Guidance Document #02
Sub-chronic oral toxicity testing in laboratory animals
March 2019
At Step 7: Steering Committee Endorsement

SUB-CHRONIC ORAL TOXICITY TESTING IN LABORATORY ANIMALS

Endorsed by the Steering Committee in
March 2019

It is recommended for the companies willing to submit applications/dossiers for pre-market authorization, to contact the jurisdictions of the countries concern to confirm their acceptance of the current guidance document.

The International Cooperation for Convergence of Technical Requirements for the Assessment of Feed Ingredients (ICCF) was launched in 2017 and aims to develop and establish common guidance documents to provide technical recommendations for the assessment of feed ingredients, including new uses of existing feed ingredients.

This guidance document has been developed by the appropriate ICCF Experts Working Group and was subject to consultation by the Parties, in accordance with the ICCF Process.

The founding members of the ICCF include the Canadian Food Inspection Agency (CFIA), the European Commission (DG SANTE), the U.S. Food and Drug Administration (FDA), as well as the American Feed Industry Association (AFIA), the Animal Nutrition Association of Canada (ANAC), the EU Association of Specialty Feed Ingredients and their Mixtures (FEFANA) and the International Feed Industry Federation (IFIF).

Table of content

SUB-CHRONIC ORAL TOXICITY TESTING IN LABORATORY ANIMALS

1. INTRODUCTION

1.1 Objective of the Guidance

In the assessment and evaluation of potential toxic characteristics of feed ingredients, sub-chronic toxicity studies in rodents are typically carried out for a period of 90 days (3 months). The 90-day study provides information on possible health effects arising from repeated exposure over the period covering post-weaning maturation and development into adulthood of animals.

This document provides guidance to applicants on the study design for conducting a sub-chronic oral toxicity study supporting risk evaluation of feed ingredients. It has been developed with an international team of experts and considers the best practices for the provision of meaningful results.

While the guidance provided supports the acceptability of the study protocol, applicants are advised to consult the appropriate regulatory authorities or guidelines during the development phase of new feed ingredients or a new use of an authorized feed ingredient, for further determination whether the study is needed for pre-market evaluation.

1.2 Initial Considerations

This guideline was developed after consideration of the current practices for evaluating feed safety in the United States of America (USA), European Union (EU), and Canada. The current guideline is one of a series of guidelines developed to facilitate the mutual acceptance of data necessary for the determination of the safety of feed ingredients. The harmonization of regulatory requirements for the assessment of feed ingredients strives to eliminate repetitious and unnecessary animal testing. Existing guidance from national and international jurisdictions were reviewed for best practices:

- OECD: Test no. 408: Repeated dose 90-day oral toxicity study in rodents [2]
- VICH: Studies to evaluate the safety of residues of veterinary drugs in human food: General approach to testing [8]
- VICH: Studies to evaluate the safety of residues of veterinary drugs in human food: Repeat-dose (90 days) toxicity testing [9]

- U.S.FDA: Redbook2000 Chapter iv.C.4.A. Subchronic toxicity studies with rodents [10]
- U.S.EPA: OPPTS 870.3100 Health Effects Test Guidelines - 90-day oral toxicity in rodents [11]
- EFSA: Guidance on conducting repeated-dose 90-day oral toxicity study in rodents on whole food/feed and supporting documents [12, 13, 14]

A gap analysis of existing toxicity data should be conducted before the decision is made to carry out this toxicity test designed to generate data to address identified relevant concerns. The test should provide an adequate amount of toxicological data to ensure animal health and food safety, while reducing the number of animals used in testing and conserving resources. In all cases, the number of animals used should be justified scientifically and take into consideration the tenets of the 3R's principle (replacement, refinement, and reduction) of animal testing.

Due regard for the welfare of the study animals should be given when designing and carrying out the study. The use of animals in the study should adhere to these protocols and should conform to general ethical standards and to the national standards for the use and care of experimental animals.

Note that there may be an obligation in certain jurisdictions for this study to be conducted in accordance with good laboratory practices (GLPs). It is important that the applicant is aware of this requirement.

1.3 Definitions[1]

Feed (Feedingstuff): Any single or multiple materials, whether processed, semi-processed or raw, which is intended to be fed directly to animals.

Feed Ingredient: A component part or constituent of any combination or mixture making up a feed, whether or not it has a nutritional value in the animal's diet. Ingredients are of plant, animal, microbial or aquatic origin, or other organic or inorganic substances.

[1] Note : adapted from the Code of Practice on Good Animal Feeding (CAC/RCP 54-2004) [1]

2. GENERAL PRINCIPLES

The purpose of this test is to provide information on major toxic effects, indicate target organs of toxicity and the possibility of accumulation, and provide an estimate of a no-observed-adverse-effect level (NOAEL) of a feed ingredient, which can be utilized for:

1. Establishing appropriate dose levels for subsequent longer-term studies in rodents (chronic or carcinogenicity studies) or specialized studies that further evaluate potential target organ toxicity including neurotoxicity, immunotoxicity, endocrine activity, or developmental and reproductive studies.
2. Extrapolating suitable dose levels for non-rodent testing, including other appropriate laboratory animal species (e.g., canine or mini-swine) or specific target species (e.g., avian, bovine, swine) as part of the overall risk assessment.
3. Evaluating applicable human health risks for feed ingredients metabolites that may be present in products from food producing animals (i.e., meat, milk, and eggs).

3. DESCRIPTION OF THE METHOD

3.1 Selection of Test Animals

This guidance document is applicable for rodent studies. While a variety of rodent species may be used, the rat is the preferred species. Commonly used laboratory strains of young healthy adult male and female animals should be employed. The females should be nulliparous and non-pregnant.

3.1.1 Strains

It is important to consider the test animals' general sensitivity and the responsiveness of particular organs and tissues to toxic chemicals when selecting rodent species, strains, and sub-strains for toxicity studies. The use of inbred, outbred, or hybrid rodent strains for toxicity studies should be based upon the scientific questions to be answered. Additionally, it is important that test animals come from well-characterized and healthy colonies. Since survivability problems exist for some strains of rats, test animals should be selected that have a lifespan consistent with the recommended duration of the study. Where the study is conducted as a preliminary to a long-term chronic toxicity study, animals from the same strain and source should be used in both studies.

