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### § 799.9620 TSCA neurotoxicity screening battery.

(a) *Scope.* This section is intended to meet the testing requirements under section 4 of TSCA. 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.

(b) *Source.* The source material used in developing this TSCA test guideline is the OPPTS harmonized test guideline 870.6200 (June 1996 Public Draft). This source is available at the address in paragraph (g) of this section.

(c) *Definitions.* The following definitions apply to this section.

*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) shall be used.

(iii) *Sex.* Both males and females shall be used. Females shall 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 shall be randomly assigned to treatment and control groups.

(3) *Control groups.* (i) A concurrent (vehicle) control group is required. Subjects shall 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 shall 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 shall 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 incidence of fatalities that would preclude a meaningful evaluation of the data. This dose may be estimated by a BD procedure as described under paragraph (e)(4) of this section, 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. Initially only one route is 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 shall 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 shall be made before the initiation of exposure, at the estimated time of peak effect within 8 hrs 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 shall 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 shall 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 shall 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 shall 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 the references under paragraphs (g)(5), (g)(6), and (g)(9) of this section. The functional observational battery shall include a thorough description of the subject's appearance, behavior, and functional integrity. This shall 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 shall 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 (the procedure described in the reference under paragraph (g)(8) of this section may be used).

(*8*) Quantitative measure of landing foot splay (the procedure described in the reference under paragraph (g)(3) of this section may be used).

(*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 shall 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 shall 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 shall be tested individually. The test session shall be long enough for motor activity to approach asymptotic levels by the last 20% of the session for nontreated control animals. All sessions shall have the same duration. Treatment groups shall 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 shall 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 glial fibrillary acidic protein (GFAP) immunohistochemistry and/or methods known as Bodian's or Bielchowsky's silver methods 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 the reference under paragraph (g)(10) of this section.

(A) *Fixation and processing of tissue.* The nervous system shall be fixed by in situ perfusion with an appropriate

aldehyde fixative. Any gross abnormalities should be noted. Tissue samples taken should adequately represent all major regions of the nervous system. The tissue samples should be postfixed and processed according to standardized published histological protocols (protocols described in the references under paragraphs (g)(1), (g)(2), or (g)(11) of this section may be used). 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 (some of these protocols are described in the references under paragraphs (g)(1) and (g)(11) of this section).

(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 shall 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. 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 shall be performed for the purpose of evaluating dose-response relationships. All regions of the nervous system exhibiting any evidence of 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 shall include the following information:

(1) *Description of equipment and test methods.* A description of the general design of the experiment and any equipment used shall be provided. This shall 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 shall be arranged by test group dose level.

(i) In tabular form, data for each animal shall 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 shall 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, and 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 shall include the relationship between the doses of the test substance and the presence or absence, incidence and severity, of any neurotoxic effects. The evaluation shall 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.* For additional background information on this test guideline, the following references should be consulted. These references are available for inspection at the TSCA Nonconfidential Information Center, Rm. NE-B607, Environmental Protection Agency, 401 M St., SW., Washington, DC, 12 noon to 4 p.m., Monday through Friday, except legal holidays.

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(10) O'Callaghan, J.P. Quantification of glial fibrillary acidic protein: Comparison of slot-immunobinding assays with a novel sandwich ELISA. *Neurotoxicology and Teratology*. 13:275–281 (1991).

(11) Pender, M.P. A simple method for high resolution light microscopy of nervous tissue. *Journal of Neuroscience Methods*. 15:213–218 (1985).

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### § 799.9630 TSCA developmental neurotoxicity.

(a) *Scope*—(1) *Applicability*. This section is intended to meet the testing requirements *under* section 4 of the Toxic Substances Control Act (TSCA).

(2) *Source*. The source material used in developing this TSCA test guideline is the OPPTS harmonized test guideline 870.6300 (August 1998).

(b) *Purpose*. In the assessment and evaluation of the toxic characteristics of a chemical substance or mixture (test substance), determination of the potential for developmental neurotoxicity is important. This study is designed to develop data on the potential functional and morphological hazards to the nervous system which may arise in the offspring from exposure of the mother during pregnancy and lactation.

(c) *Principle of the test method*. The test substance is administered to several groups of pregnant animals during gestation and early lactation, one dose level being used per group. Offspring are randomly selected from within litters for neurotoxicity evaluation. The evaluation includes observations to detect gross neurologic and behavioral abnormalities, determination of motor activity, response to auditory startle, assessment of learning, neuropathological evaluation, and brain weights. This protocol may be used as a separate study, as a followup to a standard developmental toxicity and/or adult neurotoxicity study, or as part of a two-generation reproduction study, with assessment of the offspring conducted on the second ($F^2$) generation.

(d) *Test procedure*—(1) *Animal selection*—(i) *Species and strain*. Testing must be performed in the rat. Because of its differences in timing of developmental events compared to strains that are more commonly tested in other developmental and reproductive toxicity studies, it is preferred that the Fischer 344 strain not be used. If a sponsor wishes to use the Fischer 344 rat or a mammalian species other than the rat, ample justification/reasoning for this selection must be provided.

(ii) *Age*. Young adult (nulliparous females) animals must be used.

(iii) *Sex*. Pregnant female animals must be used at each dose level.

(iv) *Number of animals*. (A) The objective is for a sufficient number of pregnant rats to be exposed to the test substance to ensure that an adequate number of offspring are produced for neurotoxicity evaluation. At least 20 litters are recommended at each dose level.

(B) On postnatal day 4, the size of each litter should be adjusted by eliminating extra pups by random selection to yield, as nearly as possible, four male and four females per litter. Whenever the number of pups of either sex prevents having four of each sex per litter, partial adjustment (for example, five males and three females) is permitted. Testing is not appropriate for litters of less than seven pups. Elimination of runts only is not appropriate. Individual pups should be identified uniquely after standardization of litters. A method that may be used for identification can be found under paragraph (f)(1) of this section.

(v) *Assignment of animals for behavioral tests, brain weights, and neuropathological evaluations*. After standardization of litters, one male or