

Practices for documents on veterinary drugs

**Section 4 of the attachment to Director General Notification,
No. 12-33, March 31, 2000,
Food Safety and Consumer Affairs Bureau,
Ministry of Agriculture, Forestry and Fisheries of Japan**

Note: This translation is made by Ministry of Health, Labour and Welfare.
In the case of any discrepancy between the Japanese original and the
English translation, the former will take priority.

4. Guidelines

1. Guidelines for toxicity studies of new animal drugs

The purpose of the present guidelines is to outline standard methods for safety studies aimed at application for approval of new animal drugs and to contribute to the appropriate evaluation of animal drugs.

However, it is not practical to apply a uniform test method to all animal drugs and it often becomes necessary to perform additional experiments along with the progress of studies. Therefore, strict application of the methods described below is not mandatory providing that observations obtained contribute to the evaluation of clinical safety.

(1) Acute toxicity studies, subacute toxicity studies and chronic toxicity studies

All new animal drugs should be studied, in principle, using small animals.

A. Animals

- (a) Animal species and strain should be selected in consideration of the life span, incidences of naturally occurring diseases, and sensitivity to known toxic substances.
- (b) If acute, subacute and chronic toxicity studies are to be conducted on the same substance, it is advisable to use the same species and strain.

B. Methods

(a) Acute toxicity study

- (i) Animals:
Animals of one or more species showing normal growth should be used. Nulliparous, non-pregnant female rats are commonly used.
- (ii) Number of animals:
An appropriate number of animals for the purpose of the study should be used.
- (iii) Route of administration:
Oral administration is used, in principle. If the intended clinical route of administration is parenteral, the intended clinical route should also be tested. If the intended clinical route is special and not applicable to the animals, another appropriate route should be used.
In principal, oral administration should be given by gavage and animals should be fasted for a fixed period of time before administration of the test substance by gavage.
- (iv) Dose levels:
Sufficient number of dose groups should be employed for determination of dose-response relationship and an approximate lethal dose 50% (LD50). Usually the upper limit for the administration should be set at 2000mg/kg.

- (v) Frequency of administration:
In principle, the test substance should be administered in a single dose.
- (vi) Observation period:
In principle, the observation period is 14 days.
- (vii) Experimental procedures:
 - a. For all animals, general condition should be observed at least once within 30 minutes after administration and at regular intervals until 24 hours, and every day thereafter.
 - b. Animals should be weighed just before administration and at least once a week.
 - c. All animals are necropsied at the end of the observation period (or at the time of death), and all organs and tissues are macroscopically examined, and the findings recorded.

(b) Subacute toxicity study

- (i) Animals:
Male and female animals of one or more species at the same weeks of age showing normal growth should be used. The rat or mouse is commonly used as a small animal.
- (ii) Number of animals:
At least 5 males and 5 females should be used per group. When a special examination that causes a heavy burden on animals or when interim sacrifice or a recovery test is scheduled, the number of animals necessary for these tests should be added beforehand.
- (iii) Route of administration:
The intended clinical route of administration should be used in principle. In the case of oral administration, oral gavage or free consumption from diet or drinking water may be used.
- (iv) Dose levels:
Three or more dose groups and a control group should be employed for both male and female animals.
Referring to the results of an acute toxicity study or a preliminary short-term repeated administration study, dose levels and the number of groups necessary to observe adverse reactions and their severity and to determine the toxic level, minimum toxic level and no observable adverse effect level (NOAEL) are selected. The toxic level is a dose at which death in some animals or other evident toxic changes occur and the minimum toxic level is a dose that causes some toxic changes. NOAEL is a dose at which no toxic change is observed in any animal. If the test substance is administered in diet or drinking water, the test substance intake should be calculated from the food and water consumption.
- (v) Control group:
A negative control group should be employed. The negative control group is administered only the solvent and/or emulsifier used for administration of the test substance. In addition, it is desirable to employ a separate untreated control group.
- (vi) Administration period:

The administration should be continued for at least 3 weeks, 7 days a week.

(vii) Experimental procedures:

- a. General condition of all animals in each group should be closely examined every day and the body weight should be recorded once a week or more frequently.
- b. During the administration period, individual or group food consumption should be measured once a week or more frequently.
- c. During the administration period, urinalysis and ophthalmologic examination should be performed once or more on all or a portion of animals in each group. It is desirable to add appropriate laboratory tests in consideration of the chemical structure and pharmacological actions of the test substance and the general condition of the animals.
- d. Animals that die during the administration period should be immediately necropsied for macroscopic examinations of the organs and tissues.
- e. Moribund animals during the administration period should be immediately necropsied to macroscopically examine the organs and tissues.
At the time of sacrifice, it is desirable to collect blood for hematology and blood chemistry tests.
- f. Surviving animals at the completion of the administration period should be necropsied after 24 hours and macroscopic examinations of the organs and tissues should be performed for all cases. At the time of sacrifice, blood should be collected for hematology and blood chemistry tests. It is desirable to perform these tests for as many items as possible and the methods and units commonly used worldwide should be used for the tests.
It is desirable to analyze the amount of residual test substance in muscle, fat, liver and kidney.

(c) Chronic toxicity study

(i) Animals:

Male and female animals of one or more species of the same weeks of age showing normal growth should be used. The rat or mouse is commonly used as a small animal.

(ii) Number of animals:

Male and female animals in groups of at least 10 animals each are used, in principle. When a special examination that causes a heavy burden on animals or when interim sacrifice or recovery tests are scheduled, the number of animals necessary for these tests should be added beforehand.

(iii) Route of administration:

The intended clinical route of administration or oral administration should be used. In the case of oral administration, oral gavage or free consumption from diet or drinking water may be used.

(iv) Dose levels:

Three or more dose groups and a control group should be employed for both male and female animals.

Referring to the results of a subacute toxicity study, dose levels and the number of groups should be selected to determine a dose related to some toxic changes and no observable adverse effect level (NOAEL).

If the test substance is administered in diet or drinking water, the test substance intake should be calculated from the food or water consumption.

(v) Control group:

A negative control group should be employed. The negative control group is administered only the solvent and/or emulsifier used for administration of the test substance. In addition, it is desirable to separately employ an untreated control group.

(vi) Administration period:

The administration should be continued for more than 3 months, 7 days a week.

(vii) Experimental procedures:

a. For all animals, general condition should be observed every day, and body weight measured once or more a week for the first 3 months of the treatment and once or more every 4 weeks thereafter.

b. During the administration period, individual or group food consumption should be recorded once or more a week during the first 3 months of the treatment and once or more every 4 weeks thereafter.

c. During the administration period, urinalysis and ophthalmologic examination should be performed once or more on a fixed number of animals randomly selected from each group. Other laboratory tests should be performed, if necessary.

d. Animals that die during the administration period should be immediately necropsied to perform macroscopic examinations, weight measurement and histopathological examinations of the organs and tissues.

Organs and tissues subjected to histopathological examinations are listed below, but some of them may be omitted if it is considered unnecessary to perform the examination from the results of macroscopic examination.