3.1.2 Age of Test Animals

Dosing should begin as soon as possible after weaning and acclimatization of animals; no earlier than six weeks and before nine weeks of age.

3.2 Housing and Feeding Conditions

3.2.1 Housing

Animals should be housed in pairs or small groups of the same sex. Animals may be housed individually if scientifically justified and the duration of the single housing shall be limited to the minimum period necessary. In group housing, observation frequency may need to be increased at times to prevent the loss of organs and tissues from moribund and dead animals due to cannibalism.

3.2.2 Bedding

Care should be taken to avoid diets or animal bedding that may contain unacceptably high levels of hormonally active substances prone or likely to interfere with the interpretation of the study results (e.g., phytoestrogens).

3.2.3 Temperature

The temperature in the experimental animal room should be 22 ± 3°C (72 ± 5°F).

3.2.4 Humidity

The relative humidity should be at least 30% and preferably not exceed 70% (50 ± 20 %).

3.2.5 Lighting

Lighting should be artificial, the sequence being 12 hours light, 12 hours dark.

3.2.6 Diet

In general, feed and water should be provided ad libitum to animals in toxicity studies. Control and test animals should be fed from the same production lot. The feed should be analyzed to assure adequacy of nutritional requirements of the species tested and for impurities that might influence the outcome of the test. For feeding studies, conventional laboratory diets should be used with an unlimited supply of drinking water.

Study Design Consideration

- The amount of feed consumed by each animal in the study cannot be determined when more than one animal is housed in each cage. This information may be necessary to evaluate feed efficiency – the relationship of feed consumed to body weight gained.
- Social interactions in group housing design may impact equal accessibility to feed and water impacting exposure to test substance and possibly confounding analyses related to determining whether decreases in body weight gain are due to feeding or substance mediated toxicity.

Unless special circumstances apply which justify otherwise, care should be taken to ensure that the diets of the test substance treated groups of animals are isocaloric (equivalent in caloric density) with and contain the same levels of nutrients (e.g., fiber, micronutrients) as the diets of the control group. Unrecognized or inadequately controlled dietary variables may result in nutritional imbalances or caloric deprivation that could confound interpretation of the toxicity study results (e.g., lifespan, background rates of tumor incidences) and alter the outcome and reproducibility of the studies. The choice of diet may be influenced by the need to ensure a suitable admixture of the test substance when administered by this method.

High levels of phytoestrogens in laboratory diets have been known to increase uterine weights in rodents. As a guide, dietary levels of phytoestrogens should not exceed 350 µg of genistein equivalents/gram of rodent laboratory diet.

3.3 Preparation of Animals

3.3.1 Animal Identification

Healthy animals which have not been subjected to previous experimental procedures should be used. The animals' species, strain and if applicable sub-strain, source, sex, age, and body weight should be documented. The animals should have been acclimatized to laboratory conditions for at least five (5) days and should have been assigned to a unique identification number. An approved method of animal identification should be used such as ringing, tagging, or biometric identification.

3.3.2 Assigning Treatment and Control

The animals should be assigned to control and test substance treated groups in a stratified random manner which will help minimize bias and assure comparability of pertinent variables

across test substance treated and control groups. The randomization procedure should be documented. Most commonly randomization is performed *via* body weight stratification; therefore, any alternative randomization procedures should be justified. At the commencement of the study the weight variation of animals used should be minimal and not exceed ± 20 % of the mean weight of each sex.

3.4 Preparation of Doses

3.4.1 Identity of Control and Test Substance

The test substance should be representative of the feed ingredient intended to be marketed. In the case of chemicals, a single lot of the test substance should be used throughout the study. Lot number, date of manufacture, expiry date, and certificate of analysis should be provided. In the case of feed ingredients that are considered novel due to genetic, manufacturing, or other changes, the anticipated differences from the parent material should be expressed. Where necessary, the test substance can be dissolved or suspended in a suitable vehicle.

3.4.2 Composition and Purity of Control Test Substance

Prior to the initiation of the study, there should be a characterization of the test substance, including the purity of the test substance and, if technically feasible, the names and quantities of contaminants and impurities. The compositional analysis is recommended due to the potential presence of inherent anti-nutritional components or minerals from the feed ingredient. A high incorporation level of such a feed ingredient in the diet of the test animals can result in a nutritional or even toxic effect.

3.4.3 Formulation of Dosage Form

The test substance is administered orally with the diet, drinking water, or gavage according to the nature of the test substance and the purpose of the study. Consideration should be given to the following characteristics of the vehicle and other additives, as appropriate: effects on the absorption, distribution, metabolism, or retention of the test substance; effects on the chemical properties of the test substance which may alter its toxic characteristics; and effects on the food or water consumption or the nutritional status of the animals. It is recommended that wherever possible the usage of an aqueous solution be considered first, followed by consideration of a

solution in oil, and then solution in other vehicles. Table 1 details formulation issues that are important to consider when establishing diets for animals in toxicity studies.

Table 1 – Formulation issues that are important to consider when establishing diets for animals in toxicity studies.

Stability and Homogeneity
If the test or control substance is to be incorporated into feed or another vehicle, the period during which the test substance is stable in such a mixture should be determined prior to the initiation of the study. Its homogeneity and concentration should be determined prior to the initiation of the study and periodically during the study. Representative samples of the mixture should be analyzed to ensure that proper mixing, formulation, and storage procedures are being followed, and that the appropriate concentration of the test or control substance is contained in the diet mixture.
Test substance with low caloric value
When the test substance has no caloric value and constitutes a substantial amount of the diet (e.g., more than 5%), both caloric and nutrient densities of the high dose diet may be diluted in comparison to the diets of the other groups. As a consequence, some high dose animals may receive higher test substance doses than expected because animals fed such diluted diets *ad libitum* may eat more than animals in other dosed groups to compensate for the differences in energy and nutrient content of the high dose diets. Such circumstances make it especially important that feed consumption of these animals be as closely and accurately monitored as possible in order to determine whether changes observed could be due to overt toxicity of the test substance or to a dietary imbalance. To further aid in this assessment, two control groups can be used; one group would be fed the undiluted control diet and a second group would be fed the control diet supplemented with an inert filler (e.g., methylcellulose) at a percentage equal to the highest percentage of the test substance in the diet.
Vehicle with caloric or nutritional value
When the vehicle for the test substance is expected to have caloric and/or nutritional value, which are greater than that of the control ration, an adjustment in the caloric and/or nutritional components may be necessary.

