



"Acute Inhalation Toxicity"

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 acute inhalation toxicity is an initial step. It provides information on health hazards likely to arise from short-term exposure by the inhalation route. Data from an acute study may serve as a basis for classification and labelling. It is an initial step in establishing a dosage regimen in subchronic and other studies and may provide additional information on the mode of toxic action of a substance.

- Definitions

Acute inhalation toxicity is the total of adverse effects caused by a substance following a single uninterrupted exposure by inhalation over a short period of time (24 hours or less) to a substance capable of being inhaled.

The LC50 (median lethal concentration) is a statistically derived concentration of a substance that can be expected to cause death during exposure or within a fixed time after exposure in 50 per cent of animals exposed for a specified time. The LC50 value is expressed as weight of test substance per standard volume of air (mg/l), or as parts per million (ppm).

- Principle of the test method

Several groups of experimental animals are exposed for a defined period to the test substance in graduated concentrations, one concentration being used per group. Where a vehicle is used to help generate an appropriate concentration of the substance in the atmosphere a vehicle control group should be used. Subsequently, observations of effects and deaths are made. Animals which die during the test are necropsied, and at the conclusion of the test surviving animals are sacrificed and necropsied as necessary.

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 test substance in the atmosphere.

- Experimental animals

Selection of species

Although several mammalian test species may be used, the preferred species is the rat. Commonly used laboratory strains should be used. The weight variation in animals or between groups used in a test should not exceed ± 20 per cent of the mean weight.

Number and sex

At least 10 animals (5 female and 5 male) at each concentration level. The females should be nulliparous and non-pregnant.

Housing and feeding conditions (before and after exposure)

The temperature of the animal room should be 22°C ($\pm 3^\circ$) and the relative humidity 30-70 per cent. Where the lighting is artificial, the sequence should be 12 hours light, 12 hours dark.

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For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. The animals may be group-caged by sex, but the number of animals per cage should not interfere with clear observation of each animal.

- Equipment

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 per cent and an evenly distributed exposure atmosphere. 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. Alternatively, oro-nasal, head only, or whole body individual chamber exposure may be used.

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.

- Test conditions

Exposure concentrations

These should be sufficient in number, at least three, and spaced appropriately to produce test groups with a range of toxic effects and mortality rates to produce a concentration mortality curve and permit an acceptable determination of an LC50. In the case of potentially explosive test substances, care should be taken to avoid generating explosive concentrations. To establish suitable exposure concentrations, a trial test is recommended.

Limit test

If a test at an exposure concentration of 5 mg/l (actual concentration of respirable substances) for 4 hours or, where this is not possible due to physical or chemical properties of

the test substance, the maximum attainable concentration, using the procedures described for this study, produces no compound-related mortality, then a full study using three dose levels may not be necessary.

An inhalation hazard test (see Annex) may be considered for volatile materials.

Exposure time

The duration of exposure should be at least 4 hours after equilibration of the chamber concentrations. Other durations may be needed to meet specific requirements.

Observation period

The observation period should be at least 14 days. However, the duration of observation should not be fixed rigidly. It should be determined by the toxic reactions, rate of onset and length of recovery period, and may thus be extended when considered necessary. The time at which signs of toxicity appear and the time of death are important, especially if there is a tendency for deaths to be delayed.

• P r o c e d u r e

Shortly before exposure, the animals are weighed and then exposed to the test concentration in the designated apparatus for 4 hours. The temperature at which the test is performed should be maintained at 22° C (\pm 2°). Ideally, the relative humidity should be maintained between 30 and 70 per cent, but in certain instances (e.g. tests of aerosols) even this may not be practicable. Food should be withheld during exposure. Water may also be withheld in certain cases.

• P h y s i c a l m e a s u r e m e n t s

Measurements or monitoring should be made of the following:

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

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- (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 made as often as necessary to determine the consistency of particle size distribution.
- (d) Temperature and humidity (preferably continuously).

