Designation: E 1372 – 95 (Reapproved 2003)

Standard Test Method for Conducting a 90-Day Oral Toxicity Study in Rats[1]

This standard is issued under the fixed designation E 1372; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This test method has been designed to examine the subchronic oral toxicity of a test substance during a 90-day period of continuous oral exposure to rats (*Rattus norvegicus*) following initial acute toxicity tests.

1.2 The values stated in SI units are to be regarded as the standard. The values given in parentheses are for information only.

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 Definitions of Terms Relating to Pesticides[2]

E 1163 Test Method for Estimating Acute Oral Toxicity in Rats[2]

2.2 *Federal Standards:*

Title 40, Code of Federal Regulations (CFR), Environmental Protection Agency, Part 798, Health Effects Testing Guidelines, Subpart C, Subchronic Exposure, Oral Toxicity[3]

Title 40, Code of Federal Regulations (CFR), Environmental Protection Agency, Part 798, Health Effects Testing Guidelines, Subpart B, General Toxicity Testing, Acute Oral Toxicity[3]

Title 40, Code of Federal Regulations (CFR), Environmental Protection Agency, Subchapter E, Pesticide Programs; Part 160, Good Laboratory Practice Standards[3]

Title 21, Code of Federal Regulations (CFR), Food and Drug Administration, Part 58, Good Laboratory Practice for Nonclinical Laboratory Studies[3]

Title 40, Code of Federal Regulations (CFR), Toxic Substance Control Act, Part 792, Good Laboratory Practice Standards[3]

3. Terminology (see Definitions E 609)

3.1 *Definitions:*

3.1.1 *concentration*—The weight of the test substance per unit weight of the diet (expressed as mg/kg of diet). The weight of the test substance per volume of H_2O (expressed as mg/ml of H_2O), or at a constant rate in the diet (expressed as ppm).

3.1.2 *dose, dosage*—The quantity of a substance applied per unit treated or applied to or entered into organism. This is expressed as the weight of the test substance per unit weight of test animal (mg/kg).

3.1.3 *feed efficiency*—This value is a measure of the efficiency with which the animals convert food to body weight. The calculation is the total body weight gain per total food consumed.

3.1.4 *gavage*—forced feeding, as by tube that is passed down the throat to the stomach.

3.1.5 LD_{50}—The statistically derived estimate of the dose of a test substance that would be expected to cause 50 % mortality to the test population under the specified test conditions.

3.1.6 *no observed effect dose (NOED)*—The highest tested dose of a substance at which the measured biological variables of a specific group under test conditions show no statistically significant dose-related adverse difference from the control treatment group.

3.1.7 *nulliparous*—Having never borne an offspring.

3.1.8 *subchronic oral toxicity*—The adverse effects occurring as a result of the daily exposure of experimental rats to a test substance by diet, water, capsule or gavage for a 90-day period.

3.1.9 *test substance*—Pesticide or other material (element, chemical compound, formulation, known mixture) administered orally for the purpose of determining subchronic oral toxicity.

4. Summary of Test Method

4.1 Equal numbers of rats (minimum of 80), 40 of each sex, of the same source, age, and strain are randomly divided into at least four test groups (control, low, medium, and high dosage

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

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

[3] Available from U.S. Government Printing Office, Superintendent of Documents, Washington, DC 20402.

groups). If it is determined that an interim sacrifice is necessary, the number of animals per test group shall be increased (at least 5 male and 5 female per dosage group), before the initiation of the study. The highest dosage level shall result in toxicological effects, but not cause more than 20 % fatalities. The lowest dosage level should be one that does not induce any evidence of toxicity. This level should be (if possible) higher than that expected for human exposure. The intermediate dosage should produce a minimal observable effect. If more than one intermediate dose is used, the dose levels should be spaced to produce a gradation of toxic effects. Where appropriate, a vehicle control (the volume of which corresponds to the volume of vehicle at the highest exposure level) should be used. The selection of test substance dosages may be estimated from a preliminary 14-day range finding study (see Section 12).

4.2 Daily observations of all individual animals for signs of toxicity and mortality are recorded.

4.3 A sample of test animals may be sacrificed at 45 days and the remaining animals at 90 days into the study. Urine, hematology, and blood samples are collected for analysis prior to necropsy.