Skin, mammary gland, lymph gland, salivary gland, sternum, femur (including bone marrow), thymus, trachea/lung/bronchi, heart*, thyroid gland/parathyroid gland, tongue, esophagus, stomach/duodenum, small intestine, large intestine, liver*, pancreas, spleen*, kidney*, adrenal gland*, bladder, seminal vesicle, prostate*, testis*, ovary*, uterus, vagina, brain*, pituitary gland*, spinal cord, eye ball, Harder's gland, and other organs/tissues exhibiting macroscopic changes.

Of these organs/tissues, those marked with* are weighed.

e. Moribund animals during the administration period should be immediately necropsied and the organs and tissues should be macroscopically examined, weighed then histopathologically examined as described in section d.

At the time of sacrifice, it is desirable to collect blood for hematology and blood chemistry tests.

- f. Surviving animals at the completion of the administration period should be necropsied and macroscopic examinations and weight measurement of the organs and tissues listed in section d should be performed for all cases. Histopathological examinations should be performed on all animals in the control group and the highest dose group, in principle. If there is any macroscopic change in organs/tissues in other dose groups, or if it is considered necessary from the changes observed in the highest dose group, histopathological examination of the relevant organs/tissues should be performed on all animals of other groups. At the time of sacrifice, blood should be collected for hematology and blood chemistry tests. It is desirable to perform these tests of as many items as possible and the methods and units commonly used worldwide should be used for the tests.

(2) Reproductive/developmental toxicity study

In principle, '(a) Teratogenicity study' should be performed for all new animal drugs.

'(b) One-generation reproductive toxicity study' should be performed if it is considered necessary from the results of this study or if it is suspected from known findings that the test substance has adverse effects on fertility of male or female animals or on reproductive processes including parturition. As a study method to accurately investigate the adverse effects on reproduction/development, the reproduction processes from the pregestation period to weaning may be divided into 3 segments of administration to perform 3 administration studies, namely: '(I) Administration before and in the early stage of pregnancy', '(II) Administration during the organogenetic period' and '(III) Administration during the perinatal and lactation periods'.

A. Animals

- (a) Species and strains should be selected in consideration of information regarding reproduction including fertility, incidence of spontaneous malformation and susceptibility to substances known to have adverse effect on reproduction and development.
- (b) It is desirable to select a species and strain with a low incidence of spontaneous malformations.
- (c) It is desirable that the animals used in the above (a) and (b) are of the same strain and species.

B. Methods

- (a) Teratogenicity study
 - (i) Animals:

Female animals of one or more species selected from rodents such as the rat and the mouse and one from non-rodents such as the rabbit should be used.

Usually, animals easy to use in studies and having known metabolic profiles are selected.

(ii) Number of animals:

At least 20 animals per group are required for rats and mice, and 8 or more animals per group for rabbits.

The number of animals means the number of those with established pregnancy.

If any animal species other than the rat, mouse and rabbit is to be used, a number of animals considered sufficient to obtain necessary findings for evaluation should be used.

(iii) Route of administration:

The route of administration should be the intended clinical route in principle.

In the case of oral administration, forced oral administration by gavage is used, in principle.

Forced administration can ensure intake of a fixed dose and is considered superior to free consumption from diet or drinking water.

If it is difficult to use the intended clinical route, other routes of administration may be used.

(iv) Dose levels:

Study groups should consist of 3 or more dose levels and a control group should be employed separately.

The highest dose should be set at a level at which some evident toxic signs can be seen, such as a decrease in food consumption or reduction of weight gain. If no toxic sign is observed even at the highest technically possible dose, the dose should be used as the highest dose of the study. The lowest dose should be set at a level at which no disorder occurs either in the dams or in the fetuses. Medium dose(s) should be set at the geometric mean of the highest and the lowest doses, in principle. It is desirable to include a dose level at which pharmacological action is exhibited in the test animal or a dose close to the estimated usual clinical dose.

(v) Control group:

A negative control group should be employed. A positive or reference control group should be employed, if necessary.

When a solvent or an emulsifier is used in the administration of the test substance, a negative control group should be given the vehicle or emulsifier alone, in principle. A positive control group should receive a substance known to have teratogenicity, and a reference control group should receive an existing drug with similar chemical structure or pharmacological actions.

(vi) Administration period:

The test substance should be administered every day during the fetal organogenetic period.

(vii) Experimental procedures:

- a. During the study period, all dams in each group should be examined for mortality and general signs, and body weight and food consumption should be recorded.
 - b. All dams should be necropsied at term and examined for successful pregnancy and mortality of fetuses. Surviving fetuses should be weighed and morphologically examined. Dead fetuses should be recorded with findings based on which the time of death can be estimated. Macroscopic observations of organs and tissues should be performed for the dams.
- (b) One-generation reproduction toxicity study
- (i) Animals:
Male and female animals of one or more species selected from rodents should be used. Usually the rat or the mouse is used.
 - (ii) Number of animals:
A group should consist of at least 20 male animals and sufficient number of females to assure 20 pregnant animals at term.
 - (iii) Route of administration:
The intended clinical route of administration should be used in principle.
In the case of oral administration, forced oral gavage or free intake from food or drinking water may be used. If it is difficult to use the intended clinical route, other appropriate routes may be used.
 - (iv) Dose levels:
Three or more dose levels and a separate control group should be employed.
The highest dose should be set at a level at which some evident toxic signs are observed, such as a decrease in food consumption or reduction of weight gain. If no toxic sign is observed even at the highest technically possible dose, the dose should be used as the highest dose for the study. The lowest dose should be set at a level at which no disorder occurs either in the dam, fetuses or neonates. Medium dose(s) should be set at geometric mean of the highest and the lowest doses, in principle.
It is desirable to include a dose level at which pharmacological action is exhibited in the test animal or a dose close to the estimated usual clinical dose.
 - (v) Control group:
A negative control group should be employed. A positive or reference control group should also be employed, if necessary.
When a solvent or an emulsifier is required for administration of the test substance, a negative control group should be administered the vehicle or emulsifier alone, in principle. A positive control group should receive a substance known to have adverse effects on reproduction, and a reference control group should receive an existing drug with similar chemical structure or pharmacological actions.
 - (vi) Administration period:
Both the male and female animals should be administered every day for at least 8 weeks from 8 weeks of age before they are subjected to

mating. For mating, the same pair of a male and a female should be housed together for a maximum period of 3 weeks.

The males should be continually administered during the mating period, and for the females the administration should be continued over the periods of mating, pregnancy and until weaning of offspring at 3 weeks after parturition.

(vii) Experimental procedures:

a. During the experimental period, all animals in each group should be examined for mortality and general signs, and body weight and food consumption should be recorded for the dams.

b. After completion of mating, the males should be necropsied for macroscopic examinations of organs and tissues. For pairs without evidence of copulation, the reason should be investigated.

The copulation index and fertility index should be calculated usually with the following equations.

Copulation index = (Number of animals with successful copulation/Number of mated animals) × 100

Fertility index = (Number of pregnant animals/Number of animals with successful copulation) × 100

c. All dams in each group should be allowed to give birth.

