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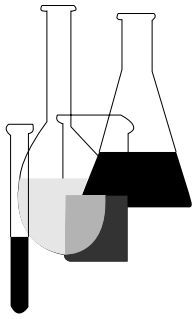
Health Effects Test Guidelines

OPPTS 870.3050 Repeated Dose 28–Day Oral Toxicity Study in Rodents



INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations.

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD).

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U. S. Environmental Protection Agency under the Toxic Substances Control Act (15 U.S.C. 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U.S.C. 136, *et seq.*).

Final Guideline Release: This guideline is available from the U.S. Government Printing Office, Washington, DC 20402 on disks or paper copies: call (202) 512–0132. This guideline is also available electronically in PDF (portable document format) from EPA's World Wide Web site (http://www.epa.gov/OPPTS_Harmonized) under the heading ''Information Resources/Test Methods/OPPTS Harmonized Test Guidelines.''

OPPTS 870.3050 Repeated dose 28–day oral toxicity study in rodents.

(a) **Scope**—(1) **Applicability**. This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U.S.C. 136, *et seq.*) and the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601).

(2) **Background**. (i) The source material used in developing this harmonized OPPTS test guideline is OECD guideline 407, Repeated Dose 28–Day Oral Toxicity Study in Rodents. While editing the OECD source guideline has resulted in some minor changes in format and style, all essential elements of this guideline and its OECD counterpart are equivalent.

(b) **Purpose**. (1) In the assessment and evaluation of the toxic characteristics of a chemical, the determination of oral toxicity using repeated doses may be carried out after initial information on toxicity has been obtained by acute testing. This study provides information on the possible health hazards likely to arise from repeated exposure over a relatively limited period of time. The method comprises the basic repeated dose toxicity study that may be used for chemicals on which a 90–day study is not warranted (e.g. when the production volume does not exceed certain limits) or as a preliminary to a long term study. The duration of exposure should normally be 28 days although a 14–day study may be appropriate in certain circumstances; justification for use of a 14–day exposure period should be provided.

(2) This guideline places emphasis on neurological effects as a specific endpoint, and the need for careful clinical observations of the animals, so as to obtain as much information as possible, is stressed. The method should identify chemicals with neurotoxic potential, which may warrant further in-depth investigation of this aspect. In addition, the method may give an indication of immunological effects and reproductive organ toxicity.

(c) **Definitions**. The definitions in section 3 of the TSCA and in 40 CFR Part 792—Good Laboratory Practice Standards (GLP) apply to this test guideline. The following definitions also apply to this test guideline.

Dosage is a general term comprising of dose, its frequency and the duration of dosing.

Dose is the amount of test substance administered. Dose is expressed as weight (g, mg) or as weight of test substance per unit weight of test animal (e.g. mg/kg), or as constant dietary concentrations (parts per million (ppm)).

No-observed-adverse-effect-level (NOAEL) is the maximum dose used in a study which produces no adverse effects. The NOAEL is usually ex-

pressed in terms of the weight of a test substance given daily per unit weight of test animals (milligrams per kilograms per day).

(d) **Principle of the test**. The test substance is orally administered daily in graduated doses to several groups of experimental animals, one dose level per group for a period of 28 days. During the period of administration the animals are observed closely, each day for signs of toxicity. Animals which die or are killed during the test are necropsied and at the conclusion of the test surviving animals are killed and necropsied.

(e) **Description of the method**—(1) **Selection of animal species**. The preferred rodent species is the rat, although other rodent species may be used. Commonly used laboratory strains of young healthy adult animals should be employed. The females should be nulliparous and non-pregnant. Dosing should begin as soon as possible after weaning and, in any case, before the animals are 9 weeks old. 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. Where a repeated dose oral study is conducted as a preliminary to a long term study, preferably animals from the same strain and source should be used in both studies.

(2) **Housing and feeding conditions**. The temperature in the experimental animal room should be 22°C (±3°C). Although the relative humidity should be at least 30% and preferably not to exceed 70% other than during room cleaning, the aim should be 50–60%. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. The choice of diet may be influenced by the need to ensure a suitable admixture of a test substance when administered by this method. Animals may be housed individually, or be caged in small groups of the same sex; for group caging, no more than five animals should be housed per cage.

