found dead during the observation/examination period should be necropsied as soon as possible. Organs and/or tissues should be observed macroscopically, and examined histopathologically.
The descriptions of neoplastic lesions must include related preneoplastic changes (findings of the examinations directed below must be described in the same manner).

Histopathological examination is performed on the following organs/tissues and any others deemed necessary based on macroscopic findings: skin, mammary gland, lymph nodes (cervical and mesenteric lymph nodes, etc.), aorta, salivary glands, bone and bone marrow (sternum and femur), thymus, trachea, lungs and bronchi, heart, thyroids and parathyroids, tongue, esophagus, stomach, duodenum, small intestine (jejunum, and ileum), large intestine (cecum, colon, and rectum), liver and gall bladder, pancreas, spleen, kidneys, adrenals, urinary bladder, seminal vesicles, prostate, testes, epididymides, ovaries and oviduct, uterus, vagina, brain, pituitary, sciatic nerve, skeletal muscle, spinal cord, nasal cavity (turbinate), eyeballs and their accessory organs, Zymbal gland, and any other organs and/or tissues having exhibited macroscopic changes.

② Animals which become moribund during the observation/examination period should immediately be isolated, or sacrificed and necropsied. Observation and examination should be performed in the manner described above.

③ All surviving animals at the termination of the observation/examination period should immediately be sacrificed and necropsied. Organs and/or tissues should be observed macroscopically. All animals of the highest-dose group and control group(s) should be examined histopathologically in the manner described above. If a significant difference is found in the tumor incidence of any organ and/or tissue between the highest-dose group and the control group(s), such organs and/or tissues of all animals from the remaining groups should be examined histopathologically.
Conduction histopathological examinations on all test animals would further improve evaluation of the data.

[7] Combined one-year toxicity/carcinogenicity study

This study is designed to provide information concerning the chronic toxic effects and the potential carcinogenicity induced when a test substance is administered repeatedly.

1. Animal species and sex

One rodent species (usually the rat) should be used. Equal numbers of young healthy males and females should be used.

Dosing of animals should begin as soon as possible after weaning and acclimation, usually before the animals are 5 to 6 weeks of age.

The nature of species and strains to be selected, i.e. their particular sensitivities or susceptibilities, should be thoroughly known, including resistance to infectious diseases, life

span, and susceptibilities to known carcinogenic substances. Also, the species and strains selected should be widely used as experimental animals. Preference in selection should be given to strains with accumulated data concerning the incidence of spontaneous tumors (tumor profile).

2. Number of animals

For the assessment of potential carcinogenicity, each group should consist of at least 50 animals per sex. Animals should be allocated into each group by appropriate randomized stratification, such as on the basis of body weight.

It is desirable that mortality due to causes other than tumors be within 50% at 24 months after the start of administration for rats, and at 18 months after the start of administration for mice.

More than 10% of any group should not be lost due to cannibalism or problems of animal husbandry management.

If interim sacrifices are planned, the initial number of animals should be increased by the number of animals required for interim sacrifices.

Satellite groups should be included for the evaluation of chronic toxicity. Each satellite group should consist of at last 10 animals per sex. However, the highest-dose group should consist of at least 20 animals per sex.

3. Route of Administration

Administration should be oral, usually by incorporating the test substance into the diet or into the drinking water. Gavage administration may be adopted in the following cases where administration in the diet or in water is difficult: (1) the test substance is unstable in the diet or in water, (2) there is an analytical problem with the substance in the diet or in water, or (3) test animals reject the test diet or the water. Administration of the microcapsulated of the test substance may be applied under certain experimental conditions.

4. Duration of administration

The administration period should be not less than 24 months and not more than 30 months for rats, and not less than 18 months and not more than 24 months for mice and hamsters for the carcinogenicity component of the study. The duration of administration for the chronic toxicity component may be one year.

The test substance is, in principle, administered daily, i.e. 7 days per week. Observation/examination should be terminated at the end of the administration period, or in 1 to 3 months after the end of administration period. The maximum duration of observation/examination should be 30 months for rats and 24 months for mice and hamsters.

