参考資料4

412

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OECD GUIDELINE FOR TESTING OF CHEMICALS

"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.

- 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

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 (± 3°) and the relative humidity 30-70 per cent. When the lighting is artifical 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.

- Equipment

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.

- Test conditions

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

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 morethan 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 (± 2°). 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.

(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.

- Pathology

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.

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* Now known as serum alanine aminotransferase.

** Now known as serum aspartate aminotransferase.

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:

(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;

– a detailed description of all histopathological findings; and

– statistical treatment of results where appropriate.

• Interpretation of results

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. LITERATURE

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.

OECD GUIDELINE FOR TESTING OF CHEMICALS

DRAFT PROPOSAL FOR A REVISED GUIDELINE: 412

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

INTRODUCTION

1. OECD Guidelines for the Testing of Chemicals are periodically reviewed in the light of scientific progress, changing assessment practices and animal welfare considerations. The original repeated dose inhalation Test Guideline 412 was adopted in 1981. Development of a revised TG 412 with more thorough histopathological examinations of the respiratory tracts including the draining lymph nodes was considered appropriate, in addition to an extended histopathological evaluation of other target organs. Recommendations to perform functional tests have also been added to the Test Guideline in accordance with the revised Test Guideline 407 on Repeated Dose 28-day Oral Toxicity Study in Rodents (1).

2. In the assessment and evaluation of the toxic characteristics of an inhalable material, such as a gas, dust, mist, vapor, volatile substance or aerosol or particulate suspensions, 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.

3. 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 Test Guideline to cover both test durations. The main differences lie in the time over which dosing takes place and in the extent of clinical and pathological investigations which might be considered appropriate for the shorter test duration.

4. This guideline provides information both for hazard assessment and for hazard classification purposes. It provides information on hazardous properties and allows the substance to be ranked and classified according to the United Nations (UN) Globally Harmonized System of Classification and Labeling of Chemicals (GHS) for the classification of chemicals which cause acute toxicity (2). Test substances that are known to cause marked pain and distress due to corrosive[1] or severely irritant actions does not need to be tested, since these substances will be classified as GHS Category I by default. Moribund animals or animals obviously in pain or distress, or showing evident signs of toxicity shall be humanely killed, and are considered in the interpretation of the test results in the same way as animals that died on test. Criteria for making the decision to humanely kill moribund or severely suffering animals, and guidance on the recognition of predictable or impending death, are the subject of a separate Guidance Document (6).

[1] Determined using a validated test method (*e.g.,* TG 430, 431 or 435 (3, 4, 5)) or an accepted prediction.

5. Definitions used in the context of this Guideline are set out in Annex 1.

INITIAL CONSIDERATIONS

6. All available information on the test substance should be considered by the testing laboratory prior to conducting the study. Such information will include the identity and chemical structure of the substance; its physico-chemical properties; the results of any other *in vitro* or *in vivo* toxicity tests on the substance; available (Q)SAR data and toxicological data on structurally related substances; the anticipated use(s) of the substance and the potential for human exposure. This information will assist in the selection of an appropriate starting concentration. If there are no suitable data available, a range finding study may be performed to aid the determination of the doses to be used.

PRINCIPLE OF THE TEST

7. Groups of animals are exposed daily for a defined period to the test substance using graduated concentrations for vapors, dusts/mists or gases, 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. A 28-day or 14-day study provides 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.

DESCRIPTION OF THE METHOD

Selection of animal species

8. The preferred rodent species is the rat, although on occasion other rodent species may be used. Justification should be provided for the use of other rodent or non-rodent species. Healthy young adult animals of commonly used laboratory strains should be used. Females should be nulliparous and non-pregnant. Each animal, at the commencement of testing, should be between 8 and 12 weeks old and its weight should fall within an interval of ±20% of the average body weight of any previously dosed animal recorded at the laboratory for the particular strain used. 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

9. At least ten animals (five female and five male) should be used for each test group. The females should be nulliparous and non-pregnant. In cases of interim sacrifices, justifications should be provided and the planned number of animals should be increased by the number of animals scheduled to be sacrificed before the completion of the study. In addition, a satellite group of ten animals (five 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. In respect of animal welfare, justifications should be given for the use of a satellite group.

Housing and feeding conditions (before and after exposure)

10. The temperature in the experimental animal room where the animals are housed before and after exposure should be 22±3C°. Although the relative humidity should be at least 30% and preferably not exceed 70% other than during room cleaning, the aim should be 50-60%. Lighting should be artificial, the

sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. Before and after exposure, animals may be caged in groups by sex and concentration, but the number of animals per cage should not interfere with clear observation of each animal.

11. During the exposures, the animals are housed individually without access to diet and drinking water.

Preparation of animals

12. The animals are acclimatised to the laboratory conditions for at least five days prior to the start of the exposure. Animals are randomly selected for use in the study and marked to provide individual identification. Animals should also be acclimatized to the test apparatus for a short period prior to testing, as this will reduce the stress caused by introduction to a foreign environment.