Test Substance with unpleasant taste or texture – oral gavage or paired feeding

When administration of the test substance is expected to have an effect on feed intake because of its unpleasant taste or texture, oral gavage may be appropriate. However, while resource intensive, paired feeding may be used to eliminate the differences in consumption between control and substance treated groups. When a paired feeding study design is to be employed, pairs of littermate weanling rats of the same sex and approximate size are selected and fed the control or the experimental diet. Animals should be single-caged so that feed consumption can be determined daily, and the control animal is then fed an amount of food equal to that which the paired experimental animal ate on the preceding day. If the test substance is non-nutritive and composes a significant proportion of the diet, the pair-fed control animals should be fed an amount of its feed such that it consumes a nutritionally equivalent amount of diet as the paired experimental animal. Additionally, the study should include a second group of control animals fed ad libitum to ensure that the impact on any observed experimental result is due to differences in energy or nutrient intake.

Test substance interferes with nutrient absorption

When the test substance interferes with the absorption of nutrients, leading to nutritional deficiencies or changes in nutrient ratios, this can confound assessment of the toxicological endpoints under consideration. For example, fat soluble vitamins may preferentially partition with a mineral oil or fat substitute which is largely unabsorbed, such that a potential deficiency in these vitamins may result. This potential may be eliminated by additional nutrient fortification of the feed for those groups receiving the test substance.

4. PROCEDURE

4.1 Placement of Animals

Animals in all groups should be placed on study on the same day; if this is not possible because of the large number of animals in a study, animals may be placed on study over several days. If the latter recommendation is followed, during the randomization process an allocated number of control animals and experimental animals from each dose level should be placed on the study each respective starting day in order to maintain concurrence (staggered start).

4.2 Number and Sex of Animals

A minimum of 20 animals (10 per sex) should be used at each dose level. If interim kills are planned, the number should be increased by the number of animals scheduled to be euthanized

before the completion of the study. If there is a need to include a recovery (non-dosing) phase to the study, a minimum of five (5) animals/sex/group should be included. Finally, the use of a single gender in a testing protocol should also be justified scientifically.

4.3 Dosage

4.3.1 Treatment Groups

At least three dose levels and a concurrent control shall be used, except where a limit test is conducted (see Section 4.3.5).

4.3.2 Selection of Treatment Doses

Dose levels may be based on the results of repeated dose or range finding studies and should take into account any existing toxicological and toxicokinetic data available for the test substance or related materials. When designing and conducting toxicity studies, unless limited by the physical-chemical nature or biological effects of the test substance the following should be considered:

- The highest dose level should be chosen with the aim to induce toxicity but not death or severe suffering - no dose should cause an incidence of fatalities that prevents meaningful evaluation of the data,
- The low dose should not induce toxic responses in test animals and would represent a NOAEL,
- A descending sequence of dose levels should be selected with a view to demonstrating any dosage related response,
- The intermediate dose levels to produce a gradation of toxic effects and be sufficiently high to elicit minimal toxic effects in test animals (such as alterations in enzyme levels or slight decreases in body weight gains)

Two to four-fold intervals are frequently optimal for setting the descending dose levels and addition of a fourth test group is often preferable to using very large intervals (e.g., more than a factor of about 6-10) between dosages.

4.3.3 Control Group

A concurrent control group of test animals is required. The control group shall be an untreated group or a vehicle-control group if a vehicle is used in administering the test substance. Except for treatment with the test substance, animals in the control group should be handled in

an identical manner to those in the test groups. If a vehicle is used, the control group shall receive the vehicle in the highest volume used. If a test substance is administered in the diet, and causes reduced dietary intake, then a pair-fed control group may be useful in distinguishing between reductions due to palatability or toxicological alterations in the test model.

4.3.4 Satellite Groups

Certain endpoints including but not limited to blood collection for exposure or tissue collection for specialized assays may dictate the need for satellite groups. Satellite groups should include the minimum numbers of animals in both sexes to achieve the desired endpoints. A satellite group of 10 animals (5 per sex) may be treated with the high dose level for 90 days and observed for reversibility, persistence, or delayed occurrence of toxic effects for a post-treatment period of appropriate length, normally not less than 28 days. In addition, a control group of 10 animals (5 animals of each sex) should be added to the satellite study.

Types of Satellite Groups – Proposed Endpoints Evaluated and Disposition

In life data (body weights, food consumption and clinical observations) should be collected for all animals (main study or satellite groups) to properly evaluate the health status and calculate proper dosages. Overall disposition for each group should be carefully detailed in the Study Protocol.

Toxicokinetic Group – Designated animals to be used for determination of blood levels of the test substance or associated metabolites. As these animals will be subjected to repeated blood sampling and therefore treated differently than the main study animals, they can be euthanized without gross examination and tissue collection following the final blood collection timepoint.

Recovery Group - Designated animals to be maintained following the completion of the dosing period, to evaluate the persistence or reversal of toxic effects or perhaps the delayed onset of toxicity. These animals should be treated the same as main study animals with regards to endpoints evaluated and samples collected, including full gross necropsy and histopathology (as appropriate).

Interim Kill Group – Designated animals to be humanely euthanized prior to the completion of the dosing period to evaluate shorter term toxicity. These animals should be treated the same as the main study animals with regards to endpoints evaluated and samples collected, including full gross necropsy and histopathology (as appropriate).

Other Groups – Additional groups may be added for specialized endpoints requiring animals treated for the same duration as the main study animals. Potential uses for these animals include but are not limited to, metabolism endpoints, imaging, *ex vivo* assays requiring tissue samples or immunology endpoints.

4.3.5 Limit Test

If a test at one dose level, equivalent to at least 1000 mg/kg body weight/day, using the procedures described for this study, produces no observed adverse effects and if toxicity would not be expected based upon data from structurally related compounds, then a full study using three dose levels may not be considered necessary. The limit test applies except when target species exposure indicates the need for a higher dose level to be used (i.e., greater than 1000 mg/kg body weight/day).