- Clinical examinations

During and following exposure, observations are made and recorded systematically; individual records should be maintained for each animal. 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. Cageside observations should include, but not be limited to, changes in the skin and fur, eyes, mucous membranes, respiratory, circulatory, autonomic and central nervous system, and somatomotor activity and behaviour pattern. Particular attention should be directed to observation of tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma. The time of death should be recorded as precisely as possible. Individual weights of animals should be determined weekly after exposure, and at death. Changes in weight should be calculated and recorded when survival exceeds one day. At the end of the test the surviving animals are weighed and sacrificed.

- Pathology

Consideration should be given to performing a gross necropsy of animals where indicated by the nature of the toxic effects observed with particular reference to any changes in the respiratory tract. Where there are significant signs of toxicity indicating the possible involvement of other organs, these should be examined and all gross pathological changes recorded. Microscopic examination of target organs should be considered since it may yield useful information.

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, time of death of individual animals at different exposure levels, number of animals displaying other signs of toxicity, description of toxic effects and necropsy findings.

The LC50 may be determined by any accepted method, e.g. Bliss (5), Litchfield and Wilcoxon (4), Finney (6), Weil (7), Thompson (8), Miller and Tainter (9).

- **Evaluation of results**

The LC50 value should be considered in conjunction with the observed toxic effects and the necropsy findings. The LC50 value is a relatively coarse measurement, useful only as a reference value for classification and labelling purposes and an expression of lethal potential of the test substance following inhalation. Reference should always be made to the experimental animal species in which the LC50 value was obtained. An evaluation should include the relationship, if any, between the animals' exposure to the test substance and the incidence and severity of all abnormalities, including behavioural and clinical abnormalities, gross lesions, body weight changes, mortality and other toxic effects.

- **Test report**

The test report should include the following information:

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 the animals in a test chamber when this is used.

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

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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 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).

Animal data:

- species/strain used;
- tabulation of response data by sex and exposure level (i.e. number of animals dying, number of animals showing signs of toxicity, number of animals exposed);
- time of death during or following exposure;
- LC50 for each sex determined at the end of the observation period (with method of calculation specified);
- 95 per cent confidence interval for the LC50;
- dose-mortality curve and slope (where permitted by the method of determination); and
- necropsy and histopathological findings including a record of lesions and abnormalities observed.

• Interpretation of the results

Determination of an LC50 provides an estimate of the relative toxicity of a substance by the inhalation route. Extrapolation of the results of LC50 and acute toxicity studies in animals to man is valid only to a very limited degree.

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

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2. VCI Draft: Prüfanforderungen zur 6. Änderung der EG, Richtlinie für gefährliche Stoffe. November 1978.
3. National Academy of Sciences, Committee for the Revision of NAS Publication 1138, *Principles and Procedures for Evaluating the Toxicity of Household Substances*, Washington DC, 1977.
4. Litchfield, J.T. and F. Wilcoxon, *J. Pharmacol. Exp. Ther.*, 96, 99-113, 1949.
5. Bliss, C.U., *Quart. J. Pharm. Pharmacol.*, II, 192-216, 1938.
6. Finney, D.G., *Probit Analysis* (3rd Ed.) London, Cambridge University Press, 1971.
7. Weil, C.S., *Biometrics*, 8, 249-263, 1952.
8. Thompson, W., *Bact. Rev.*, 11: 115-141, 1947.
9. Miller, L.C. and Tainter, M.L., *Proc. Soc. Exp. Biol. Med. NY*, 57: 261-264, 1944.

5. A N N E X**I N H A L A T I O N H A Z A R D T E S T****1. I N T R O D U C T O R Y I N F O R M A T I O N****• P r e r e q u i s i t e s**

- Volatile liquid or sublimable solid test substance
- Chemical identification of test substance
- Purity (impurities) of test substance
- Vapour pressure
- Boiling point
- Flash point
- Explosivity

• S t a n d a r d d o c u m e n t s

There are no relevant international standards.