At parturition, signs of disorders and delay of parturition should be observed, if any.

Birth index should be calculated usually with the following equation.

Birth index = (Number of females giving birth to live offspring/Number of pregnant females) × 100

d. For neonates, the litter size, mortality, sexes and external changes should be examined and the body weights recorded.

If it is necessary to standardize the litter size, a fixed number of offspring consisting of the same number of male and female animals from one dam should be randomly selected at a relatively early stage after birth, and the remaining offspring culled. For rats and mice, the litter size should be decreased to about 8 usually at 4 days of age.

e. Liveborn offspring should be examined for growth and development and presence or absence of specific symptoms and abnormal behavior.

If any abnormality is found in offspring, and additional nursing study should be performed, if necessary, to determine at what stage after birth the animals were affected.

Growth and development should be investigated by analyzing the morphology, functions and behavior.

The observation period should be extended, if necessary. Birth index, viability index and weaning index should be determined during the period from parturition to weaning usually with the following equations.

Birth index = (Number of offspring born alive/Number of implantations) × 100

Viability index on day 4 = (Number of offspring alive on day 4 after birth/ Number of offspring born alive) × 100

Weaning index = (Number of live weanlings/Number of offspring alive on Day 4 or just after culling) × 100

- f. Treated dams should be necropsied at an appropriate time to perform macroscopic examinations of the organs and tissues. If necessary, the administration of the test substance should be continued to investigate the effect on multiple generations.

(3) Mutagenicity studies

In principle, every new animal drug should be subjected to at least “(A) Bacterial reverse mutation study” using the induction of gene mutation as an indicator, and “(B) Chromosomal aberration study on mammalian culture cells” using chromosomal aberrations as an indicator. However, if mutagenicity is suspected from the results of studies (A) or (B), “(C) Micronucleus test in mice” should also be performed.

It is desirable to perform additional mutagenicity tests if it is considered necessary from the results of these or other toxicity studies, or pharmacological studies.

Methods

(a) Bacterial reverse mutation test

(i) Strains:

Several bacterial strains should be used including *Salmonella typhimurium* TA1535, TA1537, TA98 and TA100 and *Escherichia coli* WP2*uvrA*.

(ii) Dose levels:

Five to six dose groups and an additional control group should be employed. In principle, the highest dose should be no more than 5 mg/plate and a dose with antimicrobial activity should be used for antibiotics.

(iii) Controls:

A negative control group and a positive control group should be employed.

In principle, the negative control should be administered the solvent. Known mutagens which require S9 mix as well as those do not require S9 mix should be employed as positive controls.

(iv) Metabolic activation:

Tests in the presence and absence of S9 mix should be performed in parallel.

Animals of a mammalian species (usually rats) should be given an appropriate metabolic enzyme inducer to prepare S9 from the liver. S9 mix consisting of the S9 and a coenzyme is used.

(v) Experimental procedures:

Either the preincubation method or plate incorporation method should be used. For antibiotics and other drugs with specifically strong antimicrobial activity, it is desirable to wash the organisms after incubation with the test substance and to suspend the cells again to calculate the frequency of mutation from the number of cells with mutation and the viable cell count.

(vi) Presentation of results:

The actual number and mean value of revertants should be presented (including figures).

(b) Chromosomal aberration test on mammalian culture cells

- (i) Cells:
Primary or established cell lines of mammalian cells in culture should be used.
It is desirable to use Chinese Hamster fibroblasts (CHL, CHO) or others with as high sensitivity as possible.
- (ii) Dose levels:
At least three dose levels should be tested.
The highest dose should be a dose close to the 50% inhibitory concentration for cell growth (or proliferation).
If no cytotoxicity is observed, a concentration of 10mM or 5mg/mL should be used as the upper limit of the dose.
- (iii) Controls:
A negative control group and a positive control group should be employed.
In principle, a solvent should be used as a negative control. A substance known to cause chromosomal aberration should be employed as a positive control.
- (iv) Metabolic activation:
Test should also be performed with a suitable metabolic activation. Animals of a mammalian species (usually rats) should be administered an appropriate metabolic enzyme inducer to prepare S9 from the liver. S9 is mixed with coenzyme and used as an S9 mix.
- (v) Experimental procedures:
Chromosome preparations should be prepared at an appropriate time after treatment.
At least two plates should be used for each dose level.
A hundred metaphase cells per plate should be examined for structural chromosomal aberration and polyploid cells.
For the structural aberrations, abnormalities in chromatid or chromosome structures should be described.
- (vi) Presentation of results:
The incidence of cells with chromosomal aberrations or frequency of chromosomal aberrations per cell should be presented (including figures).

(c) Micronucleus test in mice

This test may be substituted with a chromosomal aberration test using bone marrow cells from rodents.

- (i) Animals:
Pure line or homogenous male animals should be used in principle.
- (ii) Number of animals:
At least 5 animals per group should be used.
- (iii) Route of administration:

The test substance should be administered intraperitoneally or orally. Oral administration should be given by gavage in principle.

- (iv) Dose levels:

At least three dose groups should be employed.
The highest dose should be a dose at which some toxic signs such as reduction of weight gain are observed. If no toxic sign is observed, 2000mg/kg should be used as the highest dose.
- (v) Control groups:

A negative control and a positive control should be employed.
In principle, a control group administered the solvent should be used as the negative control. A positive control group should receive a substance known to induce micronuclei.
- (vi) Frequency of administration:

A single dose or 4 to 5 repeated dose should be given. An appropriate uniform dose should be selected for repeated administration.
- (vii) Experimental procedures:
 - a. All animals in each group should be sacrificed at an appropriate time after administration of the test substance to prepare bone marrow smears.

It is desirable to prepare the specimens 18 to 30 hours after the administration.
 - b. In principle, 1000 polychromatic erythrocytes per animal should be observed for presence or absence of micronuclei. The frequency of polychromatic erythrocytes in the total erythrocytes should also be calculated. It is acceptable to observe the rate of reticulocytes instead of polychromatic erythrocytes.
- (viii) Presentation of results:

The incidences of polychromatic erythrocytes with micronuclei and the rate of polychromatic erythrocytes in the total erythrocytes should be presented.
If positive results are obtained, the dose dependency should be graphically shown.

(4) Carcinogenicity studies

Carcinogenicity studies should be conducted if carcinogenicity is suspected from the following.

- (a) Chemical structure or pharmacological action
- (b) Results of toxicity studies
- (c) Others

A. Animals

- (i) Animal species and the strain should be selected in consideration of resistance against infectious diseases, life span, spontaneous tumor incidence and sensitivity to known carcinogens.

- (ii) Animals of the same species and strain should be used for preliminary and full-scale carcinogenicity studies on the same test substance.

B. Methods

- (i) **Animals:**

At least two species of animal of both sexes should be used. It is desirable to use animals showing normal growth of the same age up to 6 weeks.

Usually the rat, the mouse and the hamster are presently used.