4.3.6 Administration of doses

The animals are dosed with the test substance daily seven days each week for a period of 90 days. If the test substance is administered in the drinking water, or mixed in the diet, then exposure should be on a 7– day per week basis. All animals should be dosed by the same method during the entire experimental period.

4.3.7 Route of Administration

The route of administration of the test substance should approximate that of normal animal exposure, if possible. For feed ingredients the oral route of administration is required and should be added to the diet, drinking water, or by gavage.

4.3.7.1 In the Diet

Dietary administration is preferred. The test substance will be consumed as solid feeds or a combination of solid and liquid feeds. If the test substance is added to the diet, animals should not be able to selectively consume either basal diet or test substance in the diet on the basis of color, smell, or particle size. If the test substance is mixed with ground feed and pelleted, nothing in the pelleting process should affect the test substance (e.g., heat-labile substances may be destroyed during pellet production by a steam process). When the test substance is administered in the diet, either a constant dietary concentration (mg per kg in feed or parts per million) or a constant dose level in terms of the animal's body weight (mg per kg-body weight) may be used; any alternative must be specified.

4.3.7.2 Dissolved in Drinking Water

Administration through drinking water should be considered if the test substance is likely to be ingested *via* drinking water, or if administration in the diet is inappropriate for other reasons. The amount of test substance administered in drinking water should be expressed as mg of test substance per ml of water or a constant dose level in terms of the animal's body weight (mg per kg-body weight) may be used; any alternative must be specified.

4.3.7.3 Oral gavage

If the two previous methods are unsatisfactory or if exposure is expected to be through daily ingestion of single, large doses instead of continual ingestion of small doses then oral gavage should be considered. When the test substance is administered by gavage, this should be done

in a single dose to the animals using a stomach tube or a suitable intubation cannula; split dosing may be considered (e.g., a few hours apart) when there is a need to achieve a high dose in consideration of the following:

- The maximum volume of liquid that can be administered at one time depends on the size of the test animal and vehicle,
- The volume should not exceed 1 ml/100g body weight, except in the case of aqueous solutions where 2 ml/100g body weight may be used,
- Except for irritating or corrosive substances which will normally reveal exacerbated effects with higher concentrations, variability in test volume should be minimized by adjusting the concentration to ensure a constant volume at all dose levels.

For a test substance administered by gavage, the dose should be given at approximately the same time each day and adjusted at intervals (weekly or biweekly) to maintain a constant dose level in terms of body weight.

4.4 Observations

The observation period should be at least 90 days. Animals in a satellite group scheduled for follow-up observations should be kept for an appropriate period without treatment to detect persistence of, or recovery from toxic effects; normally not less than 28 days.

4.4.1 General Clinical Observation of Test Animals

General clinical observations should be made cage-side at least once a day, preferably at the same time(s) each day, taking into consideration the peak period of anticipated effects after dosing. The clinical condition of the animals should be recorded at least twice daily, usually at the beginning and end of each day. The usual interval between observations should be at least 6 hours; all animals are inspected for signs of pharmacologic and toxicologic effects, morbidity, and mortality. Moribund animals should be sacrificed, weighed, and time of death recorded. Appropriate actions should be taken to minimize loss of animals to the study (e.g., necropsy or refrigeration of those animals found dead, and isolation or sacrifice of weak or moribund animals).

Adequate animal husbandry practices should minimize animals and tissues or organs lost to a study because of autolysis. Autolysis in excess of this standard may be a cause to repeat the

study. Necropsy should be performed soon after an animal is sacrificed or found dead, so that loss of tissues due to autolysis is minimized. When necropsy cannot be performed immediately, the animal should be refrigerated at a temperature that is low enough to prevent autolysis but not so low as to cause cell damage. If histopathological examination is to be conducted, tissue specimens should be taken from the animals and placed in appropriate fixatives when the necropsy is performed. Excessive mortality due to poor animal management is unacceptable and may be the cause to repeat the study. For example, under normal circumstances, mortality in the control group should not exceed levels seen in relevant historical control data for the laboratory, species, and strain.

4.4.2 Bodyweight, Food, and Water Intake

All animals should be weighed at the start of the experiment, at least once a week during the experiment, and at termination. Measurements of feed consumption should be made at least weekly, with quantification of feed spillage. If the test substance is administered *via* the drinking water, water consumption should be measured at least weekly. Water consumption may also be considered for dietary or gavage studies during which drinking activity may be altered.

4.4.3 Clinical Testing

Ophthalmological examination, hematology profiles, clinical chemistry tests, and urinalysis should be performed as described in the following sections.

4.4.3.1 Detailed Physical Examinations

At least once prior to the first exposure (to allow for within-subject comparisons), and once a week thereafter, detailed clinical observations should be made in all animals. These observations should be made outside the home cage, preferably in a standard arena and at similar times on each occasion. They should be carefully recorded, preferably using scoring systems, explicitly defined by the testing laboratory. Effort should be made to ensure that variations in the observation conditions are minimal. Signs noted should include, but not be limited to, changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions, and autonomic activity (e.g., lacrimation, piloerection, pupil size, and unusual respiratory pattern). Changes in gait, posture and response to handling as well as the presence of clonic or tonic movements, stereotypes (e.g., excessive grooming, repetitive circling) or bizarre behavior (e.g., self-mutilation, walking backwards) should also be recorded [16, 3].

4.4.3.2 Opthalmological Examination

This examination should be performed by a qualified individual. Using an ophthalmoscope or equivalent suitable equipment, examinations should be made prior to the administration of the test substance and at the termination of the study, preferably in all animals but at least in the high dose and control groups. If changes in the eyes are detected all animals should be examined.

4.4.3.3 Neurotoxicity Screening / Testing

Towards the end of the exposure period and in any case not earlier than in week 11, sensory reactivity to stimuli of different types [16, 15] (e.g., auditory, visual and proprioceptive stimuli) [17, 18, 19] assessment of grip strength [20] and motor activity assessment [21] should be conducted. Functional observations conducted towards the end of the study may be omitted when data on functional observations are available from other studies and the daily clinical observations did not reveal any functional deficits. Exceptionally, functional observations may also be omitted for groups that otherwise reveal signs of toxicity to an extent that would significantly interfere with the functional test performance.