If cumulative mortality reaches 75% for either sex of the animals in the lowest-dose group or control group, then the examination should be terminated with sacrifices of the surviving animals of that sex.

5. Dose levels and control groups

(1) Dose levels

A. Carcinogenicity component

At least three different dose levels and appropriate controls should be used for each

component of the study. The doses selected should permit the demonstration of a dose-response relationship. In selecting doses for carcinogenicity studies, results of the 90-day toxicity study should be consulted. The basis for dose selection must be specified.

① Highest dose level

The highest dose level should induce minimum toxic effects without inducing excessive mortality due to any cause other than tumors, when compared with the control group.
Adverse nutritional effects should be avoided when the test substance is administered as dietary admixture. The concentration of the test substance in the diet does not usually exceed 5% (w/w). For gavage administration, when the technically maximum feasible dose level (but not higher than 1000 mg/kg) fails to elicit any toxic signs, that level is to be taken as the highest dose level.

② lowest dose level

The lowest dose level should be not lower than 10% of the highest dose level. However, selection of the lowest dose level should be done, considering the amount of the test substance expected to be added to food. If the lowest level normally set is extremely different from the estimated level of anticipated human exposure, an additional dose level may be set at less than 10% of the highest dose level.

③ Intermediate dose level

The intermediate dose level is usually the geometric mean between the highest level and the lowest level. Usually, the common ratio between the two groups should be 2 or 3.

B. Chronic toxicity component

At least three different dose levels and appropriate control(s) should be used. In order to establish a comprehensive profile of toxicity of the test substance and to estimate no-observed-adverse-effect level (NOAEL), the dose levels should include: the highest level which induces any significant toxicity but without inducing excessive mortality (which could preclude proper evaluation) and the lowest level which does not induce toxicity. The doses used should permit the demonstration of a dose-response relationship.

(2) Control groups

All test conditions for the control groups should be the same as for the dosed groups, except that the test substance is administered.
When a vehicle is used to administer the test substance, the vehicle to be used for the vehicle control group should be the same level as the maximum vehicle level used for the highest dosed groups. If there is insufficient information about the toxic properties of the vehicle used, and additional control group not exposed to the vehicle should be included.

6. Observation and examination

(1) General conditions, body weight, and diet (and/or water) consumption

All test animals should be observed at least once daily.
Body weights and diet and/or water consumption should be determined before the start of administration, at least once weekly during the first 3 months of the administration period, and at least once every 4 weeks thereafter. Individual measurements of diet and/or water consumption are preferred but cage measurements may be allowed.

(2) Hematological and blood biochemical examinations

In the carcinogenicity component, hematological examinations should be performed on blood samples taken from all surviving animals at termination of the administration period. At sacrifice, smears should be prepared to perform examinations for cases suggestive of hematopoietic organ tumors including anemia and swelling of lymph nodes, liver and spleen. In the satellite groups for the assessments of chronic toxicity, hematological and blood biochemical examinations should be performed on blood samples taken from all animals prior to necropsy (at month 12). Only internationally accepted procedures should be used.

Hematological examination includes erythrocyte count, total and differential leukocyte counts, thrombocyte count, platelet count, hemoglobin concentration, hematocrit, and, if necessary, reticulocyte count and clotting potential such as prothrombin time and activated partial thromboplastin time.

Blood biochemical determinations include serum (plasma) protein, albumin, A/G ratio, protein fraction, glucose, cholesterol, triglyceride, bilirubin, urea nitrogen, creatinine, transaminase [AST (GOT), ALT (GPT)], ɤ-glutamyl transpeptidase, alkaline phosphatase, and electrolytes including Na, K, Cl, Ca, inorganic P.

(3) Urinalyses

Urinalyses should be performed at the same time as analyses on blood and on all animals in the chronic toxicity component of the study. The following are usually analyzed urinary volume, pH, protein, glucose, ketones, bilirubin, urobilinogen, occult blood, sediments, specific gravity or osmotic pressure, and electrolytes such as Na and K.

(4) Ophthalmological examination

Ophthalmological examination should be performed at least once during the administration period (at month 12) on all animal in satellite groups in the chronic toxicity component of the study. The examination is performed both macroscopically and ophthalmoscopically on the anterior portion of the eye, optic media, and ocular fundus.