(3) **Preparation of animals**. Healthy young adult animals are randomly assigned to the control and treatment groups. Cages should be arranged in such a way that possible effects due to cage placement are minimized. The animals are identified uniquely and kept in their cages for at least 5 days prior to the start of the study to allow for acclimatization to the laboratory conditions.

(4) **Preparation of doses**. (i) The test compound is administered by gavage or via the diet or drinking water. The method of oral administration is dependent on the purpose of the study, and the physical/chemical properties of the test material.

(ii) Where necessary, the test substance is dissolved or suspended in a suitable vehicle. It is recommended that, wherever possible, the use of an aqueous solution/suspension be considered first, followed by consideration of a solution/emulsion in oil (e.g. corn oil) and then by possible

solution in other vehicles. For vehicles other than water the toxic characteristics of the vehicle must be known. The stability of the test substance in the vehicle should be determined.

(f) **Procedure**—(1) **Number and sex of animals**. At least 10 animals (five female and five male) 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 killed before the completion of the study. Consideration should be given to an additional satellite group of 10 animals (five per sex) in the control and in the top dose group for observation of reversibility, persistence, or delayed occurrence of toxic effects, for at least 14 days post treatment.

(2) **Dosage**. (i) Generally, at least three test groups and a control group should be used, but if from assessment of other data, no effects would be expected at a dose of 1000 mg/kg bodyweight/per day, a limit test may be performed. If there are no suitable data available, a range finding study may be performed to aid the determination of the doses to be used. Except for treatment with the test substance, animals in the control group should be handled in an identical manner to the test group subjects. If a vehicle is used in administering the test substance, the control group should receive the vehicle in the highest volume used.

(ii) Dose levels should be selected taking into account any existing toxicity and (toxico-) kinetic data available for the test compound or related materials. The highest dose level should be chosen with the aim of inducing toxic effects but not death or severe suffering. Thereafter, a descending sequence of dose levels should be selected with a view to demonstrating any dosage related response and NOAEL at the lowest dose level. 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 10) between dosages.

(3) **Limit test**. If a test at one dose level of at least 1000 mg/kg body weight/day or, for dietary or drinking water administration, an equivalent percentage in the diet, or drinking water (based upon body weight determinations), using the procedures described for this study, produces no observable toxic 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 human exposure indicates the need for a higher dose level to be used.

(4) **Administration of doses**. (i) The animals are dosed with the test substance daily 7 days each week for a period of 28 days; use of a 5–day per week dosing regime or a 14–day exposure period needs to be justified. 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. The maximum volume of liquid that can be administered at one time depends on the size of the test animal. The volume should not exceed 1ml/100g body weight, except in the case of aqueous solutions where 2ml/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.

(ii) For substances administered via the diet or drinking water it is important to ensure that the quantities of the test substance involved do not interfere with normal nutrition or water balance. When the test substance is administered in the diet either a constant dietary concentration (ppm) or a constant dose level in terms of the animals' body weight may be used; the alternative used must be specified. For a substance administered by gavage, the dose should be given at similar times each day, and adjusted as necessary to maintain a constant dose level in terms of animal body weight. Where a repeated dose study is used as a preliminary to a long term study, a similar diet should be used in both studies.

(5) **Observations**. (i) The observation period should be 28 days, unless the study duration is 14 days (see paragraph (b)(1) of this guideline). Animals in a satellite group scheduled for follow-up observations should be kept for at least a further 14 days without treatment to detect delayed occurrence, or persistence of, or recovery from toxic effects.

(ii) General clinical observations should be made at least once a day, preferably at the same time(s) each day and considering the peak period of anticipated effects after dosing. The health condition of the animals should be recorded. At least twice daily, all animals are observed for morbidity and mortality.

(iii) Once before the first exposure (to allow for within-subject comparisons), and at least once a week thereafter, detailed clinical observations should be made in all animals. These observations should be made outside the home cage in a standard arena and preferably at the same time, each time. 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 test conditions are minimal and that observations are preferably conducted by observers unaware of the treatment. 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, unusual respiratory pattern). Changes in gait, posture and response to handling as well as the presence of clonic or tonic movements, stereotypies (e.g. excessive grooming, repetitive circling) or bizarre behaviour (e.g. self-mutilation, walking backwards) should also be recorded (see paragraph (h)(2) of this guideline).