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2. M E T H O D

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

The risk in handling volatile substances is dependent not only on the toxicity but also on the volatility (e.g. boiling point in the case of liquids). For a risk assessment, toxicity based on an LC50 value may not be sufficient on its own, but should always be considered together with the volatility of the substance. Thus, an equivalent inhalation hazard may exist for highly volatile substances of low toxicity as well as for slightly volatile substances of high toxicity.

Compared with a full acute study and determination of an LC50, this test is simpler. Due to the very long period of exposure (7 hours) and the near maximum concentration of the substance in the respired air, it provides a large safety margin for the assessment of hazard provided the animals exposed show no effects. However, if deaths of test animals occur, further studies on inhalation toxicity need to be considered.

When deciding to carry out an inhalation hazard test, the possible conditions of human exposure should be taken into account, as only hazards due to volatile substances can be examined. The inhalation hazard test may be used for materials which volatilise under conditions of human exposure.

The inhalation hazard test is regarded as an empirical procedure, and its results are dependent on the details of the experimental design. This applies especially to the flow of air, the amount of test material in the generation flask, and the size of the exposure chamber.

• D e f i n i t i o n s

Acute inhalation toxicity is the total of adverse effects caused by a substance following a single uninterrupted exposure by inhalation over a short period of time (24 hours or less) to a substance capable of being inhaled.

The LC50 (median lethal concentration) is a statistically derived concentration of a substance that can be expected to cause death during exposure or within a fixed time after exposure in 50 per cent of animals exposed for a specified time. The LC50 value is expressed as weight of test substance per standard volume of air (mg/l), or as parts per million (ppm).

- Principles of the test method

Air is passed through the test substance and is thus enriched by volatilisation or sublimation depending on the vapour pressure or other properties of the test substance. The concentration of the test substance in the mixture is measured. Animals are exposed to this mixture by inhalation for a defined period of time. Subsequently, the animals are observed for 14 days. The period over which test animals can inhale the mixture without death occurring during exposure (or subsequently) is relevant for the evaluation.

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 exposure groups. Food and water are not withheld before exposure.

- Experimental animals

Selection of species

A variety of test species can be used. For comparison with data obtained in other acute tests, the rat is the preferred species. Commonly used laboratory strains of healthy young adult rats should be employed. The weight range within the test animal population should not exceed ± 20 per cent of the mean weight.

Number and sex

At least 10 animals (5 females and 5 males) should be used for each exposure time. The females should be nulliparous and non-pregnant.

Housing and feeding conditions

The temperature of the experimental animal room should be $22^{\circ}\text{C} (\pm 3^{\circ})$ and the relative humidity 30-70 per cent. Where 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.

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- E q u i p m e n t

A whole-body exposure system is used. The volume of the chamber must be defined; it should not exceed 20 litres.

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

Exposure concentration and time

This will be the maximum attainable under the conditions of the test.

The initial and maximum period of exposure is 7 hours. If deaths occur either during the exposure or observation period, the test should be repeated over decreasing periods of exposure (e.g. 1 hour, 10 minutes) until no more deaths of test animals occur during the exposure or observation period.

Observations

The observation period should be at least 14 days. However, the duration of observation should not be fixed rigidly. It should be determined by toxic reactions, rate of onset and length of recovery period, and may thus be extended when considered necessary. The time at which signs of toxicity appear and disappear and the time of death are important, especially if there is a tendency for deaths to be delayed.

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.

- P r o c e d u r e

Withdrawal of food prior to exposure of animals is not necessary. Shortly before exposure the animals are weighed and are then exposed to the substance/air mixture for a predetermined time. During exposure food and water are withheld. Cage-side observations should include changes in the skin and fur, eyes, mucous membranes, and also respiratory, circulatory, autonomic and central nervous system, circulatory, autonomic and central nervous system, and somatomotor activity and behaviour pattern. Particular attention should be paid to the observation of tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma.