4.5 Haematology and Clinical Biochemistry

Blood samples should be taken from a named site and stored, if applicable, under appropriate conditions. At the end of the test period, samples are collected just prior to or as part of the procedure for euthanizing the animals.

4.5.1 Haematology

The following haematological examinations should be made at the end of the test period and when any interim blood samples may have been collected:

- Hematocrit
- Hemoglobin concentration
- Erythrocyte count
- Reticulocyte count
- Total and differential leukocyte counts mean corpuscular hemoglobin
- Mean corpuscular volume
- Mean corpuscular hemoglobin concentration
- Platelet count
- A measure of clotting time/potential (such as clotting time, prothrombin time, thromboplastin time)

Bone marrow smear is recommended and should be prepared if treatment-related changes are suspected in tissues/organs or in peripheral blood. This activity can further diagnose, confirm, and/or stage hematologic disease, and is a diagnostic tool in non-hematologic disorders (e.g., storage disease, systemic infection) and malignancies.

4.5.2 Clinical Biochemistry

Overnight fasting of the animals prior to blood sampling is recommended. For a number of measurements in serum and plasma, most notably for glucose, overnight fasting would be preferable. The major reason for this preference is that the increased variability, which would inevitably result from non-fasting, would tend to mask more subtle effects, and make interpretation difficult. However, overnight fasting may interfere with the general metabolism of the animals and, particularly in feeding studies, may disturb the daily exposure to the test substance. If overnight fasting is adopted, clinical biochemical determinations should be performed after the conduct of functional observations of the animals.

Clinical biochemistry determinations to investigate major toxic effects in tissues and, specifically, effects on kidney and liver, should be performed on blood samples obtained from each animal just prior to or as part of the procedure for euthanizing the animals (apart from those found moribund and/or intercurrently euthanized). In a similar manner to haematological investigations, interim sampling for clinical biochemical tests may be performed. Determinations in plasma or serum should include:

- Sodium
- Potassium
- Glucose
- Total cholesterol
- High-density lipoprotein (HDL)
- Low-density lipoprotein (LDL)
- Urea
- Blood urea nitrogen
- Creatinine
- Total protein
- Albumin
- More than two enzymes indicative of hepatocellular effects such as:
  - Alanine aminotransferase,
  - Aspartate aminotransferase,
  - Alkaline phosphatase,
  - Gamma glutamyl transpeptidase
  - Sorbitol dehydrogenase

Measurements of additional enzymes (of hepatic or other origin) and bile acids, which may provide useful information under certain circumstances, may also be included.

Clinical biochemistries should be evaluated as potential markers of general tissue damage. Other determinations that should be carried out if the known properties of the test substance may, or are suspected to, affect related metabolic profiles include:

- Calcium
- Phosphorus
- Fasting triglycerides
- Methaemoglobin
- Cholinesterase

Serum totals for the following hormones should be measured on samples obtained from each animal in the main group and in satellite and/or recovery groups at study termination.

- Thyroxine (T4)
- Tri-iodothyronine (T3)
- Thyroid Stimulating Hormone (TSH)

Other hormones should be considered as on a case-by-case basis:

- Testosterone
- Oestradiol
- Follicle stimulating hormone (FSH)
- Luteinizing hormone (LH)

Serum may be stored frozen to allow time to determine the most informative hormone analyses based on results observed for other endpoints (e.g., organ weight and histology). Hormones may be measured in plasma if appropriate validation and historical control data are available.

The following factors might influence the variability and absolute concentration of the hormone determinations:

- time of humane euthanasia because of diurnal variation of hormone concentration
- stage of the oestrus cycle
- method of humane euthanasia to avoid undue stress to the animals that may affect hormone concentrations
- test kits for hormone determinations that may differ by their standard curves

Blood samples specifically intended for hormone determination should be obtained at a comparable time of the day. The numerical values obtained when analysing hormone concentrations differ with various commercial assay kits. Consequently, it may not be possible to provide performance criteria based upon uniform historical data. Control hormone levels (measured in the same lab, same rodent strain, and using the same method) should be taken into account to differentiate between incidental and treatment-related changes. Whenever possible, best practices for blood sample collection, handling and analysis should be used [22]. Laboratories should strive, as far as possible, to keep control coefficients of variation below 25 for T3 and T4 and below 35 for TSH. All concentrations are to be recorded in ng/ml. Stability of T3, T4 and TSH under selected storage conditions should be tested as part of the hormonal assay validation.

4.5.3 Urinalysis

Optionally, the following urinalysis determinations could be performed during the last week of the study using timed urine volume collection:

- appearance
- volume
- osmolality or specific gravity
- pH
- protein
- glucose
- blood/blood cells

4.5.4 Additional Tests

In addition, studies to investigate serum markers of general tissue damage should be considered. Other determinations that may be carried out if the known properties of the test

substance may, or are suspected to, affect related metabolic profiles and need to be identified for chemicals in certain classes or on a case-by-case basis.

Overall, there is a need for a flexible approach, depending on the species and the observed and/or expected effect from a given compound. If historical baseline data are inadequate, consideration should be given as to whether haematological and clinical biochemistry variables need to be determined before dosing commences; it is generally not recommended that this data be generated before treatment [23].

5. PATHOLOGY

5.1 Gross Necropsy

All animals shall be examined by a full, detailed gross necropsy conducted by or under the supervision of a qualified pathologist. Detailed gross necropsy includes careful examination of the external surface of the body, all orifices, and the cranial, thoracic, and abdominal cavities and their contents. This also applies to animals being sacrificed early and found dead and should be done as early after death as possible. In case this is not possible, animals should be refrigerated to prevent autolysis and cell damage.