(5) Examinations for other functions

If appropriate or necessary, other functional examinations such as optic function, auditory function, and renal function should be performed.

(6) Necropsy and histopathological examination

① Animals found dead during the examination/observation period should be necropsied as soon as possible. Organs and/or tissues should be observed macroscopically, and examined histopathologically.
Descriptions of neoplastic lesions must include related preneoplastic changes (findings of the examinations directed below must be described in the same manner).

Histopathological examination should be performed on the following organs/tissues and any others deemed necessary based on macroscopic findings: skin, mammary gland, lymph nodes (cervical and mesenteric lymph nodes, etc.), aorta, salivary glands, bone and bone marrow (sternum and femur), thymus, trachea, lungs and bronchi, heart, thyroids with parathyroids, tongue, esophagus, stomach, small intestine (duodenum, jejunum, and ileum), large intestine (cecum, colon, and rectum), liver and gall bladder, pancreas, spleen, kidneys, adrenals, urinary bladder, seminal vesicles, prostate, testes, epididymides, ovaries and oviduct, uterus, vagina, brain, pituitary, sciatic nerve, skeletal muscle, spinal cord, nasal cavity (turbinate), eyeballs and their accessory organs, Zymbal gland, and any other organs and/or tissues having exhibited

macroscopic changes.

② Animals which become moribund during the examination period should immediately be isolated, or sacrificed and necropsied. Observation and examination should be performed in the manner described above.

③ All surviving animals in both components should be sacrificed and necropsied immediately at the termination of the observation/examination period. Then, organs and/or tissues should be observed macroscopically. Organs should be weighted for animals of satellite groups.
All tissues from animals of the highest-dose groups and control groups should be examined histopathologically in the manner described above. If a significant difference is found in the incidence of any organ and/or tissue lesions between the highest-dose group and the control groups, such organs and/or tissues of all test animals should be examined histopathologically for the remaining dose groups.
Histopathological examinations on all test animals would further improve evaluation of the data.

[8] Antigenicity study

This study is designed to provide information concerning the potential allergenicity of a test substance.

None of the current methods used to assess the allergenic potential of orally administered substances are definitive and reliably predictive. Selection of a test including appropriate methods of sensitization and challenging should be based on the characteristics of the test substance and the conditions of exposure including the extent.

Some of the test currently used include:

Immediate type hypersensitivity
1) Active systemic anaphylaxis reaction tests in guinea pigs
2) Homologous PCA reaction tests in rabbits or guinea pigs
3) Rat-PCA reaction tests with sensitized mouse serum

Delayed type hypersensitivity
1) Contact skin reaction tests in guinea pigs
2) Foot-pad reaction or lymph node reaction tests in mice

For food additives that may bond with polymers or proteins, the following should also be considered:
1) Antibody titer of sensitized animal serum
2) Potency of bonding with protein
3) Cross-reactivity with related chemicals
4) Others

If a chemical which is similar to the test substance is known to have antigenic properties or any effects attributable to those properties, those properties and effects of the test

substance should be examined using the same method that was used for the similar chemical.

The study report must include clear descriptions of test methods (target animals, challenging antigens, and controls) and evaluations of findings.

[9] Mutagenicity study

This study is designed to provide information concerning the potential of a test substance to cause gene mutations or structural and numerical chromosomal aberrations through effects on DNA.

The test substance should be subjected to the following thee test:
1. Reverse mutation test in bacteria;
2. Chromosome aberration test in cultured mammalian cells;
3. Micronucleus test in rodents.

Additional tests should be performed if necessary.
Examples of additional tests include the following:
Tests to detect gene mutations as a genetic endpoint
1) Gene mutation test in cultured mammalian cells
2) Gene mutation test with *Drosophila melanogaster*
3) Gene mutation test with rodents

Tests to detect chromosome aberrations as a genetic endpoint
1. Chromosome aberration test in bone marrow cells of rodents
2. Chromosome aberration test in germ cells of rodents
3. Dominant lethal test with rodents

Tests to detect DNA damage as a genetic endpoint
1. DNA repair test in bacteria
2. Unscheduled DNA synthesis (UDS) test in mammalian cells
3. Sister chromatid exchange (SCE) test in mammalian cells

1. Reverse mutation test in bacteria

When tests in bacteria are deemed inappropriate for the test substance (e.g., a substance which has strong antibacterial activity or specifically affects mammalian cells), it is appropriate to perform gene mutation tests in cultured mammalian cells instead of bacterial reverse mutation tests.

