and expired air shall be determined separately at time intervals that provide accurate measurement of clearance and excretory rates. The collection of carbon dioxide may be discontinued when less than one percent of the dose is found to be exhaled as radioactive carbon dioxide in 24 hours.

(D) *Tissue distribution.* At the termination of each study, the quantities of radioactivity shall be determined in blood and in various tissues, including bone, brain, fat, gastrointestinal tract, gonads, heart, kidney, liver, lungs, muscle, skin, spleen, thymus, and residual carcass of each animal.

(E) *Change in pharmacokinetics.* Results of pharmacokinetics measurements (i.e., biotransformation, extent of absorption, tissue distribution, and excretion) obtained in rats receiving the single inhalation exposure to the low dose of the test substance (Group B) shall be compared to the corresponding results obtained in rats receiving repeated inhalation exposures to the low dose of the test substance (Group F).

(ii) *Metabolism.* At least four animals from each group shall be used for these purposes.

(A) *Biotransformation.* Appropriate qualitative and quantitative methods shall be used to assay urine, feces, and expired air collected from rats. Efforts shall be made to identify any metabolite which comprises 5 percent or more of the dose administered.

(B) *Changes in biotransformation.* Appropriate qualitative and quantitative assay methods shall be used to compare the composition of radioactive compounds in excreta from rats receiving a single inhalation exposure (Groups B and C) with that from rats receiving repeated inhalation exposures (Group F).

(d) *Data and reporting.* The final test report shall include the following:

(1) *Presentation of results.* Numerical data shall be summarized in tabular form. Pharmacokinetics data shall also be presented in graphical form. Qualitative observations shall also be reported.

(2) *Evaluation of results.* All data shall be evaluated by appropriate statistical methods.

(3) *Reporting results.* In addition to the reporting requirements as specified in 40 CFR part 792, the following information shall be reported.

(i) Strain of laboratory animals.

(ii) Chemical characterization of the test substances, including:

(A) For the radiolabeled test substances, information on the sites and degree of radiolabeling, including type of label, specific activity, chemical purity prior to mixing with the unlabeled hexane mixture, and radiochemical purity.

(B) For the unlabeled test substance, information on lot number and the percentage of MCP and *n*-hexane.

(C) Results of chromatography.

(iii) A full description of the sensitivity, precision, and accuracy of all procedures used to obtain the data.

(iv) Percent and rate of absorption of the test substance after inhalation and dermal exposures.

(v) Quantity and percent recovery of radioactivity in feces, urine, expired air, and blood. For dermal studies, include recovery data for skin and residual radioactivity in the covering apparatus.

(vi) Tissue distribution reported as quantity of radioactivity in blood, in various tissues including bone, brain, fat, gastrointestinal tract, gonads, heart, kidney, liver, lung, muscle, skin, spleen, thymus, and in residual carcass.

(vii) Biotransformation pathways, to the extent possible, and quantities of the test substances and metabolites in excreta collected after administering single high and low doses.

(viii) Biotransformation pathways, to the extent possible, and quantities of test substances and metabolites in excreta collected after administering repeated low doses.

(ix) Pharmacokinetics models to the extent they can be developed from the experimental data.

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§ 795.250 Developmental neurotoxicity screen.

(a) *Purpose.* In the assessment and evaluation of the toxic characteristics of a chemical, it is important to determine when acceptable exposures in the

adult may not be acceptable to a developing organism. This test is designed to provide information on the potential functional and morphologic hazards to the nervous system which may arise in the offspring from exposure of the mother during pregnancy and lactation.

(b) *Principle of the test method.* The test substance is administered to several groups of pregnant animals during gestation and lactation, one dose level being used per group. Offspring are randomly selected from within litters for neurotoxicity evaluation. The evaluation includes observation to detect gross neurological and behavioral abnormalities, determination of motor activity, neuropathological evaluation, and brain weights. Measurements are carried out periodically during both postnatal development and adulthood.

(c) *Test procedures*—(1) *Animal selection*—(i) *Species and strain.* Testing should be performed in the Sprague Dawley rat.

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

(iii) *Sex.* Pregnant females shall be used at each dose level.