5.2 Organ Weights

From all animals (apart from those found dead or euthanized prematurely), the wet weight of the following organs should be determined after dissection of any adherent tissue as soon as possible after dissection to avoid drying:

- Brain
- Kidneys
- Liver
- Heart
- Spleen
- Thymus
- Adrenal glands
- Pituitary Gland
- Thyroid Gland
- Ovaries
- Uterus
- Epididymides
- Testes
- Prostate + Seminal vesicles with coagulating gland as a whole

The pituitary gland may be weighted fresh, immediately after dissection, or post-fixation. For the prostate + seminal vesicles with coagulating gland as a whole, alternatively, first weigh the entire prostate with seminal vesicles/coagulation glands together, then dissect and weigh

the prostate gland separately. Care must be exercised when trimming the prostate complex to avoid puncture of the fluid filled seminal vesicles. Alternatively, seminal vesicles and prostate may be trimmed and weighed after fixation.

Consideration should be given to weigh other organs as appropriate to fulfill the purpose of the study.

5.3 Reproductive Pathology

At termination, testes and epididymides weights are recorded for all males. At least one epididymis from each male should be reserved for histopathological examination. The remaining epididymis may be used for optional enumeration [24] of:

- cauda epididymis sperm reserves,
- sperm morphology
- sperm motility.

For the optional evaluation of sperm morphology, an epididymal (or vas deferens) sperm sample should be examined as fixed or wet preparations and at least 200 spermatozoa per sample classified as either normal (both head and midpiece/tail appear normal) or abnormal. Examples of morphologic sperm abnormalities would include fusion, isolated heads, and misshapen heads and/or tails. Misshapen or large sperm heads may indicate defects in spermiation. Sperm motility can either be evaluated immediately after humane euthanasia or recorded for later analysis. The percentage of progressively motile sperm may be determined either visually or by computer-assisted motion analysis.

Analyses of sperm parameters may be restricted to control and high-dose males. However, if treatment-related effects are observed, the lower dose groups should also be evaluated.

At necropsy, the oestrus cycle of all females should be determined by taking vaginal smears. These observations will provide information regarding the stage of oestrus cycle at the time of humane euthanasia and assist in histological evaluation of oestrogen sensitive tissues (see OECD Guidance Document 106, part 3 [4]).

5.4 Preparation of Tissues for Histopathological Examination

Appropriate sections of the tissues noted in Table 2 should be prepared for histopathological examination preserved in the most appropriate fixation medium and stain for

both the type of tissue and the intended subsequent histopathological examination. It is recommended to preserve testes by immersion in Bouin's or modified Davidson's fixative and histopathological assessment should consider staging of seminiferous tubule cross sections as described [25]. See also OECD GD 106 [4] for fixation and histological evaluation of endocrine organs.

Table 2 – Tissues to be collected and prepared for histopathological examination

Organ System	**Tissues**
Digestive system:	salivary glands, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, Peyer's patches (when present) liver, pancreas, gall bladder (when present)
Nervous system:	brain (representative regions included cerebrum, cerebellum, and medulla/pons), spinal cord (cervical, mid-thoracic, and lumbar), peripheral nerve (sciatic or tibial – in close proximity to muscle), eyes (retina and optic nerve; if changes were observed during ophthalmological examinations)
Glandular system:	pituitary, adrenals, parathyroid, thyroid, Harderian gland
Respiratory system:	trachea, lungs (preserved by inflation with fixative and then immersion), pharynx, larynx, nose
Cardiovascular/ Haematopoietic system:	aorta, heart, bone marrow (and/or fresh aspirate), lymph nodes (preferably one lymph node covering the route of administration and another one distant from the route of administration to cover systemic effects), spleen, thymus
Urogenital system:	kidneys, urinary bladder, testes, epididymides, Prostate + seminal vesicles with coagulating glands as a whole complex, uterus, ovaries (including fallopian tubes), mammary gland (male and female)
Others:	all gross lesions, masses, skin, skeletal muscle, bone, and any organs considered likely to be target organ based on the known properties of the test substance

5.5 Histopathology

Full histopathology should be carried out on the preserved organs and tissues of all animals in the control and high dose groups. These examinations should be extended to target organs of animals of the affected sex of all other dosage groups, if treatment-related changes are observed in the high dose group. All gross lesions should be examined. When a satellite group is used,

histopathology should be performed on tissues and organs identified as showing effects in the treated groups.

5.6 Detection of Endocrine Activity

Results obtained for the endocrine related parameters should be evaluated in the context of the "OECD Conceptual Framework for Testing and Assessment of Endocrine Disrupting Chemicals"[5] and "Revised Guidance Document 150 on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption"[6] The revised Guideline places additional emphasis on endocrine endpoints to combine with the existing sensitivity to neurological and immunological and reproductive effects. The need for careful clinical observations of the animals, so as to obtain as much information as possible, is also stressed. Required endpoints include the measurement of thyroxine (T4), triiodothyronine (T3), thyroid stimulating hormone (TSH) and thyroid gland weight, which are responsive to thyroid pathway perturbation [7]. In addition, serum total cholesterol, low-density lipoproteins (LDL) and high-density lipoproteins (HDL) should also be determined as levels of these parameters are directly controlled by thyroid hormone action and contribute (with other thyroid endpoints) to evidence of thyroid effects[26]. Optional endpoints include other hormone measurements, as well as assessments of sperm parameters. Required and optional measures that may be altered by endocrine effects are listed in Table 3. Assessment of the optional measures may be considered if existing information for the test substance or similar substances suggests potential to influence these or can be triggered by observations from required measures collected as part of this guideline.

Table 3 – Endpoints for the detection of endocrine activity

	Required Measures	Optional Measures
Organ Weights	Testes Epididymides Adrenal glands Prostate + seminal vesicles with coagulating glands as a whole complex Uterus Ovaries Pituitary gland Thyroid gland	

	Required Measures	Optional Measures
Histopathology	Thyroid and parathyroid glands Adrenal glands Pituitary gland* Testes Epididymides Ventral and dorsolateral prostate Seminal vesicles and coagulating glands Ovaries* Cervix* Vagina* Uterus* Vaginal smear (collected at necropsy) to determine stage of oestrus cycle* Mammary glands (female and male)*	Pancreatic islets
Serum/Plasma Biochemistry and Hormone Analyses	Total cholesterol High density lipoprotein (HDL) Low density lipoprotein (LDL) Thyroxine (T4) Thyroid Stimulating Hormone (TSH) Tri-iodothyronine (T3)	Follicle Stimulating Hormone Luteinizing Hormone Oestradiol Testosterone
Sperm Measures		Cauda epididymis sperm reserves Sperm motility Sperm morphology

*The condition of the oestrogen-sensitive organs in the female should be assessed with reference to the stage of oestrus cycle at termination as endocrine active test substances may cause histological changes that, while not overtly pathological, may differ from the condition anticipated based on the stage of ovarian cycle (as per OECD Guidance Document 106, parts 3, 4 [20]).

