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Standard Test Method for Conducting a Subchronic Inhalation Toxicity Study in Rats[1]

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[1] This test method is under the jurisdiction of ASTM Committee E-35 on Pesticides and is the direct responsibility of Subcommittee E35.26 on Safety to Man.

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1. Scope

1.1 This test method determines the no- observed-effect level (NOEL) and toxic effects associated with continuous or repeated inhalation exposure to a chemical, pesticide, or mixture for a period of 90 days. The test is not capable of determining those effects that have a long latency period for development (for example, carcinogenicity and life shortening).

1.2 This test method which is applicable to a gas, vapor, aerosol, or particulate is conducted after initial information on toxicity is obtained by acute testing.

1.3 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*

2. Referenced Documents

2.1 *ASTM Standards:*

E ~~609 Definition of Terms~~ 609 Terminology Relating to Pesticides[2]

E 943 Terminology Relating to Biological Effects and Environmental Fate[2]

2.2 *Federal Standards:*

~~Title 40, Code of Federal Regulations (CFR), Environmental Protection Agency, Part 798,~~

OPPTS 870-3465, EPA 712-C98-204 90 day Inhalation Toxicity. Health Effects ~~Testing~~ Test Guidelines, ~~Subpart C, Subchronic Inhalation Toxicity: 90-Day Study~~ Office of Prevention, Pesticides and Toxic Substances , US Environmental Protection Agency (USEPA)[3]

Title 40, Code of Federal Regulations (CFR), Environmental Protection Agency, Subchapter E, Pesticide Programs; Part 160, Good Laboratory Practice Standards[4]

Title 21, Code of Federal Regulations (CFR), Food and Drug Administration, Part 58, Laboratory Practice for Nonclinical Laboratory Studies[4]

Title 40, Code of Federal Regulations (CFR), Toxic Substance Control Act, Part 792, Good Laboratory Practice Standards[4]

3. Terminology

3.1 *Definitions*—Refer to Definitions E 609 and Terminology E 943.

3.1.1 *aerodynamic diameter*—The diameter of the unit density sphere that has the same terminal settling velocity due to gravity as the particle under consideration, whatever its size, shape and density. It is used to compare particles of different size and densities and to predict where in the respiratory tract such particles may be deposited.[5,6]

3.1.2 *concentration*—The amount of test substance administered via inhalation for a period of 90 days. Express concentration as weight of the test substance per unit volume of air (for example, milligrams per liter, parts per million).

[2] *Annual Book of ASTM Standards*, Vol 11.05.

[3] Available from ~~U.S. Government Printing Office, Superintendent of Documents,~~ Environmental Protection Agency Ariel Rios Building 1200 Pennsylvania Avenue, N.W. Washington, DC 20460 (202) 260-2090

~~[4] Hinds, William C., *Aerosol Technology, Properties, Behavior, and Measurement*~~

[4] Available from U.S. Government Printing Office, Superintendent of ~~Airborne Particles, John Wiley & Sons, 1982.~~ Documents, Washington, DC 20402.

~~[5] Richard, D.,~~

[5] Hinds, William C., ~~*Handbook on Aerosols*, U.S. Energy Research~~*Aerosol Technology, Properties, Behavior, and* ~~*Development Administration, 1976.*~~ *Measurement of Airborne Particles*, John Wiley & Sons, 1982.

~~[6] Leong, Basil K. J.,~~

[6] Richard, D., ~~*Inhalation Toxicology and Technology*~~ *Handbook on Aerosols*, ~~*Ann Arbor Science, 1981.*~~ *U.S. Energy Research and Development Administration, 1976.*

3.1.3 *geometric mean diameter or median diameter*—The calculated aerodynamic diameter that divides the particles of an aerosol in half, based on the weight of the particles. Fifty percent of the particles by weight will be larger than the median diameter and 50 % of the particles will be smaller than the median diameter. The median diameter describes the particle size distribution of any aerosol based on the weight and size of the particles.