It is desirable to use animals in the as early stages as possible after weaning.
- (ii) **Number of animals:**

At least 50 males and 50 females should be used per group. Allocation of animals to each group should be made with a proper random sampling method based on body weight stratification.
- (iii) **Route of administration:**

In principle, the route of clinical application should be used.

In the case of oral administration, gavage or free intake from diet or drinking water may be used.

If the test substance is combined with the diet, the concentration of the test substance in the diet should be 5% or less.
- (iv) **Dose levels:**

Three or more dose groups and a control group should be employed for both male and female animals.

The highest dose should be a dose determined from the preliminary subacute toxicity study, and the lowest dose should be a dose at which pharmacological effect is expected in the experimental animal or determined in consideration of the intended clinical dose. It is desirable to determine the medium dose as a geometric mean of the highest and lowest doses.

Usually, the lowest dose should not be less than 10% of the highest dose. However, if the lowest dose differs from the intended clinical usual dose markedly, an additional dose below 10% of the highest dose may also be employed. If the test substance is to be administered in diet or drinking water, the individual or group consumption quantity of food or water should be measured during the administration period at least once a week for the first three months and once or more in every 3 months thereafter to calculate the amount of test substance intake. The purity, stability and contaminants of the test substance should be analyzed qualitatively or quantitatively, before and after the commencement of the study whenever possible.
- (v) **Control group:**

A negative control group should be employed.

The negative control group is administered only the solvent and/or emulsifier used for administration of the test substance. In addition, it is desirable to employ an untreated control group.
- (vi) **Administration period:**

Administration period should be 24 to 30 months for rat and 18 to 24 months for mouse and hamster, 7 days a week.

In the case of forced administration, administration at least 5 days in a week is acceptable from a practical viewpoint.

(vii) Study period:

The study is to be terminated at the end of the administration period or 1 to 3 months later. However, the study period should not exceed 30 months for rat and 24 months for mouse and hamster. If the cumulative mortality reaches 75% in the lowest dose group or in the control group, the survivors should be sacrificed at the time and the study should be discontinued.

It is required that mortality due to reasons other than tumors should be 50% or more at 24 months after starting administration for rat and at 18 months for mouse and hamster.

If any group, animal loss due to autolysis, cannibalism or housing problems should not be more than 10%.

Therefore, it is necessary to take proper measures including isolation and necropsy if any debilitated or moribund animal is found during the study.

(viii) Experimental procedures:

a. All animals of each group should be observed daily for general signs, and body weight should be recorded at least once a week during the first 3 months of administration, and more than once in every 4 weeks thereafter.

b. Animals that die during the experimental period should immediately be necropsied for macroscopic observation and histopathological examinations of the organs and tissues. Histopathological examination should be performed on the following organs and tissues.

Skin, mammary gland, lymph gland, salivary gland, sternum, vertebra or femur (including bone marrow), thymus, trachea/lung/bronchi, heart, thyroid gland/parathyroid, tongue, esophagus, stomach/duodenum, small intestine, large intestine, liver, pancreas, spleen, kidney, adrenal gland, bladder, seminal vesicle, prostate, testis, ovary, uterus, vagina, eye ball, brain, pituitary gland, spinal cord, and other organs and tissues with macroscopic neoplastic lesions.

Findings of various changes leading to tumorigenesis (precancerous lesions) should be added to the description of neoplastic lesions.

c. Moribund animals during the study period should be promptly isolated or necropsied for macroscopic and histopathological examinations of the organs and tissues as described in section b.

All the time of sacrifice, blood should be collected to measure peripheral red blood cell and white blood cell counts as well as to prepare smear specimens. The smear specimens should be examined for the cases suspected of blood dyscrasia such as anemia, swelling of lymph gland, liver and spleen.

d. All the survivors in each group at the completion of the study period should immediately be necropsied and macroscopic examinations of the organs and tissues performed as described in section b.

In principle, histopathological examination should be performed on both the dosing groups and the control group.

At the time of sacrifice, blood should be collected to measure peripheral red blood cell and white blood cell counts as well as to prepare smear specimens. The smear specimens should be examined for the cases suspected to have blood dyscrasia such as anemia, swelling of lymph gland, liver and spleen.

(5) Residue studies

In principle, new animal drugs intended for use in edible animals (including cultivated aquatic animals) shall be tested at two or more different laboratories/testing facilities including at least one that is located in Japan.

The Study Part 2 shall apply to veterinary medicinal products that contain one or more of the following substances as active ingredients: those listed in the first column of the table in Paragraph 6 (1) in Section A *Compositional Standards for Foods*, Part 1 *Food* under the heading of *Specifications and Standards for Foods, Food Additives* (Ministry of Health and Welfare Announce No. 370, December 28, 1959) (hereinafter referred to as “table of the food compositional standards”). The Study Part 1 shall apply to other veterinary medicinal products.

(a) Studies Part 1

(i) Animals:

Target animals of application of the test substance (including cultivated aquatic animals) for which the history of the use of diet and animal drugs and the method of rearing before the study have been established should be used.

It is required for cultivated aquatic animals that the water temperature during the study period remain within the range shown below.

Yellowtail, red sea bream, carp, eel: 18-24°C

Rainbow trout: 8-14°C

Ayu: 15-21°C

(ii) Number of Animals:

The number of animals necessary to elucidate elimination of the test substance should be used.

- (iii) Route of administration:

The intended clinical route of administration should be used in principle. If there are multiple routes, a route with which the test substance is expected to remain for the longest time may be used so long as the basis for selection is clear.
- (iv) Dose levels:

Two or more dose levels should be employed including the maximum clinical dose as the low dose level for the study, and a separate control group should also be used. A dose level twice the maximum clinical dose should be employed for cultivated aquatic animals.
- (v) Administration period:

The maximum period of clinical application should be employed. However, if the clinical application extends for a long period, the administration period may be reduced to a period which can assure that the residual test substance reaches a fixed level.
- (vi) Sample collection:
 - a. Sampling points necessary to demonstrate the accumulation and elimination profile of the test substance should be used.
 - b. Samples requiring animal sacrifice should be collected once at the beginning of the elimination phase of the test substance after completion of administration, once when the analyte is not detected in tissues and at least once between the two periods.
 - c. Samples which do not require animal sacrifice (e.g. blood, egg, milk) should be collected as frequently as possible.
 - d. Samples should be collected from edible parts (e.g. muscle, fat, liver, kidney, small intestine, egg, milk, injection site) where the test substance is expected to remain.
 - e. Blood, eggs and milk should be sampled for the test substances which are applied to livestock buildings and possibly absorbed by animals or chickens. In such a case, if there is no residual test substance detected in these parts, sampling of other edible parts is not required.
 - f. Sufficient attention should be paid in sampling to the possibility that residual concentration of the test substance may differ between sampling sites even in the same organ or tissue.
 - g. Samples should immediately be subjected to analysis.

When it is necessary to store samples for a long time, they should be kept frozen and care should be taken to avoid decomposition of the samples in the process of freezing/thawing. For eggs, it is desirable to keep the yolk and albumen separate.
- (vii) Analysis:
 - a. To conduct the study, it is necessary to establish an analytical method with considerable sensitivity, accuracy and reproducibility. Considerable sensitivity, accuracy and reproducibility here mean a detection limit of 0.05 ppm or less, a recovery rate of 70% or more in a spiked recovery test with test substance at 1-2 ppm, and a coefficient of variance ((standard deviation/mean) × 100) of about 10%.