(1) Bacterial strain

The following five strains should be used:
1) *Salmonella typhimurium* TA98,
2) *Salmonella typhimurium* TA100,
3) *Salmonella typhimurium* TA1535,
4) *Salmonella typhimurium* TA1537 or TA97 or TA97a, and
5) *Escherichia coli* WP2 *uvrA* or *Escherichia coli* WP2 *uvrA* pKM101 or *Salmonella*

typhimurium TA102.

(2) Dose levels

At least 5 dose groups should be tested.

(3) Highest dose level

On the basis of preliminary tests for antibacterial activity, the highest level exhibiting overt antibacterial activity should be selected, regardless of the solubility of a test substance. If no antibacterial or mutagenic activity is found, 5mg/plate or the lowest precipitating concentration should be the upper limit

(4) Controls

The negative control group consisting of a solvent or vehicle alone should be included in each test. For the positive control, substances known to be mutagenic (both substances requiring S9 mix and substances not S9 mix) should be used.

(5) Metabolic activation

Tests with S9 mix should be performed as well. S9 mix is a mixture of S9 and cofactors. S9 should be prepared with the liver of mammals (usually rats) previously treated with appropriate inducers for drug-metabolizing enzymes.

(6) Test method

The method used should be either the preincubation method or the plate incorporation method. 2 to 3 plates should be used for each dose level.

(7) Results

The number of revertants per plate and mean number per dose level should be reported.

2. Chromosome aberration test in cultured mammalian cells

(1) Cells

Primary or established cell lines of mammalian cells in culture should be used.

Usually, cultured cell strains of Chinese hamsters (e.g., CHL/IU and CHO) or human cultured lymphocytes are used.

(2) Dose levels

At least 3 dose groups should be tested.

(3) Highest dose level

A dose inducing overt cytotoxicity should be determined by performing preliminary tests. The highest dose level should produce more than 50% inhibition of cell proliferation for cultured monolayer cells, or more than 50% reduction of the mitotic index for human cultured lymphocytes, regardless of the solubility of a test substance. When no cytotoxicity is observed, the upper limit should be 10 mM, 5 mg/ml, or the lowest precipitating concentration, whichever is lower.

(4) Controls

The negative control group consisting of solvent or vehicle alone should be included in each test. For the positive control, a substance known to be clastogenic (both a substance requiring S9 mix and a substance not requiring S9 mix) should be used.

(5) Metabolic activation

Test with the presence of S9 mix should be performed as well. S9 mix is a mixture of S9 and cofactors. S9 should be prepared with the liver of mammals (usually rats) previously treated with appropriate inducers of drug-metabolizing enzymes.

(6) Test method

Cells should be treated with the test substance both in the presence and absence of S9 mix for short time (e.g., 6 hours), and chromosome preparations should be made at an appropriate time (approximately 1.5 times normal cell cycle).

When both treatments give negative results, an additional test should be done with continuous treatment for 1.5 or 3 times normal cell cycle without S9 mix and sampling at the end of treatment.

1-2 plates per dose level are used. The frequency of chromosomal structural aberrations should be determined on 200 metaphases per dose level. Different types of structural aberrations should be separately recorded for chromatid-type aberrations and chromosomal-type aberrations.

When numerical aberrations (polyploidy or edoreduplication) are found, the frequency should be recorded.

(7) Results

The frequency of cells with structural aberrations or the number of structural aberrations per cell, and the frequency of ell with numerical aberrations should be reported.

3. Micronucleus test with rodents

This test may be replaced by chromosome aberration tests in bone marrow cells of rodents.

(1) Animals

Mice or rats are used. If there are no substantial differences in toxicity between sexes, then testing with a single sex, preferably males, will suffice.