3.1.~~3~~4 *geometric standard deviation*—The measure of dispersion for a log-normal distribution, the ratio of the 84.13 percentile to the 50 percentile and 50 percentile to the 15.8 percentile.

3.1.~~4~~5 *inhalable diameter*—The aerodynamic diameter of a particle that is considered to be inhalable for the organism. It is used to refer to particles that are capable of being inhaled and deposited anywhere within the respiratory tract from the trachea to the alveoli. For man, inhalable diameter is considered as 15 µm or less (see CFR Title 40, Part 798).

3.1.~~5~~6 *cumulative toxicity*—The adverse effects caused when the inactivation or excretion, or both, of a substance is slower than the rate at which the substance is being administered. Since there is more of the substance entering the body than is being removed, a build up results, causing repeated dosage of the substance to produce a more marked response than after the first dose.

3.1.7 *no-observed-effect-level (NOEL)*—The maximum concentration used in a study that produces no observed effects.

3.1.8 *subchronic inhalation toxicity*—The adverse effects occurring as a result of the repeated daily exposure of experimental animals to a chemical by inhalation for a part (approximately 10 %) of a life span.

4. Summary of Test Method (see ~~CFR Title 40, Part 798 and~~ Literature[7])

4.1 Three groups of 20 rats (10 female, 10 males) each are used for at least three dose levels. Doses should be spaced appropriately to produce test groups with a range of toxic effects and mortality rates.

4.2 A concurrent control group of 20 rats should be untreated or sham treated.

4.3 An additional group of 20 rats may be treated with the high concentration level for 90 days and then observed for a post-treatment period normally not less than 28 days.

4.4 Rats are exposed to the test substance, ideally for 6 h/day on a 7-day/week basis, for a period of 90 days. However, based primarily on practical considerations, exposure on a 5-day/week basis is considered to be acceptable.

4.5 Rats are placed in inhalation equipment with a dynamic air flow of 12 to 15 air changes/h. Nasal or head only exposure may be used if it is desirable to avoid exposure by the dermal or oral route.

4.6 The test substance is introduced into the chamber air supply. A suitable analytical control system should be used.

4.7 Daily observations of all individual rats for signs of toxicity and mortality are recorded.

4.8 All rats are weighed at least once every week and these weights recorded throughout the study.

4.9 Urine and blood samples are collected for analysis just prior to necropsy. After 90 days of exposure the surviving rats are weighed, sacrificed, and a complete necropsy done. Histopathological examinations are also performed on selected tissues.

5. Significance and Use

5.1 This test method determines the subchronic inhalation toxicity that is the adverse effect occurring as a result of the repeated daily exposure of experimental rats to a chemical by inhalation for part (approximately 10 %) of a life span.

5.2 This test method provides information on health hazards likely to arise from repeated exposures by the inhalation route over a limited period of time. It will provide information on target organs, the possibilities of accumulation, and can be of use in selecting dose levels for chronic studies and for establishing safety criteria for human exposure.

5.3 Hazards of inhaled substances are influenced by their inherent toxicity and by physical factors such as volatility and particle size.

6. Exposure Apparatus[8]

6.1 The inhalation chamber should sustain a dynamic air flow of ~~12 to 15~~ 10 air changes/h and ensure adequate oxygen content of 19 % and an evenly distributed exposure atmosphere. Animals should occupy less than 5 % of the chamber volume.

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

6.3 The exhaust from the chambers must be treated appropriately to prevent the release of the test materials into the environment.

6.4 The temperature at which the test is performed should be maintained at 22°C (±2°C). Ideally, the relative humidity should be maintained between 40 to 60 %, but in certain instances (for example, tests of aerosols, use of water vehicles), this may not be practicable.