4.4 Data collected from treatment and control groups are compared statistically to detect changes in food or water consumption, body weights, organ-to-body weight and organ-to-brain weight ratios, hematology and clinical blood and urine values. Histopathological examinations are also performed on selected tissues.

5. Significance and Use

5.1 This test method is intended to permit the determination of a no observed effect level and the toxic effects associated with the repeated oral exposure to a substance for a period of 90 days. This subchronic test is not capable of determining toxic effects requiring an extended latency period to develop (for example, carcinogenicity and life shortening). It will provide information on target organs, the possibilities of accumulation, and can be used in selecting dosage levels for chronic studies and for establishing safety criteria for human exposure. It provides information on potential human health hazards likely to arise from repeated exposure over a limited period of time.

6. Hazards

6.1 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. Information on acute mammalian toxicity and special handling procedures should be known before this test method is used.

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

6.3 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.

6.4 Periodic medical examinations should be considered for all personnel caring for animals or handling test substances.

7. Test Animals

7.1 This test method is intended for use with young adult male and female rats. The females should be nulliparous and nonpregnant. The Sprague-Dawley is an example of a strain frequently used. Rodents other than rats may be used with appropriate modifications and justifications.

7.2 At least 20 animals (10 male and 10 females) should be used at each dosage level. An additional group of rats (10 male and 10 female) can be treated with the high dosage level for 90 days and observed for reversibility, persistence, or delayed occurrence of toxic effects for a posttreatment period of appropriate length, normally not less than 28 days. If it is determined that interim sacrifices are necessary, the number of animals per test group shall be increased (at least 5 male and 5 female per dosage group), before the initiation of the study.

7.3 All animals for a given test must come from one source and strain and be of approximately the same age to minimize variability. Test animals may be obtained from commercial sources or reared in laboratory colonies, but they must not have been used in a previous test. Animals should be healthy and disease free and those that are deformed, injured, emaciated, or phenotypically different from normal animals must not be used as test subjects. The population of animals from which the test subjects (treated and control) are selected shall be considered unsuitable for testing if mortality exceeds 5 % during the acclimation period. At the beginning of the study the weight variation of the rats used should not exceed ±20 % of the mean weight for each sex. Dosing should begin as soon as possible after weaning, ideally before the rats are 6-weeks old, and in any case, not more than 8-weeks old.

8. Facilities

8.1 No precise physical requirements concerning the facility are set forth. However, the animal facility shall meet the established standard 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).

TABLE 1 Minimum Space Recommendations for Laboratory Rats[4]

Weight of Rat (g)	Floor area/rat (cm^2)[A]
<100	109.68 (17.0 $in.^2$)
100–200	148.40 (23.0 $in.^2$)
200–300	187.11 (29.0 $in.^2$)
300–400	258.08 (40.0 $in.^2$)
400–500	387.15 (60.0 $in.^2$)
>500	451.64 (70.0 $in.^2$)

[A] Height should be at least 17.8 cm (7 in.).

9. Test Cages

9.1 The test and control rats shall be housed individually in all metal cages designed to hold laboratory rats and should

TABLE 2 Recommended Relative Humidity and Dry-Bulb Temperature for Common Laboratory Rat[3]

Relative Humidity (%)	Dry-Bulb Temperature C°	F°
40–70	18–26	64.4–78.8

provide for proper water and food consumption. Table 1[4] suggests the minimum cage sizes for rats.

9.2 Animal cages should be suspended in racks and have wire-mesh bottoms. This will permit excrement to fall through the floor of the cage. Cages and food containers should be designed to minimize spillage of food and water.

10. Diets

10.1 Diets must be formulated in accordance with the nutrient requirements of the test species. Any unmedicated commercial diet that meets the minimum nutritional standards of the test species is acceptable.

11. Acclimation

11.1 Fourteen days prior to initiation of the test, animals that have been evaluated according to paragraph 7.3 will be randomly placed into special feeding cages and acclimated to the food and water (minus test material), temperature, humidity, and lighting, of the test facility. On the first day of the study (or at least no more than 3 days before the study begins), rats will be weighed and divided into at least four treatment groups (low, medium, high and control) of each sex so that the mean body weight of each group is similar and the group variation in weights are similar. Table 2 describes the recommended environmental temperature and humidity ranges for the common laboratory rat.