6. DATA AND REPORTING

Individual animal data of all the protocol-specified endpoints should be presented in the study report. All numerical data should be summarized in tabular form, clearly identifying the test group and number of animals in each group at each collection point. All qualitative data should also be summarized to indicate overall incidence of findings to permit interpretation of test substance related effects. Additional detail should be provided for all early deaths, including

animals euthanized *in extremis* and animals found dead. This detail should include the individual clinical signs, onset and severity of toxicity, applicable clinical pathology, gross pathology, and microscopic pathology in order to define the cause of moribundity or mortality. Omission of data collected in the report is strongly discouraged as it may be counter to the requirements of Good Laboratory Practices or other applicable quality standards.

When applicable, numerical data should be evaluated by appropriate and generally accepted statistical methods. When group housing is used and the substance is administered in the feed or drinking water, the experimental units is the cage, not the animal. These statistical methods should be detailed in the study protocol as well as the study report.

For quality control it is proposed that control data are compared to historical control values originating from the same laboratory, species, strain, and collected under similar conditions. In addition, coefficients of variation are calculated for the continuous parameters in Table 3 for endocrine activity. These data can be used for comparison among studies. Differences between rat strains should be taken into account when evaluating historical control data.

7. TEST REPORT

The test report must include the following information:

Test substance:

- chemical identification, such as IUPAC or CAS name(s), CAS registry number(s), SMILES or InChI code, structural formula, and/or other identifiers
- source, lot number, limit date for use (if available)
- stability of chemical, if known
- physical nature and, where relevant, physiochemical properties
- identification including CAS number if known/established; and
- purity
- certificate of analysis

Mono-constituent substance:

- Physical appearance, water solubility, and additional relevant physicochemical properties

Multi-constituent substance, mixtures, and chemical substances of unknown or variable composition, complex reaction products and biological materials (UVBCs):

- Characterised as far as possible by chemical identity (see above), quantitative occurrence and relevant physicochemical properties of the constituents

Vehicle if appropriate:

- Justification for choice of vehicle, if other than water

Test animals:

- species and strain used
- number, age, and sex of animals
- source, housing conditions, diet, etc.
- individual weights of animals at the start of the test; and
- justification for species if not rat

Test conditions:

- rationale for dose level selection
- details of test substance formulation/diet preparation, achieved concentration, stability, and homogeneity of the preparation
- details of the administration of the test substance
- actual doses (mg/kg body weight/day), and conversion factor from diet/drinking water test substance concentration (ppm) to the actual dose, if applicable; and
- details of food and water quality

Results:

- body weight and body weight changes
- food consumption, and water consumption, if applicable
- toxic response data by sex and dose level, including signs of toxicity
- nature, severity, and duration of clinical observations (whether reversible or not)
- results of ophthalmological examination
- sensory activity, grip strength and motor activity assessments (when available)
- haematological tests with relevant baseline values
- clinical biochemistry tests with relevant baseline values
- circulating thyroid hormones (T4, T3, TSH; required)
- other hormone measures (optional)

- method of determining hormone values (assay type, supplier, protocol, etc.)
- terminal body weight, organ weights and organ/body weight ratios
- necropsy findings
- terminal vaginal cytology
- a detailed description of all histopathological findings
- total cauda epididymal sperm number, percent progressively motile sperm, percent morphologically normal sperm, and percent of sperm with each identified abnormality (optional)
- absorption (e.g., absorption, distribution, metabolism, excretion (ADME) or toxicokinetic information) data if analysed
- statistical treatment of results, where appropriate
- for animals euthanized pre-terminally, the rationale behind the decision should be reported; and
- for animals found dead during the study, the cause of death should, when possible, be established

Discussion of results

Conclusions

8. BIBLIOGRAPHY

8.1 CODEX Alimentarius

1. Codex Alimentarius, Code of Practice on Good Animal Feeding (CAC/RCP 54-2004) http://www.fao.org/3/i1379e/i1379e06.pdf

8.2 OECD

2. OECD, *Test No. 408: Repeated Dose 90-Day Oral Toxicity Study in Rodents*, in *OECD Guidelines for the Testing of Chemicals*, Organisation for Economic Co-operation and Development (OECD), Editor. 2018, OECD Publishing: Paris https://www.oecd-ilibrary.org/content/publication/9789264070707-en.
3. OECD, Guidance Document on the Recognition, Assessment and Use of Clinical Signs as Human Endpoints for Experimental Animals Used in Safety Evaluation, in OECD Guidelines for the Testing of Chemicals, Organisation for Economic Co-operation and Development (OECD), Editor. 2000. https://www.oecd-ilibrary.org/content/publication/9789264078376-en.

4. OECD, Guidance Document for Histologic Evaluation and Reproductive Tests in Rodents., in Environmental Health and Safety Publications Series on Testing and Assessment No. 106. Parts 1-6., Organisation for Economic Co-operation and Development (OECD), Editor. 2009, OECD Publishing: Paris. http://www.oecd.org/env/ehs/testing/43411534.pdf.
5. OECD, Guidance document on standardised test guidelines for evaluating chemicals for endocrine disruption in Series on Testing and Assessment Organisation for Economic Co-operation and Development (OECD), Editor. 2012, OECD Publishing: Paris. http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono%282012%2922&doclanguage=en.
6. OECD, *Revised Guidance Document 150 on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption*. 2018. https://www.oecd-ilibrary.org/content/publication/9789264304741-en.
7. OECD, *Detailed review paper on thyroid hormone disruption assays* in *Series on Testing and Assessment* Organisation for Economic Co-operation and Development (OECD), Editor. 206, OECD Publishing: Paris. http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2006)24&doclanguage=en.