6.5 Measurements or monitoring should be made as follows:

6.5.1 The rate of air flow may be monitored continuously but should be recorded at least every 60 min,

~~[7] Silver, S. D., "Constant Gassing Chambers: Principles Influencing Design and Operation,"~~

[7] Leong, Basil K. J., ~~*Journal of Laboratory Clinical Medicine*~~*Inhalation Toxicology and Technology*, ~~*Vol 31, 1946, pp. 1153–1161.*~~ *Ann Arbor Science, 1981.*

[8] Silver, S. D., "Constant Gassing Chambers: Principles Influencing Design and Operation," *Journal of Laboratory Clinical Medicine*, Vol 31, 1946, pp. 1153–1161.

6.5.2 The actual concentration of the test substance should be measured in the breathing zone. During the exposure period the actual concentration of the test substance should be held as constant as practicable, monitored continuously if possible or at least measured at the beginning, at an intermediate time and at the end of the exposure period, and

6.5.3 During the development of the generating system, particle size analysis should be performed to establish the stability of the aerosol particle size generation in the inhalable range. During exposure, analysis should be conducted as often as necessary (at least weekly) to determine the consistency of particle size distribution.

7. Hazards

7.1 In case of potentially explosive test substances, care should be taken to avoid generating explosive concentrations.

7.2 Contact with all test substances, solutions, and mixed diets should be minimized with appropriate protective clothing, gloves, eye protection, etc. The use of fume hoods and increased ventilation in test rooms is necessary when handling volatile substances. Exhaust from fume hoods and chambers must be treated appropriately to prevent the release of the test material into the environment. Information on acute mammalian toxicity and special handling procedures should be known before this test method is used.

7.3 Disposal of excess test substances, solutions, mixed diets, excreta, and treated rats should be done with consideration for health and environmental safety, and in accordance with all federal, state, and local regulations.

7.4 Cleaning and rinsing of glassware, feeders, and other equipment with volatile solvents should be performed only in well ventilated areas. The use of fume hoods may be necessary when handling volatile substances.

7.5 Periodic medical examinations should be considered for all personnel caring for rats or handling test substances.

8. Test Animals

8.1 A variety of rodent species may be used although the rat is the preferred species. This test method is intended for use with young adult male and female rats. The females should be nulliparous and nonpregnant.

8.2 At least 20 rats (10 females and 10 males) should be used for each test group.

8.3 If interim sacrifices are planned, the number of rats should be increased by the number of rats scheduled to be sacrificed (at least 5 male and 5 female/dosage group) before initiation of the study.

8.4 All rats for a given test must come from one source and strain and be of approximately the same age to minimize variability. Test rats may be obtained from commercial sources or reared in laboratory colonies, but they must not have been used in a previous test.

8.5 Rats should be healthy and disease free and those that are deformed, injured, emaciated, or phenotypically different from normal rats must not be used as test subjects. The population of rats from which the test rats are selected shall be considered unsuitable if mortality exceeds 5 % during the acclimation period.

8.6 At the beginning of the study the weight variation of the rats should not exceed ±20 % of the mean weight for each sex. Dosing should begin as soon as possible after weaning and acclimation, ideally before the rats are 6-weeks-old, and in any case, not more than 8-weeks-old.

9. Facilities

9.1 No precise physical requirements concerning animals are set forth. However, the animal facility shall meet the established standards that may be required by law or regulations. It is desirable that the animal facilities meet the guidelines suggested by the Institute of Laboratory Animals Resources or facilities that have been approved by such organizations as the American Association of Accreditation of Laboratory Animal Care (AAALAC).

10. Concentrations Levels

10.1 In subchronic toxicity tests, it is desirable to have a concentration-response relationship as well as a ~~no-observed-toxic-effect level.~~ NOEL. Therefore, at least three concentration levels with a control and, where appropriate, a vehicle control corresponding to the concentration of vehicle at the highest exposure level should be used. Concentrations should be spaced appropriately to produce test groups with a range of toxic effects and mortality rates. The data should be sufficient to produce a concentration response curve.