11.2 The food consumption during the acclimation period can be used as a maximum indication of how much the animals will eat during the test period. Usually during this period food consumption is at high level. It can also be used to estimate the quantity of food to prepare during the study.

11.3 The photoperiod should be 12 h of light daily.

12. Range Finding Test

12.1 A preliminary 14-day toxicity oral study to calculate an LD_{50} (mg/kg) may be necessary to determine the test dosages to be used during the subchronic study.

12.2 Groups of six male and six female rats between 6 and 8 weeks of age should be used for this portion of the study. All animals should be randomized, numbered, and placed in appropriate cages for a 5-day acclimation period. During this period, all rats will receive rodent diet (Section 10) minus the test compound. Dietary levels of the test chemical to be administered may approximate the acute oral LD_{50} dosages (see CFR, Title 40, Part 798 and Test Method E 1163) (if available) and fractions thereof (such as 2×, 1×, 0.5×, 0.25×, 0.125×, 0.0625×, 0.03125× of the LD_{50}). One additional group of each sex will serve as a solvent or untreated control.

12.3 It is strongly recommended that a dietary dosage group be removed from testing for humane purposes when food consumption is markedly reduced. If consumption, as compared to controls or acclimation period values, or both, is reduced by 90 % or more, continued exposure usually will result in mortality of test animals in that group.

12.4 Base the no-effect and effect levels in the 14-day study on changes in the following parameters: body weight, organ-to-body weight and organ-to-brain weight ratios, hematology (13.4.2), clinical chemistry (13.4.3), gross necropsy, and food or water consumption, or both, if necessary. Histology on a limited selection of organs may be appropriate in some circumstances.

12.5 If a lethal dose is not found, set the highest dietary dosage at 1000 mg/kg (expected human exposure may indicate the need for a higher dose level), since dosage below this value is assumed to be nontoxic.

13. Procedure (see 2.2)

13.1 *Test Substance Administration*— The test substance may be administered in the diet, the drinking water, by capsule or by gavage. If the test substance is administered by gavage, a 5-day/week dosing regimen is acceptable.

13.1.1 Dose all animals by the same method during the entire experimental period.

13.1.2 When necessary the test substance is dissolved or suspended in a suitable vehicle. If a vehicle or diluent is needed, ideally it should not elicit toxic effects itself nor substantially alter the chemical or toxicological properties of the test substance. It is recommended that, when ever possible, the use of an aqueous solution be considered first, followed by consideration of a solution in acetone or next a solution of oil, or then by solution in other vehicles. For nonaqueous vehicles, know the toxic characteristics, and if not known, determine them before the test.

13.1.3 Administer the test substance (if in the diet or the drinking water) ad libitum throughout the study and depending on the stability of the test material replace at least weekly.

13.1.4 Perform the chemical analysis of test mixtures (depending on stability of test material) at least once on each new batch of test food or water prepared.

13.2 *Diet Preparation*—Test material food-mixture concentrations for the first 2 weeks will be calculated using the mean body weights and mean food consumption weights computed during the acclimation period. Thereafter, the mixtures will be prepared from the mean body weights and mean food consumption weights computed from the first week of the previous 2-week period. Test material food-mixture concentrations will be computed for each dosage using the following formula:

$$X = 100\,K/G$$

where:

X = percent of active test material in the diet (g of test material/100 g of ground food).

K = dosage of compound that is desired and is expressed as g of test material/kg of body weight/day.

[4] Institute of Laboratory Animal Resources, National Research Council,“ Guide for the Care and Use of Laboratory Animals,” *DHEW Publication No. (NIH) 85-23*, 1985, pp. 13.

G = amount of food consumed/day over a 1-week period and is expressed as g of food consumed/kg of body weight/day. Food (and water if necessary) consumption will be recorded at least once/week throughout the study.

13.2.1 An alternative to this test method would be to determine the concentration of test material in the feed prior to study initiation and then have it remain constant throughout the study.

