

Food Safety Assessment. (Columbia: Food Safety Council, 1978) pp. 83-96.

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(5) World Health Organization. "Part I. Environmental Health Criteria 6," *Principles and Methods for Evaluating the Toxicity of Chemicals.* (Geneva: World Health Organization, 1978).

[50 FR 39397, Sept. 27, 1985, as amended at 52 FR 19074, May 20, 1987; 53 FR 49150, Dec. 6, 1988; 54 FR 21064, May 16, 1989]

Subpart D—Chronic Exposure

§ 798.3260 Chronic toxicity.

(a) *Purpose.* The objective of a chronic toxicity study is to determine the effects of a substance in a mammalian species following prolonged and repeated exposure. Under the conditions of the chronic toxicity test, effects which require a long latency period or which are cumulative should become manifest. The application of this guideline should generate data on which to identify the majority of chronic effects and shall serve to define long term dose-response relationships. The design and conduct of chronic toxicity tests should allow for the detection of general toxic effects, including neurological, physiological, biochemical, and hematological effects and exposure-related morphological (pathology) effects.

(b) *Test procedures—(1) Animal selection—(i) Species and strain.* Testing should be performed with two mammalian species, one a rodent and another a non-rodent. The rat is the preferred rodent species and the dog is the preferred non-rodent species. Commonly used laboratory strains should be employed. If other mammalian species are used, the tester should provide justification/reasoning for their selection.

(ii) *Age.* (A) Dosing of rats should begin as soon as possible after weaning, ideally before the rats are 6, but in no case more than 8 weeks old.

(B) Dosing of dogs should begin between 4 and 6 months of age and in no case later than 9 months of age.

(C) At commencement of the study the weight variation of animals used should not exceed ± 20 percent of the mean weight for each sex.

(iii) *Sex.* (A) Equal numbers of animals of each sex should be used at each dose level.

(B) The females should be nulliparous and non-pregnant.

(iv) *Numbers.* (A) For rodents, at least 40 animals (20 females and 20 males) and for non-rodents (dogs) at least 8 animals (4 females and 4 males) should be used at each dose level.

(B) If interim sacrifices are planned, the number should be increased by the number of animals scheduled to be sacrificed during the course of the study.

(C) The number of animals at the termination of the study must be adequate for a meaningful and valid statistical evaluation of chronic effects.

(2) *Control groups.* (i) A concurrent control group is suggested. This group should be an untreated or sham treated control group or, if a vehicle is used in administering the test substance, a vehicle control group. If the toxic properties of the vehicle are not known or cannot be made available, both untreated and vehicle control groups are strongly suggested.

(ii) In special circumstances such as in inhalation studies involving aerosols or the use of an emulsifier of uncharacterized biological activity in oral studies, a concurrent negative control group should be utilized. The negative control group should be treated in the same manner as all other test animals except that this control group should not be exposed to either the test substance or any vehicle.

(3) *Dose levels and dose selections.* (i) In chronic toxicity tests, it is necessary to have a dose-response relationship as well as a no-observed-toxic-effect level. Therefore, at least three dose levels should be used in addition to the concurrent control group. Dose levels should be spaced to produce a gradation of effects.

(ii) The high dose level in rodents should elicit some signs of toxicity without causing excessive lethality; for non-rodents, there should be signs of

toxicity but there should be no fatalities.

(iii) The lowest dose level should not produce any evidence of toxicity. Where there is a usable estimation of human exposure the lowest dose level should exceed this even though this dose level may result in some signs of toxicity.

(iv) Ideally, the intermediate dose level(s) should produce minimal observable toxic effects. If more than one intermediate dose is used, the dose level should be spaced to produce a gradation of toxic effects.

(v) For rodents, the incidence of fatalities in low and intermediate dose groups and in the controls should be low to permit a meaningful evaluation of the results. For non-rodents, there should be no fatalities.

(4) *Exposure conditions.* The animals are dosed with the test substance ideally on a 7-day per week basis over a period of at least 12 months. However, based primarily on practical considerations, dosing on a 5-day per week basis is considered to be acceptable.