(iv) In the fourth exposure week sensory reactivity to stimuli of different types (see paragraph (h)(2) of this guideline) (e.g. auditory, visual and proprioceptive stimuli) (see paragraphs (h)(3), (h)(4), and (h)(5) of this guideline), assessment of grip strength (see paragraph (h)(6) of this guideline) and motor activity assessment (see paragraph (h)(7) of this guideline) should be conducted. Further details of the procedures that could be followed are given in the respective references. However, alternative procedures than those referenced could also be used.

(v) Functional observations conducted in the fourth exposure week may be omitted when the study is conducted as a preliminary study to a subsequent subchronic (90–day) study. In that case, the functional observations should be included in this follow-up study. On the other hand, the availability of data on functional observations from the repeated dose study may enhance the ability to select dose levels for a subsequent subchronic study.

(vi) 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.

(6) **Body weight and food/water consumption**. All animals should be weighed at least once a week. Measurements of food consumption should be made at least weekly. If the test substance is administered via the drinking water, water consumption should also be measured at least weekly.

(7) **Hematology**. (i) The following hematological examinations should be made at the end of the test period: hematocrit, haemoglobin concentration, erythrocyte count, total and differential leukocyte count, platelet count and a measure of blood clotting time/potential.

(ii) Blood samples should be taken from a named site just prior to or as part of the procedure for killing the animals, and stored under appropriate conditions.

(8) **Clinical Biochemistry**. (i) Clinical biochemistry determinations to investigate major toxic effects in tissues and, specifically, effects on kidney and liver, should be performed on blood samples obtained of all animals just prior to or as part of the procedure for killing the animals (apart from those found moribund and/or intercurrently killed). Overnight fasting of the animals prior to blood sampling is recommended.[1] Investiga-

[1] 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. On the other hand, 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

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tions of plasma or serum shall include sodium, potassium, glucose, total cholesterol, urea, creatinine, total protein and albumin, at least two enzymes indicative of hepatocellular effects (such as alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, gamma glutamyl transpeptidase, and sorbitol dehydrogenase). Measurements of additional enzymes (of hepatic or other origin) and bile acids may provide useful information under certain circumstances.

(ii) 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 and blood and blood cells.

(iii) In addition, studies to investigate serum markers of general tissue damage should be considered. 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, phosphate, fasting triglycerides, specific hormones, methemoglobin and cholinesterase. These need to be identified for chemicals in certain classes or on a case-by-case basis.

(iv) Overall, there is a need for a flexible approach, depending on the species and the observed and/or expected effect with a given compound.

(v) If historical baseline data are inadequate, consideration should be given to determination of hematological and clinical biochemistry variables before dosing commences.

(9) **Pathology**—(i) **Gross necropsy**. (A) All animals in the study shall be subjected to a full, detailed gross necropsy which includes careful examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents. The liver, kidneys, adrenals, testes, epididymides, thymus, spleen, brain and heart of all animals (apart from those found moribund and/or intercurrently killed) should be trimmed of any adherent tissue, as appropriate, and their wet weight taken as soon as possible after dissection to avoid drying.

(B) The following tissues should be preserved in the most appropriate fixation medium for both the type of tissue and the intended subsequent histopathological examination: all gross lesions, brain (representative regions including cerebrum, cerebellum and pons), spinal cord, stomach, small and large intestines (including Peyer's patches), liver, kidneys, adrenals, spleen, heart, thymus, thyroid, trachea and lungs (preserved by inflation with fixative and then immersion), ovaries, uterus, testes, epididymides, accessory sex organs (e.g., prostate, seminal vesicles), uri-

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fasting is adopted, clinical biochemical determinations should be performed after the conduct of functional observations in week 4 of the study.

nary bladder, lymph nodes (preferably one lymph node covering the route of administration and another one distant from the route of administration to cover systemic effects), peripheral nerve (sciatic or tibial) preferably in close proximity to the muscle, and a section of bone marrow (or, alternatively, a fresh mounted bone marrow aspirate). The clinical and other findings may suggest the need to examine additional tissues. Also any organs considered likely to be target organs based on the known properties of the test substance should be preserved.