(2) Animal number

At least 5 animals per group should be used.

(3) Route of administration

Gavage or intraperitoneal injection should be used.

(4) Dose levels

At least 3 dose levels should be tested.

(5) Highest dose level

The highest dose level should exhibit some signs of bone marrow cytotoxicity, such as a decrease of the proportion of immature erythrocytes. Or, it is defined as the upper limit which is expected not to produce lethality when the same dosing regimen is used. If necessary, preliminary tests are performed. When no toxic sign is exhibited, the upper limit should be 2 g/kg.

(6) Controls

The negative control group consisting of a solvent or vehicle alone should be included in each test. For the positive control, a substance known to induce micronucleus *in vivo*

should be used.

(7) Number of administrations

Administration should be conducted in a single dose or in multiple doses.

(8) Test method

Bone marrow or peripheral blood erythrocytes are used. Slide preparations should be made twice at appropriate times after administration of a single dose, and once at an appropriate tie after the last administration of multiple doses. The most possible sensitive time should be selected, based on findings from preliminary tests if necessary.

The slides should be stained by either acridine-orange or Giemsa.

The frequency of micronucleated cells should be determined for 2000 immature erythrocytes per animal. Also, the percentage of immature erythrocytes among total erythrocytes should be determined.

(9) Results

The frequency of micronucleated immature erythrocytes and the percentage of immature erythrocytes among total erythrocytes should be reported individually and in groups.

[10] General pharmacological studies

These studies are designed to provide information on the pharmacological effects of the test substance; i.e. to identify effects of the test substance on physiological functions using pharmacological measures.

Category A includes those studies which should be conducted for all test substances. Category B includes those studies which should be performed when deemed necessary or appropriate, in the light of the results of the category A studies. Additional test may be performed, when deemed necessary on the basis of available data concerning the chemical structure and toxicity. The properties of the test substance should determine the appropriateness of the test methods used.

1. Animal species and test systems

Animals species which are appropriate for the particular study should be employed. Consideration should be given to the strain, sex, and age of the animals selected.

Test systems may be classified as follows:
(1) Whole animals
(2) Isolated organs and tissues
(3) Blood and its components
(4) Cells and subcellular constituents

In order to obtain the most meaningful information, special attention should be given to the sensitivity, reproducibility, general acceptance, and predictiveness to humans of the test animals and the test systems selected.

2. Administration method

(1) Route of administration

Since the study should simulate human exposure conditions, oral administration or a suitable substitute should be used in studies involving whole animals.

Other routes, appropriate for the test system employed, can be used; for example, direct application of the test substance to media in isolated organ/tissue baths.

When the test substance is poorly absorbed, it may be necessary to use other routes to ensure adequate exposure.

(2) Dosage regimen

Administration of single dose should be used for examination using a whole animal. If any effects attributable to repeated administration are expected, adequate multiple dosing should be adopted.

(3) Dose levels

The following should be considered:

① The doses selected should permit the demonstration of a dose-response relationship.

② The doses should encompass the dose range demonstrated to exhibit adverse effects in previously conducted toxicity studies. The doses selected should also be based on data from metabolism and study including concentrations of the test substance and/or its metabolites in blood.

(4) Control groups

A negative or vehicle control group and/or a positive (reference or known analogous compound) control group should be included in the study.

3. Studies

Category A

Pharmacological studies to be conducted normally for all test substances to develop an overall profile of effects on physiological functions.

(1) Effects on general conditions

The general activity of the animals following administration of the test substance should be carefully observed by making detailed observations of changes in appearance and behavior.

(2) Effects on the central nervous system (CNS)

① Effects of the test substance on spontaneous locomotor activity should be evaluated.

② General anesthetic effects should be evaluated.

Effects of the test substance in conscious intact animals, and if necessary synergism with and antagonism to anesthetics, such as barbiturate derivatives, should be examined.

③ Convulsive effects should be examined.

Effects of the test substance in the conscious intact animals, and if necessary synergism with and antagonism to convulsion-inducing treatment, such as electric shock or pentetrazol treatment, should be examined.

④ Effects on algesthesia should be evaluated.

⑤ Effects on body temperature should be evaluated.

