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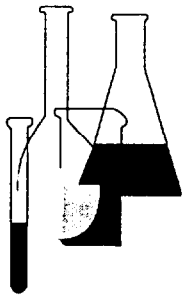
Health Effects Test Guidelines

OPPTS 870.6200 Neurotoxicity Screening Battery



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/epahome/research.htm) under the heading "Researchers and Scientists/Test Methods and Guidelines/OPPTS Harmonized Test Guidelines."

OPPTS 870.6200 Neurotoxicity screening battery.

(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.** The source material used in developing this harmonized OPPTS test guideline are 40 CFR 798.6050 Functional Observational Battery, 798.6200 Motor Activity, and 798.6400 Neuropathology; and OPP 81–8 Acute Neurotoxicity—Rat, 82–7 90–Day Neurotoxicity—Rat, and 83–1 Chronic Feeding—Two Species, Rodent and Nonrodent (Pesticide Assessment Guidelines, Subdivision F—Hazard Evaluation; Human and Domestic Animals, Addendum 10, EPA report 540/09–91–123, March 1991).

(b) **Purpose.** This neurotoxicity screening battery consists of a functional observational battery, motor activity, and neuropathology. The functional observational battery consists of noninvasive procedures designed to detect gross functional deficits in animals and to better quantify behavioral or neurological effects detected in other studies. The motor activity test uses an automated device that measures the level of activity of an individual animal. The neuropathological techniques are designed to provide data to detect and characterize histopathological changes in the central and peripheral nervous system. This battery is designed to be used in conjunction with general toxicity studies and changes should be evaluated in the context of both the concordance between functional neurological and neuropatholgical effects, and with respect to any other toxicological effects seen. This test battery is not intended to provide a complete evaluation of neurotoxicity, and additional functional and morphological evaluation may be necessary to assess completely the neurotoxic potential of a chemical.

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

ED is effective dose.

Motor activity is any movement of the experimental animal.

Neurotoxicity is any adverse effect on the structure or function of the nervous system related to exposure to a chemical substance.

Toxic effect is an adverse change in the structure or function of an experimental animal as a result of exposure to a chemical substance.

(d) **Principle of the test method.** The test substance is administered to several groups of experimental animals, one dose being used per group.

The animals are observed under carefully standardized conditions with sufficient frequency to ensure the detection and quantification of behavioral and/or neurologic abnormalities, if present. Various functions that could be affected by neurotoxicants are assessed during each observation period. Measurements of motor activity of individual animals are made in an automated device. The animals are perfused and tissue samples from the nervous system are prepared for microscopic examination. The exposure levels at which significant neurotoxic effects are produced are compared to one another and to those levels that produce other toxic effects.

(e) **Test procedures**—(1) **Animal selection**—(i) **Species.** In general, the laboratory rat should be used. Under some circumstances, other species, such as the mouse or the dog, may be more appropriate, although not all of the battery may be adaptable to other species.

(ii) **Age.** Young adults (at least 42 days old for rats) should be used.

(iii) **Sex.** Both males and females should be used. Females should be nulliparous and nonpregnant.

(2) **Number of animals.** At least 10 males and 10 females should be used in each dose and control group for behavioral testing. At least five males and five females should be used in each dose and control group for terminal neuropathology. If interim neuropathological evaluations are planned, the number should be increased by the number of animals scheduled to be perfused before the end of the study. Animals should be randomly assigned to treatment and control groups.

(3) **Control groups.** (i) A concurrent (vehicle) control group is required. Subjects should be treated in the same way as for an exposure group except that administration of the test substance is omitted. If the vehicle used has known or potential toxic properties, both untreated or saline treated and vehicle control groups are required.

(ii) Positive control data from the laboratory performing the testing should provide evidence of the ability of the observational methods used to detect major neurotoxic endpoints including limb weakness or paralysis, tremor, and autonomic signs. Positive control data are also required to demonstrate the sensitivity and reliability of the activity-measuring device and testing procedures. These data should demonstrate the ability to detect chemically induced increases and decreases in activity. Positive control groups exhibiting central nervous system pathology and peripheral nervous system pathology are also required. Separate groups for peripheral and central neuropathology are acceptable (e.g. acrylamide and trimethyl tin). Permanently injurious substances need not be used for the behavioral tests. Historical data may be used if the essential aspects of the experimental procedure remain the same. Periodic updating of positive control data is recommended. New positive control data should also be collected when

personnel or some other critical element in the testing laboratory has changed.