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Signs of narcosis and of irritant effects on the respiratory tract and mucous membranes are significant, especially during the exposure period. The time of death should be recorded as precisely as possible. Weighing of the animals should be carried out at weekly intervals. At the end of the test the animals are weighed and then sacrificed.

The concentration of the test substance in the air mixture should be known.

The test animals inhale an atmosphere enriched with the test substance at 20°C. For this purpose a volume of 600 litres of air per hour is passed through a fritted glass D₁ (pore size 90-150 µm) into a glass bottle containing the test substance to a depth of 50 mm above the fritted glass, corresponding to a volume of approximately 120 ml. The diameter of the fritted glass is chosen so that the air will pass through the entire substance as uniformly as possible. The glass bottle is kept in a thermostat-controlled water bath at 20°C ± 1°.

The test may also be performed using smaller amounts of test substance and air, but the same relative proportions should be maintained (e.g. 200 litres of air per hour and approximately 40 ml of test substance). The frit diameter should be selected so that depth of test substance remains at 50 mm.

The mixture of air and test substance is passed into the exposure system. After the first 30 minutes of exposure, the glass bottle should be replaced by one filled with fresh test substance, and this bottle is used for the remaining test period.

If a highly volatile substance is used up in less than 30 minutes, fresh substance should be added frequently enough to ensure that at least during the first hour of exposure a consistent mixture of test substance and air is produced.

- Pathology

Consideration should be given to a gross necropsy of animals that die during the test and of those sacrificed at the termination of the test. Where necropsy is performed, gross pathological changes of the intestinal tract and the major organs, such as liver, kidney, heart, brain and spleen should be recorded. The respiratory tract should be examined carefully. Microscopic examination of liver, kidney, the respiratory tract and organs showing evidence of gross pathology in animals surviving 12 or more hours should be considered because it may give useful information.

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3. D A T A A N D R E P O R T I N G

- T r e a t m e n t o f r e s u l t s

The following results are reported:

- species/strain used;
- concentration of test substance;
- time of death of individual animals related to different exposure times;
- number of animals displaying other signs of toxicity;
- observation of toxic effects; and
- pathology findings.

The number of surviving animals of each exposure group is compared with that of dead animals in terms of exposure time.

- E v a l u a t i o n o f r e s u l t s

The deaths recorded should be considered together with any observed toxic effects, including weight changes and any necropsy findings.

The inhalation hazard test is regarded as an investigation which may provide an indication of a possible hazard in handling a substance under normal conditions of exposure. It does not characterise the inhalation toxicity of a substance. If the inhalation hazard test produces deaths or severe symptoms, further investigations of inhalation toxicity, including the determination of the LC50, should be considered. Since the inhalation hazard test uses long periods of exposure and near maximum concentrations of test substance in air, the likely kind of human exposure must be taken into account in any hazard assessment.

- T e s t r e p o r t

The test report should include the following information:

- tabulation of response data by sex and duration concentration of exposure;
- time of death during or after exposure;
- number of dead animals per number of animals exposed for each exposure time; and
- necropsy findings.

- Interpretation of the results

The results only indicate the inhalation hazard for an exposure to vapours. If a seven-hour exposure does not produce any deaths, it may be assumed that there is no likely inhalation hazard under normal conditions of exposure. If deaths occur during or after a seven-hour exposure, but not with a one-hour exposure, there is an indication of a possible inhalation hazard. If deaths occur during or after a one-hour exposure, but not with a ten-minute exposure, there is an inhalation hazard. If deaths are observed with a ten-minute exposure, there is a severe inhalation hazard. Where there is any evidence of hazard a full acute inhalation toxicity study with determination of an LC50 should be carried out (see Test Guideline 403).

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

1. H.F. Smyth, C.P. Carpenter and C.S. Weil, *Am. Ind. Hyg. Assoc. J.*, 23, 95, 1962.