(3) Effects on the autonomic nervous system (ANS) and smooth muscle

Evaluation should be performed using the isolated ileum preparations. Effects of the test substance itself and interactive effects with agonists (including histamine, acetylcholine, barium chloride, serotonin) should be evaluated.

(4) Effects on the respiratory and cardiovascular systems

Effects of the test substance on respiration, blood pressure, blood flow, heart rate, and electrocardiogram should be evaluated in anesthetized and/or conscious animals.

(5) Effects on the gastrointestinal system

Effects on the gastrointestinal transit should be evaluated.
The intestinal transit time, and when deemed necessary the gastric emptying time should be evaluated.

(6) Effects on water and electrolyte metabolism

Effects of the test substance on water and electrolyte balance can be evaluated by measuring renal function, urinary volume and urinary concentration of electrolytes such as sodium, potassium, and chloride.

(7) Other pharmacological effects

Effects which have been reported from chemically or pharmacologically related compounds should be evaluated using the test substance.

<u>Category B</u>

Pharmacological studies to be conducted if deemed appropriate or necessary in the light of the results of the studies described above (Category A).

(1) Effects on the central nervous system

① Effects of the test substance on the spontaneous electroencephalogram should be evaluated. Computer-assisted analyses of data may be used.

② Effects on spinal reflexes should be evaluated.

③ Effects on the conditioned avoidance response should be evaluated.

④ Effects on coordinated locomotor activity should be evaluated.

(2) Effects on the somatic nervous system

① Effects of the test substance on the neuro-muscular junction should be evaluated.

② The muscular relaxation potential should be evaluated.

③ Local anesthetic effects should be evaluated.

(3) Effects on the autonomic nervous system and smooth muscle

① Effects of the test substance on the pupillary diameter and nictitating membrane contraction should be evaluated.

② Effects on the autonomic nervous system and smooth muscle should be evaluated using isolated organs and tissues, such as blood vessels, trachea, vas deferens, and

uterus.

(4) Effects on the respiratory and cardiovascular systems

① Effects of the test substance on changes in blood pressure and heart rate induced by autonomic drugs, vagal stimulation, and occlusion of the common carotid artery should be assessed.

② Effects on the heart should be evaluated *in situ*.

③ Evaluation should be conducted using isolated organs and tissues, such as the heart, atrium, papillary muscle, and vascular bed.

(5) Effects on the gastrointestinal system

① Effects of the test substance on gastrointestinal secretions such as gastric juice, saliva, bile, and pancreatic juice should be evaluated.

② Effects on the motility of the gastrointestinal tract should be evaluated using the isolated stomach and intestine.

③ Effects on the motility of the gastrointestinal tract should be evaluated using the stomach and intestine *in situ*.

④ Effects on the gastric and duodenal mucous membranes should be evaluated.

(6) Other pharmacological effects

① Effects of the substance on blood coagulation should be evaluated.

② Effects on platelet aggregation should be evaluated.

③ Effects on hemolytic potential should be evaluated.

④ Effects on renal function should be evaluated.

[11] Metabolism and pharmacokinetic study

This study is designed to provide information about the biodisposition and pharmacokinetics of a test substance including absorption, distribution, metabolism, excretion and the kinetics of these processes by administering the test substance to animals. The information obtained should assist in the evaluation of the adequacy and significance of the results of toxicity studies.

The design of this study should consider the principles presented below. Some of the principles may be omitted or replaced depending upon the characteristics of the test substance. Appropriate metabolic and pharmacokinetic data obtained from toxicity studies using the test substance may be used in this study.

1. Test substance

A food additive or a substance intended for use as a food additive is used either as the isotope-labeled form or as the non isotope-labeled form. The isotope-labeled compound used should be fully characterized including the name of the supplier, the method of synthesis, purity, nuclide, labeled position, radioactivity, and stability.

2. Animal species and sex

At least one rodent species (usually the rat) and at least one non-rodent species (usually the dog) are used.

The same species and strain that were used for the toxicity studies should be used for this study. Both sexes should be used. Infant/immature animals should be used when deemed appropriate or necessary.