10.2 Concentration levels may be based upon acute inhalation studies or results of a pilot study.

10.3 The highest concentration should result in toxic effects but not produce an incidence of fatalities that would prevent a meaningful evaluation.

10.4 The lowest concentration should not produce any evidence of toxicity. Where there is suitable estimation of human exposure the lowest concentration should exceed this.

10.5 A satellite group of 20 rats (10 female and 10 male) may be treated with the highest concentration for 90 days and observed for reversibility, persistence, or delayed occurrence of toxic effects for a post-treatment period of appropriate length, normally, not less than 28 days.

11. Procedure

11.1 *Rat Exposure Period*—Weigh test and control rats immediately before starting the test. Compare body weights of test rats to the body weights for control rats to ascertain statistical insignificance within and among groups.

11.1.1 Place rats in the inhalation chambers and expose them to the test substance for 6 h/day, 5 days/week for a period of 90 days.

11.1.2 Withhold food and water during the exposure period.

11.1.3 Examine rats carefully at least once per day. Make additional observations daily with appropriate actions taken to minimize loss of rats to the study. Observations should include, but not be limited to, changes in the skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behavior pattern.

11.1.4 Record signs of toxicity as they are observed including time of onset, the degree and duration.

11.1.5 Weigh and record rat weights weekly. Determine food consumption weekly.

11.2 *Clinical ~~Examinations~~*—~~Make the following clinical examinations on at least five of each sex in each exposure group of rats.~~ Pathology:

11.2.1 *Urinalysis*—Perform urinalysis at the termination of the testing period. Randomly selected rats from each sex and each group may be placed in metabolism cages for urine collection. Evaluate individually each urine sample and include the following measurements: specific gravity, pH, protein, glucose, ketones, bilirubins, urobilinogen, as well as microscopic examination of formed elements.

11.2.2 *Hematology*—Make the following hematology determinations at least once during the test period on all groups of rats including concurrent controls (just prior to the terminal sacrifice at the end of the test period): hematocrit, hemoglobin concentration, erythrocyte count, total and differential leukocyte counts, and a measure of clotting potential such as prothrombin time, thromboplastin time or platelet count, and if signs of anemia are present, reticulocyte count and bone marrow cytology.

11.2.3 ~~*Blood*~~*Clinical Chemistry*—Make clinical biochemical tests that are considered appropriate to all studies, at least once during the test period on all groups of rats including concurrent controls (just prior to the terminal sacrifice at the end of the test period) as follows: 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. Suggested determinations are as follows: calcium, phosphorus, chloride, sodium, potassium, fasting glucose with period of fasting appropriate to the species/breed, serum glutamic-pyruvic transaminase (now known as serum alanine aminotransferase), serum glutamic oxaloacetic transaminase (now known as serum aspartate aminotransferase), ornithine decarboxylase, gamma glutamyl transferase (now known as gamma glutamyl transpeptidase), urea nitrogen, albumen, blood creatinine, total bilirubin, and total serum protein measurements. Other determinations may be necessary for adequate toxicological evaluation include as follows: 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.

11.3 *Ophthalmological Examination*—This examination is not recommended on a routine basis, but only when there is an indication based on expected or observed toxicity.

11.3.1 Examine the eyes of at least five animals of each sex in each group using an opthalmoscope or equivalent suitable equipment. Conduct the examination prior to exposure to the test substance and at the end of the study. If changes in the eyes are detected, examine the eyes of all animals.

11.4 *Necropsy*—At the end of the study period, sacrifice all survivors in the non-satellite treatment groups and subject to a gross necropsy. Remove and sacrifice moribund rats when noticed.

11.4.1 Gross necropsy should include examination of the external surface of the body, all orifices, and the cranial, thoracic, and abdominal cavities, and their contents. Conduct a gross examination for abnormalities; weigh brain, lungs, liver, spleen, heart, kidneys, and testes and record. Before being weighed, carefully dissect and trim organs to remove fat and other tissue in a uniform manner. Weigh organs as soon as possible to avoid drying.