- b. If the test substance is an antibiotic, the analysis should be performed by a biological method with sensitivity, accuracy and reproducibility.
Biological analysis is considered to be considerably sensitive if it has detection sensitivity under 0.1 Unit or 0.1 µg (potency)/mL. Accuracy and reproducibility requirements are as described in section a. above. However, if there is a method with a high correlation with a biological analysis and if it is superior to the biological method in sensitivity, accuracy and reproducibility, the method may be used in place of the biological method.
- c. The analyte should be an active ingredient of the test substance in principle. However, metabolites should also be analyzed if they are identified and there is a possibility of residue of the metabolite.
- d. Actual measurement data should be recorded without subtracting the control value. The data should not be corrected for the recovery rates.
- e. When a measurement is less than the detection limit (a ppm), it should be recorded as “<a ppm”, not as “not detected”.
- f. If data less than the detection limit is included, the mean should not be calculated.

(b) Studies Part 2

- (i) Animals:
As described in section (a) (i)
- (ii) Number of animals:
The number of animals necessary for statistical analysis should be used.
- (iii) Administration route:
As described in section (a) (3)
- (iv) Dose levels:
In principle, the dose level should be the the highest clinical application dose.
- (v) Administration period:
As described in section (a) (5)
- (vi) Sample collection:
 - a. Time points should be set to allow appropriate statistical analysis. However, in principle the analyte should be detected in at least 3 samples at each time point.
 - b. When test substances are those listed in the first column of the table of food compositional standards, subject animals shall be sacrificed at the following points of time and samples be collected: when the subject animals are in the elimination phase of the substances administered after the end of administration, when the residue concentrations of the test substances in the target organ or tissue come below the corresponding levels specified in the third column of the table of food compositional standards, and at least once between the above two points of time.
 - c. As described in section (a) (6) c.

- d. As described in section (a) (6) d. However, in principle the organ for the the residual standard value is set is used as the subject of analysis.

When the site of longest residue has been clearly established on scientific grounds, that site may be used as the sample to determine the washout period.

- e. As described in section (a) (6) e.
- f. As described in section (a) (6) f.
- g. As described in section (a) (6) g.

(vii) Analysis:

- a. When test substances are those listed in the first column of the table of food compositional standards, studies shall be conducted using the methods specified in the Notification of the Director General of Department of Food Safety (No. 0124001, January 24, 2005) or other methods comparable or superior in accuracy and sensitivity to the specified methods.
- b. As described in section (a) (7) d.
- c. As described in section (a) (7) e.
- d. As described in section (a) (7) f.

(viii) Statistical analysis:

- a. Washout period should be determined by using a proper statistical method.

[a] An exponential decay curve model is frequently used for the final drug excretion from tissues and other cell structures. This mathematical model has the following form.

$$C_1 = C_0 e^{-vt} \text{ -----(1)}$$

C_1 represents the measurement value at time t . If substitution in the corresponding natural logarithm is conducted: $\log_e C_1 = \log_e C_0 - vt$. If $Y_{(t)}$ denotes $\log_e C_1$, $a = \log_e C_0$, and $v = b$, then a linear regression equation for $Y_{(t)}$ in terms of t is

$$Y_{(t)} = a - bt \text{ -----(2)}$$

Appendices 3 and 4 indicate data by equation (2) and measurement data.

[b] Linear regression analysis

Verify the regression assumptions specified in (a) to (c).

[i] On the basis of scatterplots of data, examine data variation at each point and possible general linearly decreasing tendency. If outliers are detected, check process anomalies in design, sampling and drug measurements. The application form should be filled in with clear reasons for anomalies, if feasible. The outliers may be excluded from the following analysis.

You should begin the study over if the outliers are 10% or more of all data.

[ii] Concerning V_i at each point, check for homogeneity of variance by Cochran's test or Bartlett's test.

[iii] Analyze variance based on the regression, test for linearity and calculate the test statistics (see Appendix 5). Conduct F test to fit linear and nonlinear models at the 2.5% significance level ($p=0.025$). If there is a significant

linearity and no significant non-linearity, then calculate the residual variance V_e by combining residual and non-linearity sum of squares.

$$V_e = S^2 = (S_{TL} + S_R) / (N-2)$$

This will yield the test statistic for the regression.

$$\text{Regression coefficient (linearity)} \quad b = - S_{ty}/S_n$$

$$\text{Intercept} \quad a = Y_{..} - bt$$

b. How to evaluate washout period

[a] According to the following equation, estimate the upper limit of maximum permissible concentration (MPC) at the specified sampling time t_i .

$$Y(t_i) = a - bt_i + ks \quad \text{-----}(3)$$

Where s is the square root of error variance s^2 and k is the test statistic calculated by the equation $k = h \cdot t (N-2, a)$.

$$h = [1/N + (t_i - t_{..})^2 / S_n]^{1/2}$$

$$d = z_p / h$$

z_p is the value of upper 100(1- p) percentile point of the standard normal distribution. Select the upper 99 percentile ($p = 0.01$); $z_p = 2.3264$.

$t(N-2, a)$ is the value of upper 100(1- a) percentile point of the t distribution with $N-n$ degrees of freedom and noncentrality parameter d . Choose the 95 percentile at the significance level of $\alpha=0.05$. Of note, the t distribution with noncentrality parameter d is different from the t distribution with $d = 0$, so apply Owen's figures and other table of figures.

[b] Examine whether the upper limit of MPC $Y_{(t_1)}$ at any t_i exceeds the logarithm of standard for residual concentration. If so, repeat the calculation of (3) by replacing the greater value of t_i . The washout period is the period until t_i when $Y_{(t_i)}$ is below the logarithm of standard for residual concentration for the first time. The estimated concentration is confirmed by converting the antilogarithm of $e^{Y(t)}$ at the preceding t_i . Based on the assumption that the measurement concentration Y follow a normal distribution, the probability of $Y_{(t_i)}$ in (3), which falls below the logarithm of standard for residual concentration, will be above 99(1 - 0.001) percent. This probability is assured with a confidence level of 95(1-0.05) percent and this upper limit is the MPC.

(6) Safety study

For all new animal drugs, studies should be performed using target animals of the test substance in principle.

(i) Animals:

a. Target animals of the test substance (including cultivated aquatic animals) for which history of use of diet and animal drugs and the method of rearing before study have been established should be used.

It is required for cultivated aquatic animals that the water temperature during the study period remain within the range shown below.