3. Number of animals

The number of animals should be sufficient to provide meaningful data and should recognize individual differences among test animals.

4. Route of administration

The oral route should be used. When deemed appropriate or necessary, other routes may be used such as the intravenous route.

5. Dosage regimen

Both single and repeated administrations should be used. The timing (interval and duration) of repeated administrations should be based on steady state and cumulativity data. If meaningful metabolic and pharmacokinetic data can be obtained from toxicity studies, the need for repeated administrations in this study may be obviated.

6. Dose levels

At least two levels should be used. In setting those levels, the highest level for repeated dosing toxicity studies and the level not inducing toxicity (no-observed-adverse-effect level, NOAEL) should be consulted.

If possible, the setting of an additional lower dose level should be based on an estimated exposure derived from food.

7. Test method

The basis for the test methods selected, and their sensitivity, accuracy and specificity should be clarified.

8. Parameters evaluated

The parameters of absorption, distribution, metabolism, and excretion should be evaluated throughout the study. When deemed necessary, biological half-lives (or constants equivalent to half-lives), metabolic clearances, volume of distribution, and bioavailability should be determined. The test substance should be examined for the presence of non-linear regression. When the test substance is metabolized in the body, the metabolites should be examined.

(1) Absorption

The amount and rate of absorption of the test substance should be obtained. This can be obtained from the blood concentration-versus-time curve or cumulative-excretion amount curve.

Method using blood concentration (concentration in serum, plasma, or whole blood) versus time curve

The degree and rate of absorption can be obtained from parameters, such as the maximum concentration in blood after administration (Cmax), the time required for the blood concentration to reach tis maximum (Tmax), and the area under the blood concentration-versus-time curve (AUC). Also comparison of these parameters between oral route and intravenous route or other route of administration will improve the precision of estimation of the degree and rate of absorption.

Method using cumulative excretion-amount curve

The amounts of the test substance and its metabolites excreted into urine, feces, bile, and exhaled air should be determined to obtain the total amount excreted. These cumulative amounts excreted can lead to reliable indexes for the extent of absorption of the test substance.

When deemed necessary or appropriate, the following parameters which influence absorption should be examined.

① Physico-chemical form of the test substance in food
② Sites of absorption
③ Metabolism and stability in the gastrointestinal tract
④ Test diet and pH in the gastrointestinal tract

(2) Distribution

Information should be obtained on distribution of the test substance in individual organs and tissues and changes with time and cumulativity of the test substance. Determinations should be performed at several times when metabolism and pharmacokinetics can be reflexed adequately.

① Concentrations in organs and tissues

For organs and tissues in which the test substance is distributed in high concentration or expected to be accumulated or which can be targets of toxicity of the test substance, it is recommended that the chemical form of the test substance in these organs and tissues be characterized fully.
Whole body autoradiography is an effective method to obtain information on distribution of the test substance in sites where reliable information cannot be provided by determination methods using dissection of organs and tissues.

② Transfer into placenta, fetus, and milk

③ Binding with plasma proteins, distribution in blood corpuscles

(3) Metabolism

The test substance and its major metabolites should be identified and quantified to obtain information about metabolic pathways and the degree and rate of metabolism.
This test is usually performed by isolating and quantifying the test substance and its metabolites in biological specimens including blood, urine, bile, and feces. In vitro studies using specimens such as organ slices, cell suspensions, tissue homogenates, and subcellular fractions are also very useful as metabolic studies.

(4) Excretion

The excretion pathways and the degree and rate of excretion of the test substances and

its major metabolites via the following routes should be determined.

① Urine, feces, exhaled air

When single doses of the radio-labeled compound are administered, recovery tests should be performed for 7 consecutive days after administration or until at least 95% of the radioactivity is recovered.

② Bile

When bile is a major excretory pathway, enterohepatic circulation should be examined.

③ Milk

④ When necessary, at least the following parameters which can affect excretion should be examined.

Renal function

Urinary pH

(5) General considerations

Findings from these tests should be compared with data from compounds which are chemically similar to the test substance, and be discussed extensively. If the test substance is a racemate, the metabolism and pharmacokinetics of each optical isomer should be evaluated when necessary in relation to the toxicity findings.