(iv) *Number of animals.* The objective is for a sufficient number of pregnant rats to be exposed to ensure that an adequate number of offspring are produced for neurotoxicity evaluation. At least 20 litters are recommended at each dose level. This number assumes a coefficient of variation of 20 to 25 percent for most behavioral tests. If, based upon experience with historical control data or data for positive controls in a given laboratory, the coefficient of variation for a given task is higher than 20 to 25 percent, then calculation of appropriate sample sizes to detect a 20 percent change from control values with 80 percent power would need to be done. For most designs, calculations can be made according to Dixon and Massey (1957) under paragraph (e)(5) of this section, Neter and Wasserman (1974) under paragraph (e)(10) of this section, Sokal and Rohlf (1969) under paragraph (e)(11) of this section, or Jensen (1972) under paragraph (e)(8) of this section.

(A) On day 4 after birth, the size of each litter should be adjusted by eliminating extra pups by random selection to yield, as nearly as possible, 4 males and 4 females per litter. Whenever the number of male or female pups prevents having 4 of each sex per litter, partial adjustment (for example, 5 males and 3 females) is permitted. Adjustments are not appropriate for litters of less than 8 pups. Elimination of runts only is not appropriate. Individual pups should be identified uniquely after standardization of litters. A method that may be used can be found in Adams et al. (1985) under paragraph (e)(1) of this section.

(B) After standardization of litters, males and females shall be randomly assigned to one of each of three behavioral tasks. Alternatively, more than one of the behavioral tasks may be conducted in the same animal. In the latter case, a minimum of 1 to 2 days should separate the tests when conducted at about the same age.

(C) One male and one female shall be randomly selected from each litter for sacrifice at weaning as specified in paragraph (c)(8) of this section.

(2) *Control group.* A concurrent control group shall be used. This group shall be a sham treated group, or, if a vehicle is used in administering the test substance, a vehicle control group. Animals in the control groups shall be handled in an identical manner to test group animals. The vehicle shall neither be developmentally toxic nor have effects on reproduction.

(3) *Dose levels and dose selection.* (i) At least 3 dose levels plus a control (vehicle control, if a vehicle is used) shall be used.

(ii) If the substance has been shown to be developmentally toxic either in a standard developmental toxicity study or a pilot study, the highest dose level shall be the maximum dose which will not induce *in utero* or neonatal deaths or malformations sufficient to preclude a meaningful evaluation of neurotoxicity.

(iii) In the absence of standard developmental toxicity, unless limited by the physicochemical nature or biologicial properties of the substance, the highest dose level shall induce some overt maternal toxicity but shall not result in a reduction in weight gain exceeding 20 percent during gestation and lactation.

(iv) The lowest dose should not produce any grossly observable evidence of either maternal or developmental neurotoxicity.

(v) The intermediate dose(s) shall be equally spaced between the highest and lowest dose.

(4) *Dosing period.* Day 0 in the test is the day on which a vaginal plug and/or sperm are observed. The dose period shall cover the period from day 6 of gestation through weaning (21 days postnatally).

(5) *Administration of test substance.* The test substance or vehicle should be administered orally by intubation. The test substance shall be administered at the same time each day. The animals shall be weighed periodically and the dosage based on the most recent weight determination.

(6) *Observation of dams.* (i) A gross examination of the dams shall be made at least once each day, before daily treatment. The animals shall be observed by trained technicians who are blind with respect to the animal's treatment, using standardized procedures to maximize inter-observer reliability. 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 inter-observer reliability is required.

(ii) During the treatment and observation periods, cage-side observations shall include:

(A) Any responses with respect to body position, activity level, coordination of movement, and gait.

(B) Any unusual or bizarre behavior including, but not limited to headflicking, head searching, compulsive biting or licking, self-mutilation, circling, and walking backwards.

(C) The presence of:

(*1*) Convulsions.

(*2*) Tremors.

(*3*) Increased levels of lacrimation and/or red-colored tears.

(*4*) Increased levels of salivation.

(*5*) Piloerection.

(*6*) Pupillary dilation or constriction.

(*7*) Unusual respiration (shallow, labored, dyspneic, gasping, and retching) and/or mouth breathing.

(*8*) Diarrhea.