(5) *Observation period.* Duration of observation should be for at least 12 months, and may be concurrent with or subsequent to dosing. If there is a post-exposure observation period, an interim sacrifice should be performed on no fewer than half of the animals of each sex at each dose level immediately upon termination of exposure.

(6) *Administration of the test substance.* The three main routes of administration are oral, dermal, and inhalation. The choice of the route of administration depends upon the physical and chemical characteristics of the test substance and the form typifying exposure in humans.

(i) *Oral studies.* (A) The animals should receive the test substance in their diet, dissolved in drinking water, or given by gavage or capsule for a period of at least 12 months.

(B) If the test substance is administered in the drinking water, or mixed in the diet, exposure is continuous.

(C) For a diet mixture, the highest concentration should not exceed 5 percent.

(ii) *Dermal studies.* (A) The animals are treated by topical application with

the test substance, ideally for at least 6 hours per day.

(B) Fur should be clipped from the dorsal area of the trunk of the test animals. Care must be taken to avoid abrading the skin which could alter its permeability.

(C) The test substance should be applied uniformly over a shaved area which is approximately 10 percent of the total body surface area. With highly toxic substances, the surface area covered may be less, but as much of the area should be covered with as thin and uniform a film as possible.

(D) During the exposure period, the test substance may be held if necessary, in contact with the skin with a porous gauze dressing and non-irritating tape. The test site should be further covered in a suitable manner to retain the gauze dressing and test substance and ensure that the animals cannot ingest the test substance.

(iii) *Inhalation studies.* (A) The animals should be tested with inhalation equipment designed to sustain a dynamic air flow of 12 to 15 air changes per hour, ensure an adequate oxygen content of 19 percent and an evenly distributed exposure atmosphere. Where a chamber is used, its design should minimize crowding of the test animals and maximize their exposure to the test substance. This is best accomplished by individual caging. As a general rule to ensure stability of a chamber atmosphere, the total "volume" of the test animals should not exceed 5 percent of the volume of the test chamber. Alternatively, oro-nasal, head-only or whole body individual chamber exposure may be used.

(B) The temperature at which the test is performed should be maintained at 22 °C ($\pm 2^\circ$). Ideally, the relative humidity should be maintained between 40 to 60 percent, but in certain instances (e.g., tests of aerosols, use of water vehicle) this may not be practicable.

(C) Feed and water should be withheld during each daily 6 hour exposure period.

(D) A dynamic inhalation system with a suitable analytical concentration control system should be used. The rate of air flow should be adjusted to ensure that conditions throughout

the equipment are essentially the same. Maintenance of slight negative pressure inside the chamber will prevent leakage of the test substance into the surrounding areas.

(7) *Observation of animals.* (i) Each animal should be handled and its physical condition appraised at least once each day.

(ii) Additional observations should be made daily with appropriate actions taken to minimize loss of animals to the study (e.g., necropsy or refrigeration of those animals found dead and isolation or sacrifice of weak or moribund animals).

(iii) Clinical signs of toxicity including suspected tumors and mortality should be recorded as they are observed, including the time of onset, the degree and duration.

(iv) Cage-side observations should include, but not be limited to, changes in skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behavior pattern.

(v) Body weights should be recorded individually for all animals once a week during the first 13 weeks of the test period and at least once every 4 weeks thereafter unless signs of clinical toxicity suggest more frequent weighings to facilitate monitoring of health status.

(vi) When the test substance is administered in the feed or drinking water, measurements of feed or water consumption, respectively, should be determined weekly during the first 13 weeks of the study and then at approximately monthly intervals unless health status or body weight changes dictate otherwise.

(vii) At the end of the study period all survivors should be sacrificed. Moribund animals should be removed and sacrificed when noticed.

(8) *Physical measurements.* For inhalation studies, measurements or monitoring should be made of the following:

(i) The rate of air flow should be monitored continuously, but should be recorded at intervals of at least once every 30 minutes.