Yellowtail, red sea bream, carp, eel: 22-28 °C

Rainbow trout: 12-18 °C

Ayu: 19-25 °C

- b. Among biological products for use in animals, vaccines, sera and diagnostic fluids directly used on animals (hereafter referred to as 'Vaccines'), animals conforming not only to the requirement under section a, but also to the following requirements, should be used. In the case of 'Vaccines' intended for use in pregnant animals, studies should be performed in both pregnant and non-pregnant animals.
 - [a] Target animals at days of age at which disorders are likely to occur with the highest potency during the intended age of application
 - [b] Target animals of which species, strains and specifications (SPF etc.), and in which types and amounts of available antibodies are confirmed
- (ii) Number of animals:

For each dose group, at least 3 animals should be used for mammalians, 10 or more for chickens, and 20 or more for cultivated aquatic animals
- (iii) Route of administration:

The route of administration should be the intended clinical route in principle. If there are several routes for clinical application, they may be represented by one with which the most severe disorders may occur. However, vaccines containing adjuvants should be tested in all possible routes of clinical application.
- (iv) Dose levels:
 - a. At least two dose groups and a separate control group should be employed.
 - b. Dose levels of the study groups should include a dose causing adverse reactions (or the highest possible dose) and a non-effective dose. The highest possible dose of 'Vaccines' should be determined at the following doses. It should be given in divided doses, if necessary, so that the animals are not physically affected.
 - [a] Live vaccines to be prepared just before use; 100 doses
 - [b] Others: 10 doses
- (v) Administration period:
 - a. Specimens other than the 'Vaccines' should be administered for a period longer than the longest clinical administration period. Even if the clinical application is limited to 1 or 2 days, administration for 3 days or longer is necessary. However, if the clinical application extends to a longer period, the administration period for the study may be reduced.
 - b. [a] Vaccines should be administered for the period or at the frequency of intended clinical use and at least one additional dose should be given after an interval of 2 months. However, if the lifetime frequency of administration is limited to 2 times

or less, it is acceptable to use the intended clinical administration period and frequency.

[b] Serum or diagnostic fluid is to be used directly on animals should be administered twice or more at intervals of 2 months.

(vi) Observation criteria:

- a. For all animals in the study groups, multiple observations of general condition should be performed every day and all or a part of the animals should be subjected to serological and biochemical examinations, if necessary.
- b. Animals that die during the experimental period should be immediately necropsied. The organs and tissues should be macroscopically observed, then weighed and histopathologically examined, if necessary.
- c. Moribund animals during the experimental period should be immediately necropsied. The organs and tissues should be macroscopically observed, then weighed and histopathologically examined, if necessary.
At the time of sacrifice, blood should be collected to perform hematology and blood chemistry tests.
- d. All or a part of survivors at the end of the study should be necropsied (except for the cases of topical acting agents or of at the highest dose showing no abnormality in general conditions and laboratory data). The organs and tissues should be macroscopically observed, then weighed and histopathologically examined,
At the time of sacrifice, blood should be collected to perform hematology and blood chemistry tests.
- e. For 'Vaccines' intended for use in pregnant animals, offspring of the pregnant animals used in the study should also be observed in the same manner as the study groups. For the vaccines containing adjuvants, accumulation and elimination of the adjuvant or other foreign materials should be observed in addition to conventional histopathological examinations.

2. Detailed rules for the conduct of various studies required for applications for manufacturing (import) approval of animal drugs for use on aquatic animals

Objective of these detailed rules is to indicate the detailed guidelines and recommendations to be added to the application method of the tests specified in the 'Guidelines for toxicity studies of new animal drugs' (part 1 the forth) for the items requiring consideration for properties of aquatic animals and aquatic cultivation industry among the studies conducted for approval of manufacture of animal drugs for use on aquatic animals (hereafter referred to as 'aquatic drugs'). However, it is not practical to establish uniform studies for all aquatic drugs. Furthermore, addition of new experiments may become necessary in many cases during progress of the studies. Therefore, it is not mandatory to persist in the methods specified here as long as it is assured that usefulness of the drug is appropriately evaluated.

(1) Methods of each study

A. Stability study

Considering the characteristics of aquatic cultivation, the following studies should be conducted for diet additives and bathing drugs including chemical substances nonexistent in the natural environment as active ingredients.

However, drugs for aquarium fish and assumed smaller use at home may be omitted from the following studies.

(a) Degradability study in marine water and in freshwater

The test substance is dissolved or suspended in marine water or in freshwater, depending on whether the target cultivated aquatic animal lives in marine water or in freshwater, at a concentration between one and 10 $\mu\text{g/mL}$, and 1 mL of the test sample placed in 2 mL beaker is kept at room temperature around 25°C, and then the sample is examined under the following three conditions. In each study, the decrease of water by volatilization is corrected by adding distilled water.

In addition, synthetic marine water may be used instead of native marine water.

Test 1: Under dark and static conditions

Test 2: Under 20,000 lux of light with a xenon lamp etc., while stirring

Test 3: Approximate 100 g of sand or mud obtained from near the cultivation area is added to the test substance sample and under the same conditions as test 2.

The test substance concentration of the sample solution is determined at 10, 20, and 30 days after beginning of the study, and then the degradation rate is obtained against the concentration at the beginning of the study.

An analytical method officially established by Japanese Pharmacopoeia etc. is desirable, however, other analytical methods in the public domain may be applied to determine the change of the test substance concentration in marine water or in freshwater.

B. Safety study

(a) Animals

It is desirable to use animals free from administration of any substance which may affect the study such as antimicrobial agents for more than 1 month.

Breeding chambers may be a tank in a room, outdoor ponds or fish preserves. Animals should be acclimatized before the study. During this period, it should be confirmed that no death occurs, that there is no abnormality in food consumption, body color or swimming performance and other health conditions in addition to sufficient acclimatization of the animals.

Animals should be fed during the acclimatization period and the study period. The feeding should be on a satiation basis and care should be taken to avoid food remaining and to ensure uniform feeding of animals. Food should be that used in actual cultivation of the aquatic animals and the raw materials and the composition ratio should be clarified.

Breeding water temperature for cultivated aquatic animals other than yellowtail, red sea bream, carp, eel, rainbow trout and ayu should be within the following ranges.

Fish in the Order of Clupeiformes: 12-18

Fish in the Orders of Cypriniformes, Anguilliformes, Perciformes, Cottiformes, Pleuronectiformes, Tetraodontiformes, Decapoda Crustacea: 22-28°C

(b) Dose levels

For aquatic animals, dose levels in the study groups may be up to 10 times the highest clinical dose because adverse reactions are unlikely to occur in aquatic animals even at high doses.

(c) Observation criteria

As a result of multiple observations of general conditions of all animal in the study groups every day, if there is no apparent adverse reactions observed in food consumption, body color or swimming performance at a considerably high dose compared to the maximum clinical dose and the drug is considered as safe, all or a part of the examinations requiring animal sacrifice and blood collection may be omitted depending on the necessity of each examination.

C. Pharmacological test (data of the primary pharmacodynamics)

(a) Susceptibility test using target pathogenic bacteria

This test should be performed only in the cases of antimicrobial medicines. Efficacy of the test substance should be evaluated to determine the susceptibility of target pathogenic bacteria to the test substance.

(i) Test bacterial strain:

About 50 or more field-isolated bacterial strains from aforementioned cultivated aquatic animals should be used. Test bacterial strains are

best collected broadly without bias regarding the season or place of collection, for example that the collected numbers of field-isolated strains are five or less in the same season and from the same place.