(*9*) Excessive or diminished urination.

(*10*) Vocalization.

(iii) Signs of toxicity shall be recorded as they are observed, including the time of onset, the degree and duration.

(iv) Animals shall be weighed at least weekly.

(v) The day of delivery of litters shall be recorded.

(7) *Study conduct*—(i) *Observation of offspring.* (A) All offspring shall be examined cage-side daily for gross signs of mortality and morbidity.

(B) All offspring shall be examined outside the cage for gross signs of toxicity whenever they are weighed or removed from their cages for behavioral testing. The offspring shall be observed by trained technicians, who are blind with respect to the animal's treatment using standardized procedures to maximize inter-observer reliability. 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 inter-observer reliability is required. At a minimum, the end points outlined in paragraph (c)(6)(ii) of this section shall be monitored as appropriate for the developmental stage being observed.

(C) Any gross signs of toxicity in the offspring shall be recorded as they are observed, including the time of onset, the degree, and duration.

(ii) *Developmental landmarks.* Live pups should be counted and litters weighed by weighing each individual pup at birth, or soon thereafter, and on days 4, 7, 13, 17, and 21, and biweekly thereafter. The age of the pups at the time of the appearance of the following developmental landmarks shall be determined:

(A) *Vaginal opening.* General procedure for this determination may be found in Adams et al. (1985) under paragraph (e)(1) of this section.

(B) *Testes descent.* General procedure for this determination may be found in Adams et al. (1985) under paragraph (e)(1) of this section.

(iii) *Motor activity.* (A) Motor activity shall be monitored specifically on days 13, 17, 21, 45 (±2 days), and 60 (±2 days). Motor activity shall be monitored by an automated activity recording apparatus. The device used shall be capable

of detecting both increases and decreases in activity, i.e., baseline activity as measured by the device shall not be so low as to preclude decreases nor so high as to preclude increases. Each device shall be tested by standard procedures to ensure, to the extent possible, reliability of operation across devices and testing of animals within dose groups shall be balanced across devices.

(B) Each animal shall be tested individually. The test session shall be long enough to demonstrate habituation of motor activity in control animals, i.e., to approach asymptotic levels by the last 20 percent of the session. Animals' activity counts shall be collected in equal time periods of no greater than 10 minutes duration. All sessions shall have the same duration. Treatment groups shall be counter-balanced across test times.

(C) Efforts shall 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 home cage or novel test cage, and environmental distractions.

(D) Additional information on the conduct of a motor activity study may be obtained in the TSCA motor activity guideline, in § 798.6200 of this chapter.

(iv) *Auditory startle test.* An auditory startle habituation test shall be performed on the offspring on days 22 and 60. Details on the conduct of this testing may be obtained in Adams et al. (1985) under paragraph (e)(1) of this section. In performing the auditory startle task, the mean response amplitude on each block of 10 trials (5 blocks of 10 trials per session on each day of testing) shall be made. While use of prepulse inhibition is not a requirement, it may be used at the discretion of the investigator. Details on the conduct of this testing may be obtained from Ison (1984) under paragraph (e)(7) of this section.

(v) *Active avoidance test.* Active avoidance testing shall be conducted beginning at 60 to 61 days of age. Details on the apparatus may be obtained in Brush and Knaff (1959) and on the conduct of testing from Brush (1962), under paragraphs (e)(2) and (e)(4) of this section, respectively; reviews on active avoidance conditioning by Brush (1971) and McAllister and McAllister (1971) can be found under paragraphs (e)(3) and (e)(9) of this section, respectively. In performing the active avoidance task, the following measures should be made:

(A) Mean number of shuttles during the adaptation period preceding each daily session.

(B) Mean number and latency of avoidances per session, presented in blocks of 10 trials (2 blocks of 10 trials per session across 5 sessions).

(C) Mean number and latency of escapes per session, presented in blocks of 10 trials as above.

(D) Mean duration of shocks per session, presented in blocks of 10 trials as above.

(E) Mean number of shuttles during the inter-trial intervals.

(8) *Post-mortem evaluation*—(i) *Age of animals.* One male and one female per litter shall be sacrificed at weaning and the remainder following the last behavioral measures. Neuropathology and brain weight determinations shall be made on animals sacrificed at weaning and after the last behavioral measures.