13.3 *Observations*—Make observations of each animal at least once each day, with appropriate actions taken to minimize loss of animals to the study (for example, necropsy or refrigeration of animals found dead and isolation or sacrifice of weak or moribund animals).

13.3.1 Record signs of toxicity (/dosage group/sex) as they are observed, including time of onset, degree, and duration. These signs include, but are not limited to, changes in skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous systems, somatomotor activity, and unusual behavioral patterns.

13.3.2 Weigh all animals at least once a week, on the same day of each week, and record weights.

13.3.3 Record temperature and humidity continually throughout the study.

13.3.4 At the end of the 90-day period, sacrifice all surviving animals.

13.4 *Clinical Examinations*—The following clinical examinations should be made on at least five of each sex in each group of rats.

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

13.4.2 *Hematology*—Make the following hematology determinations at least twice during the test period on all groups of animals including concurrent controls (at 45 days into the study and just prior to the terminal sacrifice at the end of the test period) as follows: 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 reticulocyte count, if signs of anemia are present.

13.4.3 *Blood Chemistry*—Make clinical biochemical tests that are considered appropriate to all studies, at least twice during the test period on all groups of animals including concurrent controls (at 45 days into the study and 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.

13.5 *Necropsy*—Perform a necropsy on all mortalities and any group withdrawn from the study. At the termination of the test all surviving animals shall be sacrificed by accepted humane methods and subjected to a full gross necropsy. This includes examination of the external surface of the body, all orifices, and the cranial, thoracic, and abdominal cavities, and their contents.

13.5.1 Preserve the following organs and tissues, or representative samples in a suitable medium 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), 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.

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

13.5.3 In addition, weigh the following organs: liver, kidneys, adrenals, gonads, and brain. Prior to being weighed, carefully dissect organs and trim to remove fat and other tissue in a uniform manner. Weigh organs as soon as possible to avoid loss of weight due to drying.

13.6 *Histopathology*:

13.6.1 Perform full histopathological examinations on organs and tissues of all animals in the control and high dosage groups and all animals that died or were killed during the study.

13.6.2 Perform histopathological examinations on all gross lesions and on the lungs, liver and kidneys of all animals.

13.6.3 If an interim sacrifice group is used, perform histopathological examination on all tissues and organs showing effects in the other treated groups.

13.6.4 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.

14. Quality Assurance

14.1 In order to ensure the quality and reliability of data developed using this test method, good laboratory practices should be followed (see 2.2).

15. Interpretation of Results

15.1 Test group data (animal weights, organ-to-body weight, and organ-to-brain weight ratios or appropriate alternative means of correlation), food consumption, (water consumption if necessary), feed efficiency, hematology, and clinical chemistry and urinalysis for any given period will be statistically compared with the control group for the same period. Generally any acceptable statistical method may be used.

15.2 Choose the statistical method during the design of the study. Supplementary statistical tests may be performed. The need for and the nature of these supplementary statistical tests may be necessary based upon initial statistical analysis of data.

16. Report

16.1 Report the following information:

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

16.1.2 Name of species and strain of animal tested, including scientific name, source, and age of the animals at the beginning of the test,

16.1.3 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 and name of solvent or carrier, if used,

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

16.1.5 Name and source of feed, including description and analysis of diet,

16.1.6 The concentration of test material in food or water; predicted and calculated doses for each test group when tested material is mixed with food or water,

16.1.7 Number of animals (male and female) per dosage group, body weights, food consumption (water consumption if necessary), signs of toxicity (numbers affected and dose by sex and dosage group), abnormal behavior, urinalysis and hematology values, percent mortality (by sex and dosage group),

16.1.8 Anything unusual about the test, any deviations from the protocol, and other relevant information,

16.1.9 Statistical methods employed,

16.1.10 Significant necropsy findings, organ weights (liver, kidneys, adrenals, gonads, and brain), organ-to-body weight and organ-to-brain weight ratios (or appropriate alternative means of correlation).

17. Precision and Bias

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

18. Keywords

18.1 blood chemistry; cumulative toxicity; feed efficiency; gavage; hematology; histopathology; LD_{50}; necropsy; no observed effect dose (NOED); oral; pesticide; rat; subchronic; toxicity; urinalysis