(ii) **Histopathology**. (A) 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 animals of all other dosage groups, if treatment-related changes are observed in the high dose group.

(B) All gross lesions shall be examined.

(C) When a satellite group is used, histopathology should be performed on tissues and organs identified as showing effects in the treated groups.

(g) **Data and reporting**—(1) **Data**. (i) Individual data should be provided. Additionally, all data should be summarized in tabular form showing for each test group the number of animals at the start of the test, the number of animals found dead during the test or killed for humane reasons and the time of any death or humane kill, the number showing signs of toxicity, a description of the signs of toxicity observed, including time of onset, duration, and severity of any toxic effects, the number of animals showing lesions, the type of lesions and the percentage of animals displaying each type of lesion.

(ii) When possible, numerical results should be evaluated by an appropriate and generally acceptable statistical method. The statistical methods should be selected during the design of the study.

(2) **Test report**. The test report must include the following information:

(i) Test substance:

(A) Physical nature, purity and physicochemical properties.

(B) Identification data.

(ii) Vehicle (if appropriate): Justification for choice of vehicle, if other than water.

(iii) Test animals:

(A) Species/strain used.

(B) Number, age and sex of animals.

(C) Source, housing conditions, diet, etc.

(D) Individual weights of animals at the start of the test.

(iv) Test conditions:

(A) Rationale for dose level selection.

(B) Details of test substance formulation/diet preparation, achieved concentration, stability and homogeneity of the preparation.

(C) Details of the administration of the test substance.

(D) Conversion from diet/drinking water test substance concentration (ppm) to the actual dose (mg/kg body weight/day), if applicable.

(E) Details of food and water quality.

(v) Results:

(A) Body weight/body weight changes.

(B) Food consumption, and water consumption, if applicable.

(C) Toxic response data by sex and dose level, including signs of toxicity.

(D) Nature, severity and duration of clinical observations (whether reversible or not).

(E) Sensory activity, grip strength and motor activity assessments.

(F) Hematological tests with relevant base-line values.

(G) Clinical biochemistry tests with relevant base-line values.

(H) Body weight at killing and organ weight data.

(I) Necropsy findings.

(J) A detailed description of all histopathological findings.

(K) Absorption data if available.

(L) Statistical treatment of results, where appropriate.

(vi) Discussion of results.

(vii) Conclusions.

(h) **References.** The following references should be consulted for additional background material on this test guideline.

(1) OECD (Paris, 1992). Chairman's Report of the Meeting of the ad hoc Working Group of Experts on Systemic Short-term and (Delayed) Neurotoxicity.

(2) IPCS (1986). Principles and Methods for the Assessment of Neurotoxicity Associated with Exposure to Chemicals. Environmental Health Criteria Document No. 60.

(3) Tupper, D.E., Wallace, R.B. (1980). Utility of the Neurologic Examination in Rats. *Acta Neurobiological Exposure*, 40:999–1003.

(4) Gad, S.C. (1982). A Neuromuscular Screen for Use in Industrial Toxicology. *Journal of Toxicology and Environmental Health*, 9:691–704.

(5) Moser, V.C., McDaniel, K.M., Phillips, P.M. (1991). Rat Strain and Stock Comparisons Using a Functional Observational Battery: Baseline Values and Effects of Amitraz. *Toxicology and Applied Pharmacology*, 108:267–283.

(6) Meyer O.A., Tilson H.A., Byrd W.C., Riley M.T. (1979). A Method forthe Routine Assessment of Fore- and Hindlimb Grip Strength of Rats and Mice. *Neurobehavioral Toxicology*, 1:233–236.

(7) Crofton K.M., Howard J.L., Moser V.C., Gill M.W., Reiter L.W., Tilson H.A., MacPhail R.C. (1991). Interlaboratory Comparison of Motor Activity Experiments: Implication for Neurotoxicological Assessments. *Neurotoxicology and Teratology*, 13:599–609.