(ii) *Neuropathology.* Details for the conduct of neuropathology evaluation may be obtained in the TSCA neuropathology guideline, in § 798.6400 of this chapter. At least 6 offspring per dose group shall be randomly selected from each sacrificed group (weaning and adulthood) for neuropathologic evaluation. These animals shall be balanced across litters, and equal numbers of males and females shall be used. The remaining sacrificed animals shall be used to determine brain weight. Animals shall be perfused *in situ* by a generally recognized technique. After perfusion, the brain and spinal cord shall be removed and gross abnormalities noted. Cross-sections of the following areas shall be examined: The forebrain, the center of the cerebrum and midbrain, the cerebellum and pons, and the medulla oblongata; the spinal cord at cervical and lumbar swelling; Gasserian ganglia, dorsal root ganglia,

dorsal and ventral root fibers, proximal sciatic nerve (mid-thigh and sciatic notch), sural nerve (at knee), and tibial nerve (at knee). Tissue samples from both the central and peripheral nervous system shall be further immersion-fixed and stored in appropriate fixative for further examination. After dehydration, tissue specimens shall be cleared with xylene and embedded in paraffin or paraplast except for the sural nerve which should be embedded in plastic. A method for plastic embedding is described by Spencer et al. under paragraph (e)(12) of this section. Tissue sections shall be prepared from the tissue blocks. The following general testing sequence is recommended for gathering histopathological data:

(A) *General staining.* A general staining procedure shall be performed on all tissue specimens in the highest treatment group. Hematoxylin and eosin (H&E) shall be used for this purpose. The staining shall be differentiated properly to achieve bluish nuclei with pinkish background.

(B) *Special stains.* Based on the results of the general staining, selected sites and cellular components shall be further evaluated by use of specific techniques. If H&E screening does not provide such information, a battery of stains shall be used to assess the following components in all appropriate required samples: Neuronal body (e.g., Einarson's gallocyanin), axon (e.g., Kluver's Luxol Fast Blue), and neurofibrils (e.g., Bielchosky). In addition, nerve fiber teasing shall be used. A section of normal tissue shall be included in each staining to assure that adequate staining has occurred. Any changes shall be noted and representative photographs shall be taken. If lesions are observed, the special techniques shall be repeated in the next lower treatment group until no further lesions are detectable.

(C) *Alternative technique.* If the anatomical locus of expected neuropathology is well-defined, epoxy-embedded sections stained with toluidine blue may be used for small sized tissue samples. This technique obviates the need for special stains.

(iii) *Brain weight.* At least 10 animals that are not sacrificed for histopathology shall be used to determine brain weight. The animals shall be decapitated and the brains carefully removed, blotted, chilled, and weighed. The following dissection shall be performed on an ice-cooled glass plate: First, the rhombencephalon is separated by a transverse section from the rest of the brain and dissected into the cerebellum and the medulla oblongata/pons. A transverse section is made at the level of the ''optic chiasma'' which delimits the anterior part of the hypothalamus and passes through the anterior commissure. The cortex is peeled from the posterior section and added to the anterior section. This divides the brain into four sections, the telencephalon, the diencephalon/midbrain, the medulla oblongata/pons, and the cerebellum. Sections shall be weighed as soon as possible after dissection to avoid drying. Detailed methodology is available in Glowinski and Iversen (1966) under paragraph (e)(6) of this section.

(d) *Data reporting and evaluation.* In addition to the reporting requirements specified in part 792, subpart J of this chapter, the final test report shall include the following information.

(1) *Description of system and test methods.* (i) A detailed description of the procedures used to standardize observation and operational definitions for scoring observations.

(ii) Positive control data from the laboratory performing the test that demonstrate the sensitivity of the procedures being used. These data do not have to be from studies using prenatal exposures. However, the laboratory must demonstrate competence in testing neonatal animals perinatally exposed to chemicals and establish test norms for the appropriate age group.

(iii) Procedures for calibrating and assuring the equivalence of devices and balancing treatment groups.

(iv) A short justification explaining any decisions where professional judgement is involved such as fixation technique and choice of stains.