(ii) Methods:

Minimal inhibitory concentrations of test substance to each test bacterial strain should be determined with agar plate dilution method according to 'Standard method of Japanese Society of Chemotherapy'.

(iii) Others:

When a metabolic product having antimicrobial activity by the administration of the test substance is recognized and efficacy of the metabolic product is claimed, antimicrobial activity of the metabolic product against target pathogenic bacteria should be examined with the aforementioned susceptibility test.

(b) Dose-finding study

This study aims to find the effective dose of antimicrobial medicines for clinical study and safety study. The efficacy of administered test substance should be evaluated after inoculation of target pathogenic bacteria to test animals. In addition, this test may be omitted when dose-finding is to be determined in clinical study or when there are other data for determining the dose-finding.

(i) Animals:

Target animals of the cultivated aquatic animals for which history of use of diet and animal cultivation and the method of rearing before study have been established should be used. It is desirable to use animals free from administration of any substance which may affect the study such as antimicrobial agents for more than 1 month. Breeding chambers may be a tank in a room, outdoor ponds or fish preserves, as far as diffusion of pathogenic bacteria has no bad influence on the environment.

Animals should be acclimatized before the study. During this period, it should be confirmed that no death occurs, that there is no abnormality in food consumption, body color or swimming performance and other health conditions in addition to sufficient acclimatization of the animals.

Animals should be fed during the acclimatization period and the study period. The feeding should be on a satiation basis and care should be taken to avoid food remaining and to ensure uniform feeding of animals. Food should be that used in actual cultivation of the aquatic animals and the raw materials and the composition ratio should be clarified.

Breeding water temperature should be within the range expected for the disease in question.

(ii) Number of animals:

For each dosage group, about 20 animals or more should be used.

(iii) Infection:

A human-induced infection should be implemented.

After LD50 determination of test bacterial strain by preliminary examination, inoculation amount should be established based on it and

should be written clearly. In this study, the inoculation amount of bacterial strain should be established to be basically 70% or more mortality rate in control group without the administration and the dosage should be determined to be basically 70% or more efficacy in the highest dose group.

In addition, the efficacy in this case should be calculated from the following equation.

$$\text{Efficacy (\%)} = (1 - (\text{mortality rate in dosage group} / \text{mortality rate in control group})) \times 100$$

However, it is not necessary to use aforementioned mortality and efficacy rates when a dosage group of existing medicine is used as control group.

- (iv) Route of administration:
The route of administration should be the intended clinical route. If it is oral administration, forced oral administration may be acceptable.
- (v) Beginning time of administration:
The first administration should be performed within 2 hours after human-induced infection.
- (vi) Dose levels:
Three dosage groups including dosage groups of half and twice the intended clinical dosage and a separate control group should be employed.
- (vii) Observation criteria:
The presence or absence of death in each group should be observed every day. Dead and moribund animals during the experimental period should be immediately necropsied, and if necessary bacteriologic examination should be performed to determine whether death and moribundity were due to the pathogenic bacterium in question.

D. Absorptions study

(a) Absorption/Excretion studies for the approval of new drugs

- (i) Animals:
Target animals of the cultivated aquatic animals for which history of use of diet and animal cultivation and the method of rearing before study have been established should be used. It is desirable to use animals free from administration of any substance which may affect the study such as antimicrobial agents for more than 1 month. Breeding chambers may be a tank in a room, outdoor ponds or fish preserves. Animals should be acclimatized before the study. During this period, it should be confirmed that no death occurs, that there is no abnormality in food consumption, body color or swimming performance and other health conditions in addition to sufficient acclimatization of the animals.
Animals should be fed during the acclimatization period and the study period. The feeding should be on a satiation basis and care should be taken to avoid food remaining and to ensure uniform feeding of animals. Food should be that used in actual cultivation of the aquatic animals and the raw materials and the composition ratio should be clarified.

However, feeding may be stopped if the animals do not show feeding behavior after forced oral administration of test substance.

Breeding water temperature should be within the ranges expected for the disease in question.

- (ii) Number of animals:
Three animals or more should be used at each sampling time.
- (iii) Route of administration:
The route of administration should be the intended clinical route. If it is oral administration, forced oral administration may be acceptable.
- (iv) Dose level:
Dose level should be one dose at the highest clinical dose.
- (v) Administration period:
Administration should be once.
- (vi) Sampling of test sample:
The sampling time of the test samples should be set to detect increase and decrease of the amount of the test substance and the highest amount of it in plasma and in each organ. When metabolic products having antimicrobial activities and efficacy of the metabolic products is claimed, changes in these products over time should be also clarified. The samples obtained should be plasma, muscle, liver, and kidneys for fish, and muscle and digestive gland for Crustacea.
Each sample should be analyzed for each corresponding animal; however, samples may be mixed from a few animals if the amount of the sample is inadequate for the analysis. In this case, one sample may be used at each sampling time.
The samples should be immediately analyzed. The samples should be stored frozen when they have to be stored for a long period and care should be taken to avoid the test substance being degraded during the process of freezing/thawing and to avoid the analytical value being affected with instruments and materials for preservation.
- (vii) Sampling methods:
Muscle --- For fish, muscle from above the lateral line of the basal part of the first dorsal fin of the left of the body, without skin or dark-colored flesh meat.
Plasma --- Plasma should be obtained by immediate centrifugation after blood collection from dorsal aorta (vena cava) or heart or Cuvier's canal. Whole blood may be used for analytical sample instead of plasma.
Internal organs --- Target organs should be collected taking care to avoid injuring the gallbladder in fish. The collected internal organs should be washed well with saline etc. if the gallbladder is injured and bile leaked and the fact should be filled out in the remarks column.
- (viii) Analysis
Analytical method should be employed to clarify the change of test substance and metabolic products having antimicrobial activities *in vivo* over time.
Each analyzed value should be recorded for each test sample. In addition, average values should be recorded except the case including measured value less than detectable limit. The changes over time should be shown as a figure independently.

(b) Bioequivalence studies for approval of generic drugs

(i) Animals:

Target animals of the cultivated aquatic animals for which history of use of diet and animal cultivation and the method of rearing before study have been established should be used. It is desirable to use animals free from administration of any substance which may affect the study such as antimicrobial agents for more than 1 month. Breeding chambers may be a tank in a room, outdoor ponds or fish preserves.

Animals should be acclimatized before the study. During this period, it should be confirmed that no death occurs, that there is no abnormality in food consumption, body color or swimming performance and other health conditions in addition to sufficient acclimatization of the animals.

Animals should be fed during the acclimatization period and the study period. The feeding should be on a satiation basis and care should be taken to avoid food remaining and to ensure uniform feeding of animals. Food should be that used in actual cultivation of the aquatic animals and the raw materials and the composition ratio should be clarified. However, feeding may be stopped if the animals do not show feeding behavior after forced oral administration of test substance.

Breeding water temperature should be within the range expected for the disease in question.

(ii) Number of animals:

Five animals or more should be used at each sampling time.