(4) **Dose level and dose selection.** At least three doses should be used in addition to the vehicle control group. The data should be sufficient to produce a dose-effect curve. The Agency strongly encourage the use of equally spaced doses and a rationale for dose selection that will maximally support detection of dose-effect relations. For acute studies, dose selection may be made relative to the establishment of a benchmark dose (BD). That is, doses may be specified as successive fractions, e.g. 0.5, 0.25, ...n of the BD. The BD itself may be estimated as the highest nonlethal dose as determined in a preliminary range-finding lethality study. A variety of test methodologies may be used for this purpose, and the method chosen may influence subsequent dose selection. The goal is to use a dose level that is sufficient to be judged a limit dose, or clearly toxic.

(i) **Acute studies.** The high dose need not be greater than 2 g/kg. Otherwise, the high dose should result in significant neurotoxic effects or other clearly toxic effects, but not result in an indicence of fatalities that would preclude a meaningful evaluation of the data. This dose may be estimated by a BD procedure as described in paragraph (e)(4) of this guideline, with the middle and low dose levels chosen as fractions of the BD dose. The lowest dose should produce minimal effect, e.g. an ED10, or alternatively, no effects.

(ii) **Subchronic and chronic studies.** The high dose need not be greater than 1 g/kg. Otherwise, the high dose level should result in significant neurotoxic effects or other clearly toxic effects, but not produce an incidence of fatalities that would prevent a meaningful evaluation of the data. The middle and low doses should be fractions of the high dose. The lowest dose should produce minimal effects, e.g. an ED10, or alternatively, no effects.

(5) **Route of exposure.** Selection of route may be based on several criteria including, the most likely route of human exposure, bioavailability, the likelihood of observing effects, practical difficulties, and the likelihood of producing nonspecific effects. For many materials, it should be recognized that more than one route of exposure may be important and that these criteria may conflict with one another. In order to save resources, initially only one route is being required for screening for neurotoxicity. The route that best meets these criteria should be selected. Dietary feeding will generally be acceptable for repeated exposures studies.

(6) **Combined protocol.** The tests described in this screening battery may be combined with any other toxicity study, as long as none of the requirements of either are violated by the combination.

(7) **Study conduct**—(i) **Time of testing.** All animals should be weighed on each test day and at least weekly during the exposure period.

(A) **Acute studies.** At a minimum, for acute studies observations and activity testing should be made before the initiation of exposure, at the estimated time of peak effect within 8 hours of dosing, and at 7 and 14 days after dosing. Estimation of times of peak effect may be made by dosing pairs of rats across a range of doses and making regular observations of gait and arousal.

(B) **Subchronic and chronic studies.** In a subchronic study, at a minimum, observations and activity measurements should be made before the initiation of exposure and before the daily exposure, or for feeding studies at the same time of day, during the 4th, 8th, and 13th weeks of exposure. In chronic studies, at a minimum, observations and activity measurements should be made before the initiation of exposure and before the daily exposure, or for feeding studies at the same time of day, every 3 months.

(ii) **Functional observational battery**—(A) **General conduct.** All animals in a given study should be observed carefully by trained observers who are unaware of the animals' treatment, using standardized procedures to minimize observer variability. Where possible, it is advisable that the same observer be used to evaluate the animals in a given study. If this is not possible, some demonstration of interobserver reliability is required. The animals should be removed from the home cage to a standard arena for observation. Effort should be made to ensure that variations in the test conditions are minimal and are not systematically related to treatment. Among the variables that can affect behavior are sound level, temperature, humidity, lighting, odors, time of day, and environmental distractions. Explicit, operationally defined scales for each measure of the battery are to be used. The development of objective quantitative measures of the observational end-points specified is encouraged. Examples of observational procedures using defined protocols may be found in paragraphs (g)(6), (g)(8), and (g)(11) of this guideline. The functional observational battery should include a thorough description of the subject's appearance, behavior, and functional integrity. This should be assessed through observations in the home cage and while the rat is moving freely in an open field, and through manipulative tests. Testing should proceed from the least to the most interactive with the subject. Scoring criteria, or explicitly defined scales, should be developed for those measures which involve subjective ranking.

(B) **List of measures.** The functional observational battery should include the following list of measures:

(*1*) Assessment of signs of autonomic function, including but not limited to:

(*i*) Ranking of the degree of lacrimation and salivation, with a range of severity scores from none to severe.

(*ii*) Presence or absence of piloerection and exophthalmus.

(*iii*) Ranking or count of urination and defecation, including polyuria and diarrhea. This is most easily conducted during the open field assessment.