(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 and litter from which it came.

(B) Its body weight and score on each developmental landmark at each observation time; total session activity counts and intrasession subtotals on each day measured; auditory startle response magnitude session counts and intrasession subtotals on each day measured; avoidance session counts and intrasession counts on each day measured; time and cause of death (if appropriate); locations, nature or frequency, and severity of the lesions; total brain weight; absolute weight of each of the four sections; and weight of each section as a percentage of total brain weight. A commonly used scale such as 1+, 2+, 3+, and 4+ for degree of severity of lesions ranging from very slight to extensive may be used for morphologic evaluation. Any diagnoses derived from neurologic signs and lesions, including naturally occurring diseases or conditions, shall also be recorded.

(ii) Summary data for each group shall include:

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

(B) Body weights of the dams during gestation and lactation.

(C) Litter size and mean weight at birth.

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

(E) The percentage of animals showing each abnormal sign at each observation time.

(F) The mean and standard deviation for each continuous end point at each observation time. These will include body weight, motor activity counts, acoustic startle responses, performance in active avoidance tests, and brain weights (both absolute and relative).

(G) The number of animals in which any lesion was found.

(H) The number of animals affected by each different type of lesion, the average grade of each type of lesion, and the frequency of each different type and/or location of lesions.

(3) *Evaluation of data.* An evaluation of the test results shall be made. The evaluation shall include the relationship between the doses of the test substance and the presence or absence, incidence, and severity of any neurotoxic effect. The evaluation shall include appropriate statistical analyses. The choice of analyses shall consider tests appropriate to the experimental design and needed adjustments for multiple comparisons.

(e) *References.* For additional background information on this test guideline, the following references should be consulted:

(1) Adams, J., Buelke-Sam, J., Kimmel, C.A., Nelson, C.J., Reiter, L.W., Sobotka, T.J., Tilson, H.A., and Nelson, B.K. ''Collaborative behavioral teratology study: Protocol design and testing procedure.'' *Neurobehavioral Toxicology and Teratology*. 7: 579–586. (1985).

(2) Brush, F.R. ''The effects of inter-trial interval on avoidance learning in the rat.'' *Journal of Comparative Physiology and Psychology*. 55: 888–892. (1962).

(3) Brush, F.R. ''Retention of aversively motivated behavior.'' In: ''Adverse Conditioning and Learning.'' Brush, F.R., ed., New York: Academic Press. (1971).

(4) Brush, F.R. and Knaff, P.R. ''A device for detecting and controlling automatic programming of avoidance-conditioning in a shuttle-box.'' *American Journal of Psychology*. 72: 275–278 (1959).

(5) Dixon, W.J. and Massey, E.J. ''Introduction to Statistical Analysis.'' 2nd ed. New York: McGraw-Hill. (1957).

(6) Glowinski, J. and Iversen, L.L. ''Regional studies of catecholamines in the rat brain-I.'' *Journal of Neurochemistry*. 13: 655–669. (1966).

(7) Ison, J.R. ''Reflex modification as an objective test for sensory processing following toxicant exposure.'' *Neurobehavioral Toxicology and Teratology*. 6: 437–445. (1984).

(8) Jensen, D.R. ''Some simultaneous multivariate procedures using Hotelling's T2 Statistics.'' *Biometrics*. 28: 39–53. (1972).

(9) McAllister, W.R. and McAllister, D.E. ''Behavioral measurement of conditioned fear.'' In: ''Adverse Conditioning and Learning.'' Brush, F.R., ed., New York: Academic Press (1971).

(10) Neter, J. and Wasserman, W. ''Applied Linear Statistical Models.'' Homewood: Richard D. Irwin, Inc. (1974).

(11) Sokal, R.P. and Rohlf, E.J. ''Biometry.'' San Francisco: W.H. Freeman and Co. (1969).

(12) Spencer, P.S., Bischoff, M.C., and Schaumburg, H.H., ''Neuropathological methods for the detection of neurotoxic disease.'' In: ''Experimental and Clinical Neurotoxicology.'' Spencer, P.S. and Schaumburg, H.H., eds., Baltimore, MD: Williams & Wilkins, pp. 743–757. (1980).

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