



"Repeated Dose Inhalation Toxicity: 28-day or 14-day Study"

1. INTRODUCTORY INFORMATION

• Prerequisites

- Gas, volatile material or aerosol/particulate test substance
- Chemical identification of test substance
- Purity (impurities) of test substance
- Liquid: vapour pressure, boiling point
- Aerosol/particulate: particle size, shape and density distribution
- Flash point
- Explosivity

• Standard documents

There are no relevant international standards.

2. METHOD

A. INTRODUCTION, PURPOSE, SCOPE, RELEVANCE, APPLICATION AND LIMITS OF TEST

In the assessment and evaluation of the toxic characteristics of an inhalable material, such as a gas, volatile substance or aerosol/particulate, determination of inhalation toxicity using repeated exposures may be carried out after initial information on toxicity has been obtained by acute testing. It provides information on health hazards likely to arise from repeated exposure by the inhalation route over a limited period of time. Hazards of inhaled substances are influenced by the inherent toxicity and by physical factors such as volatility and particulate size.

There is sufficient similarity between the considerations involved in the conduct of a 28-day or 14-day repeated dose inhalation study to allow one Guideline to cover both test durations. The main differences lie in the time over which dosing takes place (indicated in the text) and in the extent of clinical and pathological investigations which might be considered appropriate for the shorter test duration.

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- Principle of the test method

Several groups of experimental animals are exposed daily for a defined period to the test substance in graduated concentrations, one concentration being used per group, for a period of 28 days or 14 days. Where a vehicle is used to help generate an appropriate concentration of the test substance in the atmosphere, a vehicle control group should be used. During the period of administration the animals are observed daily to detect signs of toxicity. Animals which die during the test are necropsied, and at the conclusion of the test surviving animals are sacrificed and necropsied.

B. DESCRIPTION OF THE TEST PROCEDURE

- Preparations

Healthy young adult animals are acclimatised to the laboratory conditions for at least 5 days prior to the test. Before the test, animals are randomised and assigned to the required number of groups. Where necessary, a suitable vehicle may be added to the test substance to help generate an appropriate concentration of the substance in the atmosphere. If a vehicle is used it should be shown not to influence absorption of the test substance or produce toxic effects.

- Experimental animals

Selection of species

A variety of test species may be used. This Guideline is intended primarily for use with rodents. Where a rodent is required the preferred species is the rat. Commonly used laboratory strains of young healthy animals should be employed. At the commencement of the study the weight variation of animals used should not exceed ± 20 per cent of the mean weight. Where a repeated dose inhalation study is conducted as a preliminary to a long-term study, the same species and strain should be used in both studies.

Number and sex

At least 10 animals (5 female and 5 male) should be used for each test group. The females should be nulliparous and non-pregnant. If interim sacrifices are planned the number should be increased by the number of animals scheduled to be sacrificed before the completion

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of the study. In addition, a satellite group of 10 animals (5 animals per sex) may be treated with the high concentration level for 28 days or 14 days and observed for reversibility, or persistence, or delayed occurrence of toxic effects for 14 days post-treatment.

Housing and feeding conditions (before and after exposure)

The temperature in the experimental animal room should be 22°C ($\pm 3^\circ$) and the relative humidity 30-70 per cent. When the lighting is artificial the sequence should be 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. Animals may be caged in groups by sex or individually; the number of animals per cage should not interfere with clear observation of each animal.

- E q u i p m e n t

The animals should be tested in inhalation equipment designed to sustain a dynamic air flow of 12 to 15 air changes per hour and ensure an adequate oxygen content of 19 per cent and an evenly distributed exposure atmosphere. Maintenance of a slight negative pressure inside the chamber will prevent leakage of the test substance into the surrounding area. Where a chamber is used its design should minimise crowding of the test animals and maximise their exposure to the test substance. As a general rule to ensure stability of a chamber atmosphere, the total "volume" of the test animals should not exceed 5 per cent of the volume of the test chamber. Oro-nasal or head-only exposure may be used if it is desirable to avoid concurrent exposure by the oral and dermal routes.

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.

- T e s t c o n d i t i o n s

Exposure concentrations

At least three concentrations, with a control and, where appropriate, a vehicle control (corresponding to the concentration of vehicle at the highest exposure level) should be used. Except for exposure to the test substance, animals in the control group should be handled in an

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identical manner to the test group animals. The highest concentration should result in toxic effects but not produce an incidence of fatalities which would prevent a meaningful evaluation. The lowest concentration should not produce any evidence of toxicity. Where there is a usable estimation of human exposure, the lowest concentration should exceed this. Ideally, the intermediate concentration(s) should produce minimal observable toxic effects. If more than one intermediate concentration is used the concentrations should be spaced to produce a gradation of toxic effects. In the low and intermediate groups and in the controls the incidence of fatalities should be low in order to permit a meaningful evaluation of the results.

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

Exposure time

The duration of daily exposure should be 6 hours after equilibration of the chamber concentrations. Other durations may be used to meet specific requirements.

Observations

A careful clinical examination should be made at least once each day. Additional observations should be made daily with appropriate actions taken to minimise 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.

• Procedure

The animals are exposed to the test substance ideally on a 7-day per week basis for a period of 28 or 14 days. However, based primarily on practical considerations, exposure on a 5-day per week basis is considered to be acceptable. Animals in a satellite group scheduled for follow-up observations should be kept for a further 14 days without treatment to detect recovery from, or persistence of, toxic effects. 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 30 and 70 per cent, but in certain instances (e.g. tests of aerosols) this may not be practicable. Food and water should be withheld during exposure.