(ii) During each exposure period the actual concentrations of the test substance should be held as constant as

practicable, monitored continuously and measured at least three times during the test period: at the beginning, at an intermediate time and at the end of the period.

(iii) During the development of the generating system, particle size analysis should be performed to establish the stability of aerosol concentrations. During exposure, analysis should be conducted as often as necessary to determine the consistency of particle size distribution and homogeneity of the exposure stream.

(iv) Temperature and humidity should be monitored continuously, but should be recorded at intervals of at least once every 30 minutes.

(9) *Clinical examinations.* The following examinations should be made on at least 10 rats of each sex per dose and on all non-rodents.

(i) Certain hematology determinations (e.g., hemoglobin content, packed cell volume, total red blood cells, total white blood cells, platelets, or other measures of clotting potential) should be performed at termination and should be performed at 3 months, 6 months and at approximately 6 month intervals thereafter (for studies extending beyond 12 months) on blood samples collected from all non-rodents and from 10 rats per sex of all groups. These collections should be from the same animals at each interval. If clinical observations suggest a deterioration in health of the animals during the study, a differential blood count of the affected animals should be performed. A differential blood count should be performed on samples from those animals in the highest dosage group and the controls. Differential blood counts should be performed for the next lower group(s) if there is a major discrepancy between the highest group and the controls. If hematological effects were noted in the subchronic test, hematological testing should be performed at 3, 6, 12, 18, and 24 months for a two year study and at 3, 6, and 12 months for a 1-year study.

(ii) Certain clinical biochemistry determinations on blood should be carried out at least three times during the test period: just prior to initiation of dosing (base line data), near the middle and at the end of the test period. Blood

samples should be drawn for clinical chemistry measurements from all non-rodents and at least ten rodents per sex of all groups; if possible, from the same rodents at each time interval. Test areas which are considered appropriate to all studies: electrolyte balance, carbohydrate metabolism and liver and kidney function. The selection of specific tests will be influenced by observations on the mode of action of the substance and signs of clinical toxicity. Suggested chemical determinations: calcium, phosphorus, chloride, sodium, potassium, fasting glucose (with period of fasting appropriate to the species), serum glutamic-pyruvic transaminase (now known as serum alanine aminotransferase), serum glutamic oxaloacetic transaminase (now known as serum aspartate aminotransferase), ornithine decarboxylase, gamma glutamyl transpeptidase, blood urea nitrogen, albumen, blood creatinine, creatinine phosphokinase, total cholesterol, total bilirubin and total serum protein measurements. Other determinations which may be necessary for an adequate toxicological evaluation include analyses of lipids, hormones, acid/base balance, methemoglobin and cholinesterase activity. Additional clinical biochemistry may be employed where necessary to extend the investigation of observed effects.

(iii) Urine samples from rodents at the same intervals as the hematological examinations under paragraph (b)(9)(i) of this section should be collected for analysis. The following determinations should be made from either individual animals or on a pooled sample/sex/group for rodents: appearance (volume and specific gravity), protein, glucose, ketones, bilirubin, occult blood (semi-quantitatively); and microscopy of sediment (semi-quantitatively).

(iv) Ophthalmological examination, using an ophthalmoscope or equivalent suitable equipment, should be made prior to the administration of the test substance and at the termination of the study. If changes in eyes are detected all animals should be examined.

(10) *Gross necropsy.* (i) A complete gross examination should be performed on all animals, including those which

died during the experiment or were killed in moribund conditions.

(ii) The liver, kidneys, adrenals, brain and gonads should be weighed wet, as soon as possible after dissection to avoid drying. For these organs, at least 10 rodents per sex per group and all non-rodents should be weighed.