(iii) Route of administration:

The route of administration should be the intended clinical route. If it is oral administration, forced oral administration may be acceptable.

(iv) Dose level:

Dose level should be one dose at the highest clinical dose.

(v) Administration period:

Administration should be once or the longest clinical administration period.

(vi) Sampling of test sample:

The number of test samples should be in total six samples or more as follows: one sample while the test substance's concentration is increasing, two samples around the time of expected highest value, and three samples or more during the elimination process.

The samples obtained should be plasma (or whole blood) for fish and muscle for Crustacea.

Each sample should be analyzed for each corresponding animal; however, samples may be mixed from a few animals if the amount of the sample is inadequate for the analysis. In this case, five samples or more should be used at each sampling time.

The sampling methods should be according to the methods in "Absorption/Excretion studies".

E. Clinical study

(a) Animals:

Target animals of the cultivated aquatic animals for which history of use of diet and animal cultivation and the method of rearing before study have been established should be used.

Feeding should be conducted according to the ordinary method used in cultivation of the aquatic animals. Food should be that used in actual cultivation of the aquatic animals, and the raw materials and the composition ratio should be clarified.

(b) Number of animals:

It is desirable to set the minimum unit of the cultivation management system as one study group (the smallest cultivation pond or fish preserve of about 10 m² is acceptable).

(c) Dose levels:

A single clinical dose will be applied.

If the clinical dose is variable, two dose groups of the lowest and highest should be set.

If the efficacy of the drug can be determined based on the results of the study group such as changes in the number of deaths, a control group is not required.

When no foundations for the dose can be found, or if data of pharmacological studies and other documents support variable doses for the clinical study, grounds for setting the dosage and administration described on the application for manufacturing approval should be clarified by conducting a study with multiple doses in the clinical study.

(d) Administration period:

The test substance will be administered during the period when clinical application is scheduled.

If the period is variable, two periods of the shortest and longest should be set.

(e) Number of test settings:

A study under a single test condition based on dose levels and administration period should be conducted at two sites. A study under more than one condition can be conducted at one site for each condition.

(f) Observation and measurement:

Death in each study group should be observed daily. In addition, attentions should be paid to changes in swimming performance, food consumption, body color, and symptoms. A dead body should be immediately necropsied to confirm that the death was caused by the target pathogenic bacteria, and a microbiological examination should be conducted, as necessary.

F. Residual study

The study is not required for drugs which show no adverse effects on human health and drugs for aquarium fish.

(a) Animals:

It is desirable to use animals free from administration of any substances which may affect the study such as antimicrobial agents for more than one month.

Breeding chambers may be a tank in a room, outdoor ponds or fish preserves. Animals should be acclimatized before the study. During this period, it should be confirmed that no deaths occur, that there is no abnormality in food consumption, body color, or swimming performance and other health conditions in addition to sufficient acclimatization of the animals.

Animals should be fed during the acclimatization period and the study period. The feeding should be on a satiation basis, and attention should be paid to avoid food remaining and to ensure uniform feeding of animals. Food should be that used in actual cultivation of the aquatic animals, and the raw materials and the composition ratio should be clarified.

Breeding water temperature for cultivated aquatic animals other than yellowtail, red sea bream, carp, eel, rainbow trout, and ayu should be within the following ranges.

Fish in the Order of Clupeiformes : 8-14°C

Fish in the Order of Cypriniformes, Anguilliformes, Perciformes, Cottiformes, Pleuronectiformes, Tetraodontiformes, and Decapoda Crustacea : 18-24°C

(b) Number of animals:

Five animals or more should be used at each sampling time.

(c) Sampling of test sample:

Frequency of sample collection should be 3 times or more.

Sample collection sites should be as follows in consideration of normal human consumption habits.

As an exception, in the clause of the respective articles of the first food division D (hereinafter called "food clause") in the standards for food products and food additives (MHW notification No. 370 issued in December 1959), meat listed in the first column of the (1) table of compositional standards for meat and whale meat in the section of the meat and whale meat should be included in the sampling sites.

If the sampling sites are specified in the (1) compositional standards for fishery products, or (2) test methods for compositional standards fishery products of the section of fishery products (except for oyster for eating raw, The same applies in this clause) in food clause, they should be included in the sampling sites.

If the sampling sites are specified in the (1) table for compositional standards for oyster for eating raw, or in the test methods for oyster for eating raw (test methods for compositional standards of fishery products are accorded) in the section of oyster for eating raw in food clause, they should be included in the sampling sites.

Fish in the Order of Clupeiformes (reared in marine water), Perciformes, Pleuronectiformes, Tetraodontiformes : muscle (excluding skin)

Fish in the Order of Clupeiformes excluding ayu (reared in freshwater), Cottiforms: muscle (including skin)

Ayu, Cypriniformes : muscle (including skin), internal organs (mixture of liver, kidney, spleen, stomach, and intestine)

Anguilliformes : muscle (including skin), internal organs (mixture of liver, stomach, and intestine)

Decapoda Crustacea: muscle (excluding shells), digestive gland

Muscle for fish means muscle from above the lateral line of the basal part of the first dorsal fin of the left of body including dark-colored flesh meat.

When the skin is included, all the skin over the sampling site of muscle should be included, and attention should be paid to avoid large scattering of the rate of skin involved. The internal organ sample should be prepared by mixing the organs after isolating them separately. A sample mixture of multiple sites or organs should be prepared by mincing to make a homogenous sample and a fixed amount should be subjected to analysis. Samples should be collected separately from individual animals, but if the amount of samples collected from one animal is not sufficient for detection of the test substance, a mixture of samples from multiple animals may be acceptable. Also in this case, at least three samples should be prepared at individual sampling periods.

(2) Classification of the fish

A. Antimicrobial drug for cultivated aquatic animals

Data of safety, pharmacological, absorptions, clinical and residue studies for animals listed in column A of the attached table 3 can be represented by those indicated in the corresponding column B. In this case, safety, pharmacological and absorptions studies should be conducted at one site for any one species, and clinical and residue studies should be conducted at two sites for one species, or at one site each for two species. However, residue studies for Clupeiformes (reared in freshwater) should be conducted at one site for rainbow trout and ayu respectively, and residue studies for Perciformes should include that conducted at one or more sites for yellowtail.

Regarding pharmacological and clinical studies, objects could be limited to drugs that have the same target pathogen specified in the indications.

For whole Osteichthyes, data of sensitivity studies using the target pathogen among pharmacological studies can be represented by study data using the different Orders of fish indicated in the B column of the attached table if the target pathogen specified in the indications is identical.

B. Non-antimicrobial drug for cultivated aquatic animals (excluding biological products)

Study data for the different Orders of fish indicated in the B column of the attached table can represent the data for whole Osteichthyes.

Study data for prawn can represent the data for whole Decapoda Crustacea.

C. Drugs for aquarium fish

Study data for the different Orders of aquarium fish can represent the data for aquarium fish as a whole.

Data of pharmacological and absorptions studies for aquarium fish are not required if the data for other vertebrae (not limited to aquatic animals) exist.