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- Physical measurements

Measurements of monitoring should be made of the following:

- (a) The rate of air flow, preferably, should be monitored continuously.
- (b) During the exposure period, the actual concentrations of the test substance should be held as constant as practicable.
- (c) 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.
- (d) Temperature and humidity (preferably continuously).

- Clinical examinations

Animals should be observed during and following exposure. Observations should be made and recorded systematically; individual records should be maintained for each animal. All the animals should be observed daily and signs of toxicity recorded including the time of onset, the degree and duration. Cage-side observations should include, but not be limited to, changes in the skin and fur, eyes, mucous membranes and also respiratory, circulatory, autonomic and central nervous system and somatomotor activity and behaviour pattern. Measurements should be made of food consumption weekly and the animals weighed weekly. Regular observation of the animals is necessary to ensure that animals are not lost from the study due to causes such as cannibalism, autolysis of tissues or misplacement. At the end of the study period all survivors in the non-satellite treatment groups are sacrificed. Moribund animals should be removed and sacrificed when noticed.

The following examinations should be made:

- (a) Haematology, including haematocrit, haemoglobin concentration, erythrocyte count, total and differential leucocyte count, and a measure of clotting potential, such as clotting time, prothrombin time, thromboplastin time, or platelet count should be investigated at the end of the test period.

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- (b) Clinical biochemistry determinations in blood should be carried out at the end of the study. The selection of specific tests will be influenced by observations on the mode of action of the substance. Suggested determinations are calcium, phosphorus, chloride, sodium, potassium, fasting glucose (with period of fasting appropriate to the species), serum glutamic-pyruvic transaminase*, serum glutamic-oxaloacetic transaminase**, ornithine decarboxylase, gamma glutamyl transpeptidase, urea nitrogen, albumen, blood creatinine, 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, methaemoglobin, cholinesterase activity, etc. Additional clinical biochemistry may be employed where necessary to extend the investigation of observed toxic effects.
- (c) Urinalysis is not required on a routine basis but only when there is an indication based on expected or observed toxicity.

If historical baseline data are inadequate, determination of haematological and clinical biochemistry parameters before dosing commences should be considered.

- P a t h o l o g y

- *Gross necropsy*

All animals in the study should be subjected to a full gross necropsy which includes examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents. The liver, kidneys, adrenals and testes should be weighed wet as soon as possible after dissection to avoid drying. The following organs and tissues should be preserved in a suitable medium for possible future histopathological examination: lungs - which should be removed intact, weighed and treated with a suitable fixative to ensure that lung structure is maintained (perfusion with the fixative is considered to be an effective procedure), liver, kidney, spleen, adrenals, heart and any target organs, that is, those showing gross lesions or changes in size.

* Now known as serum alanine aminotransferase.

** Now known as serum aspartate aminotransferase.

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Histopathology

Histological examination should be performed on the preserved organs and tissues of the high concentration group and the control group(s). These examinations may be extended to animals of other concentration groups, if considered necessary to investigate the changes observed in the high concentration group. Animals in a satellite group should be examined histologically with particular emphasis on those organs and tissues identified as showing effects in the other treated groups.

3. DATA AND REPORTING

- Treatment of results

Data may be summarised 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 type of lesions and the percentage of animals displaying each type of lesion.

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

- Evaluation of the results

The findings of a repeated dose inhalation study should be considered in terms of the toxic effects and the necropsy and histopathological findings. The evaluation will include the relationship between the concentration of the test substance and the duration of exposure, and the presence or absence, the incidence and severity, of abnormalities, including behavioural and clinical abnormalities, gross lesions, identified target organs, body weight changes, effects on mortality and any other general or specific toxic effects. A properly conducted 28-day or 14-day study should provide information on the effects of repeated inhalation exposure and can indicate the need for further longer term studies. It can also provide information on the selection of concentrations for longer term studies.

- Test report

The test report should include the following information:

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(a) Test conditions

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 animals in a test chamber when this is used.

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:

- airflow rates through the inhalation equipment;
- temperature and humidity of air;
- nominal concentrations (total amount of test substance fed into the inhalation equipment divided by the volume of air);
- actual concentrations in test breathing zone; and
- particle size distribution (e.g. median aerodynamic diameter of particles with standard deviation from the mean).

(c) Animal data

- species/strain used;
- toxic response data by sex and concentration;
- time of death during the study or whether animals survived to termination;
- toxic or other effects;
- the time of observation of each abnormal sign and its subsequent course;
- food and body weight data;
- haematological tests employed and results with relevant baseline data;
- clinical biochemistry tests employed and results with relevant baseline data;
- necropsy findings;

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- a detailed description of all histopathological findings; and
- statistical treatment of results where appropriate.
- I n t e r p r e t a t i o n o f r e s u l t s

A repeated dose inhalation study will provide information on the effects of repeated inhalation exposure to a substance. Extrapolation from the results of the study to man is valid to a limited degree, but it can provide useful information on the toxicity and mode of action of the substance by the inhalation route.

4. L I T E R A T U R E

1. WHO Publication: Environmental Health Criteria No. 6. *Principles and Methods for Evaluating the Toxicity of Chemicals*. Part I. Geneva, 1978.
2. United States National Academy of Sciences, Committee for the Revision of NAS Publication 1138, *Principles and Procedures for Evaluating the Toxicity of Household Substances*, Washington, 1977.