(iii) The following organs and tissues, or representative samples thereof, should be preserved in a suitable medium for possible future histopathological examination: All gross lesions and tumors; brain—including sections of medulla/pons, cerebellar cortex, and cerebral cortex; pituitary; thyroid/parathyroid; thymus; lungs; trachea; heart; sternum and/or femur with bone marrow; salivary glands; liver; spleen; kidneys; adrenals; esophagus; stomach; duodenum; jejunum; ileum; cecum; colon; rectum; urinary bladder; representative lymph nodes; pancreas; gonads; uterus; accessory genital organs (epididymis, prostate, and, if present, seminal vesicles; female mammary gland; aorta; gall bladder (if present); skin; musculature; peripheral nerve; spinal cord at three levels—cervical, midthoracic, and lumbar; and eyes. In inhalation studies, the entire respiratory tract, including nose, pharynx, larynx, and paranasal sinuses should be examined and preserved. In dermal studies, skin from sites of skin painting should be examined and preserved.

(iv) Inflation of lungs and urinary bladder with a fixative is the optimal method for preservation of these tissues. The proper inflation and fixation of the lungs in inhalation studies is considered essential for appropriate and valid histopathological examination.

(v) If other clinical examinations are carried out, the information obtained from these procedures should be available before microscopic examination, since they may provide significant guidance to the pathologist.

(11) *Histopathology.* (i) The following histopathology should be performed:

(A) Full histopathology on the organs and tissues, listed above, of all non-rodents, of all rodents in the control and high dose groups and of all rodents that died or were killed during the study.

(B) All gross lesions in all animals.

(C) Target organs in all animals.

(D) Lungs, liver and kidneys of all animals. Special attention to examination of the lungs of rodents should be made for evidence of infection since this provides an assessment of the state of health of the animals.

(ii) If excessive early deaths or other problems occur in the high dose group compromising the significance of the data, the next dose level should be examined for complete histopathology.

(iii) In case the results of an experiment give evidence of substantial alteration of the animals' normal longevity or the induction of effects that might affect a toxic response, the next lower dose level should be examined fully, as described under paragraph (b)(11)(i) of this section.

(iv) An attempt should be made to correlate gross observations with microscopic findings.

(c) *Data and reporting*—(1) *Treatment of results.* (i) 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 showing lesions, the types of lesions and the percentage of animals displaying each type of lesion.

(ii) All observed results, quantitative and incidental, should be evaluated by an appropriate statistical method. Any generally accepted statistical methods may be used; the statistical methods should be selected during the design of the study.

(2) *Evaluation of study results.* (i) The findings of a chronic toxicity study should be evaluated in conjunction with the findings of preceding studies and considered in terms of the toxic effects, the necropsy and histopathological findings. The evaluation will include the relationship between the dose of the test substance and the presence, incidence and severity of abnormalities (including behavioral and clinical abnormalities), gross lesions, identified target organs, body weight changes, effects on mortality and any other general or specific toxic effects.

(ii) In any study which demonstrates an absence of toxic effects, further investigation to establish absorption and

bioavailability of the test substance should be considered.

(3) *Test report.* (i) In addition to the reporting requirements as specified under 40 CFR part 792 subpart J, the following specific information should be reported:

(A) *Group animal data.* Tabulation of toxic response data by species, strain, sex and exposure level for:

(1) Number of animals dying.

(2) Number of animals showing signs of toxicity.

(3) Number of animals exposed.

(B) *Individual animal data.* (1) Time of death during the study or whether animals survived to termination.

(2) Time of observation of each abnormal sign and its subsequent course.

(3) Body weight data.

(4) Feed and water consumption data, when collected.

(5) Results of ophthalmological examination, when performed.

(6) Hematological tests employed and all results.

(7) Clinical biochemistry tests employed and all results.

(8) Necropsy findings.

(9) Detailed description of all histopathological findings.

(10) Statistical treatment of results, where appropriate.

(ii) In addition, for inhalation studies the following should be reported:

(A) *Test conditions.* (1) Description of exposure apparatus including design, type, dimensions, source of air, system for generating particulates and aerosols, method of conditioning air, treatment of exhaust air and the method of housing the animals in a test chamber.

(2) The equipment for measuring temperature, humidity, and particulate aerosol concentrations and size should be described.

(B) *Exposure data.* These should be tabulated and presented with mean values and a measure of variability (e.g., standard deviation) and should include:

(1) Airflow rates through the inhalation equipment.