11.4.2 Preserve in a 10 % formalin the following organs and tissues for future histopathological examination as follows: a sample of all tissues containing gross lesions, brain (including sections of medulla/pons, cerebellar cortex, and cerebral cortex), eye, pituitary, thyroid/parathyroid, thymus, lungs and trachea, heart, bone marrow (either femur, sternum or rib at the costochondral junction), salivary glands, liver, spleen, kidneys, adrenals, pancreas, gonads, uterus, accessory genital organs (epididymis, prostate, and if present seminal vesicles), ovaries, aorta, skin, gall bladder (non-rat), esophagus, stomach, duodenum, jejunum, ileum, caecum, colon, rectum, urinary bladder, representative lymph node, and peripheral nerve.

11.4.3 The following tissues need be preserved only if indicated by signs of toxicity or target organ involvement: mammary gland, thigh musculature, femur (including articular surface), spinal cord at three levels (cervical, midthoracic, and lumbar), and exorbital lachrymal glands.

11.5 *Histopathology*—Perform full histopathological examinations on organs and tissues of all rats in the control and high dosage groups and all rats that died or were killed during the study.

11.5.1 Perform histopathological examinations on all gross lesions and on the lungs, liver and kidneys of all rats.

11.5.2 Carry out further histopathology in other dosage groups on organs that show lesions in the high dosage group or for which clinical observations indicate such a need.

12. Quality Assurance

12.1 In order to ensure the quality and reliability of data developed using this test method, good laboratory practices should be followed (see CFR Title 40, Parts 160, 792, and Title 21, Part 58).

13. Interpretation of Results

13.1 Compare statistically test group data (rat weights, organ-to-body weight, and organ-to-brain weight ratios) to the control group. Generally any acceptable statistically method may be used.

13.2 The statistical method should be chosen during the design of the study. Supplementary statistical tests may be performed. The need for and the nature of these tests may be determined only when the analytical results have been subjected to a preliminary examination.

14. Report

14.1 Report the following information:

14.1.1 Name of investigator(s), laboratory, laboratory address, location of raw data, and date of initiation and termination of test,

14.1.2 Name of species tested, including scientific name, source, and age of the rats at the beginning of the test,

14.1.3 Detailed description of the test substance including its chemical name, Chemical Abstract Services (CAS) number, synonyms, structure, formulations, purity, source batch, lot number, physical/chemical properties,

14.1.4 Description of the test facilities and housing conditions, including cages, temperature, humidity and photoperiod,

14.1.5 Name and source of feed, including description and analysis of diet.

14.1.6 Description of exposure apparatus to include the following: design, type, dimensions, source of air, system for generating particulates and aerosols, method of conditioning air, method for housing rats in a test chamber, the equipment for measuring temperature, humidity and particulate aerosol concentrations and size shall be described.

14.2 The following exposure data will be presented in a tabular form with the appropriate statistical evaluations to include mean values, measure of variability and shall include: air flow rates, temperature and humidity inside the chambers, nominal concentration (the total amount of test substance dispersed into the exposure chamber divided by the volume of air), actual analytical concentration in the breathing zone of the exposed rats, geometric mean diameter and geometric standard deviation and shape of the particles.

14.3 Rat data listed by sex and concentration shall include: individual rat body weights, pharmacotoxic signs, mortality, urinalysis, clinical chemistry, hematology, gross necropsy results, organ weights, organ-to-body weight and organ-to-brain weight ratios, histopathological results and statistical evaluation where appropriate.

15. Precision and Bias

15.1 A precision and bias statement cannot be made at this time.

16. Keywords

16.1 aerodynamic diameter; blood chemistry; cumulative toxicity; geometric mean diameter; geometric standard deviation; head-only exposure; hematology; histopathology; inhalable diameter; inhalation; inhalation chamber; median diameter; nasal exposure; necropsy; particle size distribution; pesticide; rat; subchronic; toxicity; urinalysis