8.3 VICH

8. VICH, *Studies to evaluate the safety of residues of veterinary drugs in human food: General approach to testing.* International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products, 2009. **VICH GL33**. https://vichsec.org/component/attachments/attachments/319.html?task=download.
9. VICH, *Studies to evaluate the safety of residues of veterinary drugs in human food: Repeat-dose (90 days) toxicity testing.* International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products, 2004. **VICH GL31**. https://vichsec.org/component/attachments/attachments/317.html?task=download.

8.4 United States of America

10. U.S. FDA, Chapter IV.C.4.a. Subchronic Toxicity Studies with Rodents, in Redbook 2000: Guidance for Industry and Other Stakeholders Toxicological Principles for the Safety Assessment of Food Ingredients 2003, U.S. FDA. https://www.fda.gov/downloads/food/guidanceregulation/ucm222779.pdf.
11. U.S. EPA, *90-Day Oral Toxicity in Rodents*, in *OPPTS 870.3100 Health Effects Test Guidelines*, U.S. Environmental Protection Agency, Editor. 1998. https://tinyurl.com/ycmlpox3.

8.5 European Union

12. EFSA, Outcome of the public consultation on the draft EFSA guidance on conducting repeated-dose 90-day oral toxicity study in rodents on whole food/feed. EFSA Supporting Publications, 2011. **8**(12). https://doi.org/10.2903/sp.efsa.2011.EN-205.
13. EFSA, Guidance on conducting repeated-dose 90-day oral toxicity study in rodents on whole food/feed. EFSA Journal, 2011. **9**(12). https://doi.org/10.2903/j.efsa.2011.2438.
14. EFSA, Explanatory statement for the applicability of the Guidance of the EFSA Scientific Committee on conducting repeated-dose 90-day oral toxicity study in rodents on whole food/feed for GMO risk assessment. EFSA Journal, 2014. **12**(10). https://doi.org/10.2903/j.efsa.2014.3871.
15. EEC, Council Directive 86/609/EEC on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes. Official Journal, 1986. **29** (L358 18th December 1986). https://eur-lex.europa.eu/legal-content/en/TXT/?uri=CELEX:31986L0609.

8.6 Others

16. IPCS, *Principles and Methods for the Assessment of Neurotoxicity Associated with Exposure to Chemicals.* International Program on Chemical Safety (IPCS) Environmental Health Criteria Document, 1986. **No. 60.** http://www.inchem.org/documents/ehc/ehc/ehc060.htm.
17. Tupper, D.E. and R.B. Wallace, *Utility of the neurological examination in rats.* Acta neurobiologiae experimentalis, 1980. **40**(6): p. 999-1003. http://www.ncbi.nlm.nih.gov/pubmed/7234526.
18. Gad, S.C., *A neuromuscular screen for use in industrial toxicology.* Journal of Toxicology and Environmental Health, 1982. **9**(5-6): p. 691-704. http://www.ncbi.nlm.nih.gov/pubmed/6750143.
19. Moser, V.C., K.L. McDaniel, and P.M. Phillips, *Rat strain and stock comparisons using a functional observational battery: baseline values and effects of amitraz.* Toxicology and Applied Pharmacology, 1991. **108**(2): p. 267-83. http://www.ncbi.nlm.nih.gov/pubmed/2017756.
20. Meyer, O.A., H.A. Tilson, W.C. Byrd, and M.T. Riley, *A method for the routine assessment of fore- and hindlimb grip strength of rats and mice.* Neurobehavioral Toxicology, 1979. **1**(3): p. 233-6. http://www.ncbi.nlm.nih.gov/pubmed/551317.

21. Crofton, K.M., J.L. Howard, V.C. Moser, M.W. Gill, L.W. Reiter, H.A. Tilson, and R.C. MacPhail, *Interlaboratory comparison of motor activity experiments: implications for neurotoxicological assessments.* Neurotoxicology and Teratolology, 1991. **13**(6): p. 599-609. http://www.ncbi.nlm.nih.gov/pubmed/1779947.
22. Kucheryavenko, O., G. Lurman, A. Lehman, J. Bras, L. Niemann, A. Terron, I. Chabhoud, A. Mantovani, H. Hakansson, S. Schneider, V. Ritz, and R. Solecki, *Report from the BfR Expert Hearing on Practicability of Hormonal Measurements.* Arch Toxicol. (*in prep*), 2018.
23. Weingand, K., G. Brown, R. Hall, D. Davies, K. Gossett, D. Neptun, T. Waner, T. Matsuzawa, P. Salemink, W. Froelke, J.P. Provost, G. Dal Negro, J. Batchelor, M. Nomura, H. Groetsch, A. Boink, J. Kimball, D. Woodman, M. York, E. Fabianson-Johnson, M. Lupart, and E. Melloni, *Harmonization of animal clinical pathology testing in toxicity and safety studies. The Joint Scientific Committee for International Harmonization of Clinical Pathology Testing.* Fundamental and applied toxicology : official journal of the Society of Toxicology, 1996. **29**(2): p. 198-201. http://www.ncbi.nlm.nih.gov/pubmed/8742316.
24. Seed, J., R.E. Chapin, E.D. Clegg, L.A. Dostal, R.H. Foote, M.E. Hurtt, G.R. Klinefelter, S.L. Makris, S.D. Perreault, S. Schrader, D. Seyler, R. Sprando, K.A. Treinen, D.N. Veeramachaneni, and L.D. Wise, *Methods for assessing sperm motility, morphology, and counts in the rat, rabbit, and dog: a consensus report. ILSI Risk Science Institute Expert Working Group on Sperm Evaluation.* Reproductive toxicology, 1996. **10**(3): p. 237-44. http://www.ncbi.nlm.nih.gov/pubmed/8738562.
25. Russell, L.D., R.A. Ettlin, A.P. Sinha Hikim, and E.D. Clegg, *Histological and Histopathological Evaluation of the Testis*. 1990, Cache River: Clearwater, FL.
26. Kovanen, P.T., *Regulation of plasma cholesterol by hepatic low-density lipoprotein receptors.* American Heart Journal, 1987. **113**(2 Pt 2): p. 464-9 http://www.ncbi.nlm.nih.gov/pubmed/3544761.

9. ABBREVIATIONS

EU	European Union
USA	United States of America
NOAEL	No Observed Adverse Effect Level