(2) Temperature and humidity of air.

(3) Nominal concentration (total amount of test substance fed into the inhalation equipment divided by volume of air).

(4) Actual concentration in test breathing zone.

(5) Particle size distribution (e.g., median aerodynamic diameter of particles with standard deviation from the mean).

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

(1) Benitz, K.F. "Measurement of Chronic Toxicity," *Methods of Toxicology*. Ed. G.E. Paget. (Oxford: Blackwell Scientific Publications, 1970) pp. 82-131.

(2) D'Aguanno, W. "Drug Safety Evaluation—Pre-Clinical Considerations," *Industrial Pharmacology: Neuroleptics*. Vol. I, Ed. S. Fielding and H. Lal. (Mt. Kisco: Futura Publishing Co. 1974) pp. 317-332.

(3) Fitzhugh, O.G. Third Printing: 1975. "Chronic Oral Toxicity," *Appraisal of the Safety of Chemicals in Foods, Drugs and Cosmetics*. The Association of Food and Drug Officials of the United States (1959, 3rd Printing 1975) pp. 36-45.

(4) Goldenthal, E.I., D'Aguanno, W. "Evaluation of Drugs," *Appraisal of the Safety of Chemicals in Foods, Drugs, and Cosmetics*. The Association of Food and Drug Officials of the United States (1959, 3rd Printing 1975) pp. 60-67.

(5) National Academy of Sciences. "Principles and Procedures for Evaluating the Toxicity of Household Substances," a report prepared by the Committee for the Revision of NAS Publication 1138, under the auspices of the Committee on Toxicology, National Research Council, National Academy of Sciences, Washington, DC (1977).

(6) National Center for Toxicological Research. "Appendix B," *Report of Chronic Studies Task Force Committee, April 13-21, 1972*. (Rockville: National Center for Toxicological Research, 1972).

(7) Page, N.P. "Chronic Toxicity and Carcinogenicity Guidelines," *Journal of Environmental Pathology and Toxicology*, 1:161-182 (1977).

(8) Schwartz, E. "Toxicology of Neuroleptic Agents," *Industrial Pharmacology: Neuroleptics* Ed. S. Fielding and H. Lal. (Mt. Kisco, Futura Publishing Co., 1974) pp. 203-221.

(9) United States Pharmaceutical Manufacturers Association. *Guidelines for the Assessment of Drug and Medical Device Safety in Animals*. (1977).

(10) World Health Organization. "Guidelines for Evaluation of Drugs for Use in Man," *WHO Technical Report Series No. 563*. (Geneva: World Health Organization, 1975).

(11) World Health Organization. "Part I. Environmental Health Criteria 6," *Principles and Methods for Evaluating the Toxicity of Chemicals*. (Geneva: World Health Organization, 1978).

(12) World Health Organization. "Principles for Pre-Clinical Testing of Drug Safety," *WHO Technical Report Series No. 341*. (Geneva: World Health Organization, 1966).

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§ 798.3300 Oncogenicity.

(a) *Purpose.* The objective of a long-term oncogenicity study is to observe test animals for a major portion of their life span for the development of neoplastic lesions during or after exposure to various doses of a test substance by an appropriate route of administration.

(b) *Test procedures*—(1) *Animal selection*—(i) *Species and strain.* A compound of unknown activity shall be tested on two mammalian species. Rats and mice are the species of choice because of their relatively short life spans, the limited cost of their maintenance, their widespread use in pharmacological and toxicological studies, their susceptibility to tumor induction, and the availability of inbred or sufficiently characterized strains. Commonly used laboratory strains shall be employed. If other species are used, the tester shall provide justification/reasoning for their selection.

(ii) *Age.* (A) Dosing of rodents shall begin as soon as possible after weaning, ideally before the animals are 6 weeks old, but in no case more than 8 weeks old.

(B) At commencement of the study, the weight variation of animals used shall not exceed ± 20 percent of the mean weight for each sex.

(C) Studies using prenatal or neonatal animals may be recommended under special conditions.