(*iv*) Pupillary function such as constriction of the pupil in response to light or a measure of pupil size.

(*v*) Degree of palpebral closure, e.g., ptosis.

(*2*) Description, incidence, and severity of any convulsions, tremors, or abnormal motor movements, both in the home cage and the open field.

(*3*) Ranking of the subject's reactivity to general stimuli such as removal from the cage or handling, with a range of severity scores from no reaction to hyperreactivity.

(*4*) Ranking of the subject's general level of activity during observations of the unperturbed subject in the open field, with a range of severity scores from unresponsive to hyperactive.

(*5*) Descriptions and incidence of posture and gait abnormalities observed in the home cage and open field.

(*6*) Ranking of any gait abnormalities, with a range of severity scores from none to severe.

(*7*) Forelimb and hindlimb grip strength measured using an objective procedure, e.g. that described by Meyer et al. under paragraph (g)(10) of this guideline

(*8*) Quantitative measure of landing foot splay; the procedure described in paragraph (g)(4) of this guideline is recommended.

(*9*) Sensorimotor responses to stimuli of different modalities will be used to detect gross sensory deficits. Pain perception may be assessed by a ranking or measure of the reaction to a tail-pinch, tail-flick, or hot-plate. The response to a sudden sound, e.g., click or snap, may be used to assess audition.

(*10*) Body weight.

(*11*) Description and incidence of any unusual or abnormal behaviors, excessive or repetitive actions (stereotypies), emaciation, dehydration, hypotonia or hypertonia, altered fur appearance, red or crusty deposits around the eyes, nose, or mouth, and any other observations that may facilitate interpretation of the data.

(C) **Additional measures.** Other measures may also be included and the development and validation of new tests is encouraged. Further information on the neurobehavioral integrity of the subject may be provided by:

(*1*) Count of rearing activity on the open field.

(*2*) Ranking of righting ability.

(*3*) Body temperature.

(*4*) Excessive or spontaneous vocalizations.

(*5*) Alterations in rate and ease of respiration, e.g., rales or dyspnea.

(*6*) Sensorimotor responses to visual or proprioceptive stimuli.

(iii) **Motor activity.** Motor activity should be monitored by an automated activity recording apparatus. The device used must be capable of detecting both increases and decreases in activity, i.e., baseline activity as measured by the device must not be so low as to preclude detection of decreases nor so high as to preclude detection of increases in activity. Each device should be tested by standard procedures to ensure, to the extent possible, reliability of operation across devices and across days for any one device. In addition, treatment groups must be balanced across devices. Each animal should be tested individually. The test session should be long enough for motor activity to approach asymptotic levels by the last 20 percent of the session for nontreated control animals. All sessions should have the same duration. Treatment groups should be counterbalanced across test times. Effort should be made to ensure that variations in the test conditions are minimal and are not systematically related to treatment. Among the variables which can affect motor activity are sound level, size and shape of the test cage, temperature, relative humidity, lighting conditions, odors, use of the home cage or a novel test cage, and environmental distractions.

(iv) **Neuropathology: Collection, processing and examination of tissue samples.** To provide for adequate sampling as well as optimal preservation of cellular integrity for the detection of neuropathological alterations, tissue should be prepared for histological analysis using in situ perfusion and paraffin and/or plastic embedding procedures. Paraffin embedding is acceptable for tissue samples from the central nervous system. Plastic embedding of tissue samples from the central nervous system is encouraged, when feasible. Plastic embedding is required for tissue samples from the peripheral nervous system. Subject to professional judgment and the type of neuropathological alterations observed, it is recommended that additional methods, such as Bodian's or Bielchowsky's silver methods, and/or glial fibrillary acidic protein (GFAP) immunohistochemistry be used in conjunction with more standard stains to determine the lowest dose level

at which neuropathological alterations are observed. When new or existing data provide evidence of structural alterations it is recommended that the GFAP immunoassay also be considered. A description of this technique can be found in paragraph (g)(12) of this guideline.

(A) **Fixation and processing of tissue.** The nervous system should be fixed by in situ perfusion with an appropriate aldehyde fixative. Detailed descriptions of vascular perfusions may be found in paragraphs (g)(7), (g)(13), (g)(19), and (g)(21) of this guideline. Any gross abnormalities should be noted. Tissue samples taken should adequately represent all major regions of the nervous system. Detailed dissection procedures are described in paragraphs (g)(19)(chapter 50), and (g)(13) of this guideline. The tissue samples should be postfixed and processed according to standardized published histological protocols under paragraph (g)(1), (g)(2), (g)(3), (g)(14), (g)(19), or (g)(20) of this guideline. Tissue blocks and slides should be appropriately identified when stored. Histological sections should be stained for hematoxylin and eosin (H&E), or a comparable stain according to standard published protocols under paragraphs (g)(1), (g)(2), and (g)(14) of this guideline.

(B) **Qualitative examination.** Representative histological sections from the tissue samples should be examined microscopically by an appropriately trained pathologist for evidence of neuropathological alterations. The nervous system should be thoroughly examined for evidence of any treatment-related neuropathological alterations. Particular attention should be paid to regions known to be sensitive to neurotoxic insult or those regions likely to be affected based on the results of functional tests. Such treatment-related neuropathological alterations should be clearly distinguished from artifacts resulting from influences other than exposure to the test substance. Guidance for both regions to be examined and the types of neuropathological alterations that typically result from toxicant exposure can be found in paragraph (g)(20) of this guideline. A stepwise examination of tissue samples is recommended. In such a stepwise examination, sections from the high dose group are first compared with those of the control group. If no neuropathological alterations are observed in samples from the high dose group, subsequent analysis is not required. If neuropathological alterations are observed in samples from the high dose group, samples from the intermediate and low dose groups are then examined sequentially.

(C) **Subjective diagnosis.** If any evidence of neuropathological alterations is found in the qualitative examination, then a subjective diagnosis will be performed for the purpose of evaluating dose-response relationships. All regions of the nervous system exhibiting any evidenceof neuropathological changes should be included in this analysis. Sections from all dose groups from each region will be coded and examined in randomized order without knowledge of the code. The frequency of each type and severity of each lesion will be recorded. After all samples from

all dose groups including all regions have been rated, the code will be broken and statistical analysis performed to evaluate dose-response relationships. For each type of dose-related lesion observed, examples of different degrees of severity should be described. Photomicrographs of typical examples of treatment-related regions are recommended to augment these descriptions. These examples will also serve to illustrate a rating scale, such as 1+, 2+, and 3+ for the degree of severity ranging from very slight to very extensive.

(f) **Data reporting and evaluation.** The final test report must include the following information:

(1) **Description of equipment and test methods.** A description of the general design of the experiment and any equipment used should be provided. This should include a short justification explaining any decisions involving professional judgment.

(i) A detailed description of the procedures used to standardize observations, including the arena and scoring criteria.

(ii) Positive control data from the laboratory performing the test that demonstrate the sensitivity of the procedures being used. Historical data may be used if all essential aspects of the experimental protocol are the same. Historical control data can be critical in the interpretation of study findings. The Agency encourages submission of such data to facilitate the rapid and complete review of the significance of effects seen.

(2) **Results.** The following information must be arranged by test group dose level.

(i) In tabular form, data for each animal must be provided showing:

(A) Its identification number.

(B) Its body weight and score on each sign at each observation time, the time and cause of death (if appropriate), total session activity counts, and intrasession subtotals for each day measured.

(ii) Summary data for each group must include:

(A) The number of animals at the start of the test.

(B) The number of animals showing each observation score at each observation time.

(C) The mean and standard deviation for each continuous endpoint at each observation time.

(D) Results of statistical analyses for each measure, where appropriate.

(iii) All neuropathological observations should be recorded and arranged by test groups. This data may be presented in the following recommended format:

(A) Description of lesions for each animal. For each animal, data must be submitted showing its identification (animal number, sex, treatment, dose, duration), a list of structures examined as well as the locations, nature, frequency, and severity of lesions. Inclusion of photomicrographs is strongly recommended for demonstrating typical examples of the type and severity of the neuropathological alterations observed. Any diagnoses derived from neurological signs and lesions including naturally occurring diseases or conditions, should be recorded.

(B) Counts and incidence of neuropathological alterations by test group. Data should be tabulated to show:

(*1*) The number of animals used in each group and the number of animals in which any lesion was found.

(*2*) The number of animals affected by each different type of lesion, the locations, frequency, and average grade of each type of lesion.

(3) **Evaluation of data.** The findings from the screening battery should be evaluated in the context of preceding and/or concurrent toxicity studies and any correlated functional and histopathological findings. The evaluation should include the relationship between the doses of the test substance and the presence or absence, incidence and severity, of any neurotoxic effects. The evaluation should include appropriate statistical analyses, for example, parametric tests for continuous data and nonparametric tests for the remainder. Choice of analyses should consider tests appropriate to the experimental design, including repeated measures. There may be many acceptable ways to analyze data.

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

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