Mode of exposures

13. Head/nose-only and whole-body exposure are the techniques used. The preferred mode of exposure is the nose-only or head-only exposure technique. These types of exposure minimize exposure or uptake by non-inhalation routes. Additionally, it allows testing of high concentrations without the need for large quantities of test material. Also, instability of the test compounds (*e.g.,* reactivity with excreta or humidity) and the possible heterogeneity of the test atmosphere in inhalation chambers is of less concern when head/nose-only inhalation chambers are used. The duration required to attain the inhalation chamber equilibration is considerably shorter in head/nose-only chambers compared to whole-body exposures.

14. Head/nose-only exposure requires restraining of the animals and the restraining tubes should be constructed in such a way to avoid hyperthermic effects on the animals. Urine and faeces should escape from the restrainer during the course of exposure. To provide optimal exposure of animals, a slight positive balance of air volumes supplied and extracted should be obtained. The design of the restraining tube, as well as the flow dynamics, should make it impossible for the animal to avoid inhalation exposure. However, if a negative balance of air volumes supplied and extracted cannot be avoided, a dilution of test atmosphere by bias-airflow (via exposure tubes) should be prevented. The inhalation chambers should be operated in well ventilated chemical hoods. Maintenance of slight negative pressure inside the hood will prevent leakage of the test substance into the surrounding areas. The animals should be tested with inhalation equipment designed to sustain a dynamic airflow which exceeds at least two times the respiration ventilation volume of all animals in the inhalation device. (For the young adult rat the approximate respiratory minute ventilation volume is roughly 1 L/kg/min). An adequate oxygen content of at least 19% but not higher than 24% and identical exposure conditions at each exposure port should be ensured. During sampling of test atmosphere, a significant disturbance of the airflow dynamics should be avoided. The rate of sampling airflow should be adjusted to ensure that conditions throughout the equipment are essentially the same.

15. In case of whole-body exposure, the animals should be tested using inhalation equipment designed to sustain a dynamic air flow of approximately 12 to 15 air changes/hour and ensure an adequate oxygen content of at least 19%, but not higher than 24%, and an evenly distributed exposure atmosphere should be ensured. Maintenance of a slight negative pressure inside the chamber will prevent leakage of the test substance into the surrounding area. As a general rule to ensure stability of a chamber atmosphere, the total volume of the test animals should not exceed 5% of the volume of the test chamber. The rate of sampling air flow should be adjusted to ensure that conditions throughout the equipment are essentially the same.

16. Principles of the head/nose-only and whole-body exposure techniques and their particular advantages and disadvantages have been published (7).

Exposure conditions

Exposure time

17. The duration of daily exposure should be 6 hours after equilibration of the chamber concentrations. For other durations to meet specific requirements justifications should be provided.

Exposure concentrations

18. At least three exposure concentrations and a control should be used. When appropriate, a vehicle control that corresponds to the concentration of vehicle at the highest exposure concentration level should be used. Animals in the control group should be handled in an identical manner to the test group animals. The highest concentration level should result in toxic effects but not produce an incidence of deaths which would prevent a meaningful evaluation of the study. The lowest concentration level should not produce any evidence of toxicity. When there is usable information on levels for human exposure, the lowest concentration should exceed this. Ideally, the intermediate concentration(s) should produce minimal clinical signs of toxicity. If more than one intermediate concentration is used, the concentrations should be spaced to produce a gradient of toxic responses. In the low and intermediate groups and in the controls the incidence of deaths should be negligible, or low, in order to permit a meaningful evaluation of the results. If no information is available on a suitable concentration range, a range finding study may be performed. For further guidance on dose selection for hazard classification purposes see the UN GHS (2).

19. Variability in test concentrations should be minimized to ensure constant exposures at the concentrations tested. In the case of potentially explosive test substances, care should be taken to avoid dangerous conditions. To establish suitable exposure concentrations, technical trial tests without animals are recommended.

Procedure

20. The animals should be exposed to the test substance 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 acceptable. Animals in an eventual 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.

Observations

21. 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 appropriate handling of those animals found dead for later investigations. Animals found in a moribund condition and animals showing severe pain and enduring signs of severe distress should be humanely killed.

Particle-size

22. Since it is difficult to predict the most responsive region of the respiratory tract or the most harmful particle size, the particle size distribution of dusts and aerosols should be such that exposure of all regions of the respiratory tract can be achieved. An aerosol with a mass median aerodynamic diameter (MMAD) between 1 to 4 µm and a geometric standard deviation (GSD) in the range of 1.5 to 3.0 is

recommended to ensure that comprehensive respiratory tract exposure occurs. In case of deviation from the recommended MMAD, an explanation and justification should be given. (7, 8, 9).

Generation of test atmosphere

23. Where necessary, a suitable vehicle may be added to the test substance to help generate appropriate exposure concentrations and enhance the respirability of the test substance. If a vehicle is used a vehicle group could be added to show that the vehicle does not influence absorption of the test substance or produce toxic effects. Particulate material may be subjected to mechanical processes to decrease the particle size.

MONITORING OF EXPOSURE CONDITIONS

24. As indicated previously it should be noticed that the monitoring of the test atmosphere is an integral measurement of all dynamic parameters and hence provide an indirect mean to control all relevant dynamic inhalation parameters (8). Therefore, the frequency of measurement of particle size distribution may be reduced to one single measurement per exposure concentration per week. For further information on characterization of particle-size distribution, see OECD Test Guideline 110: Particle Size Distribution / Fibre Length and Diameter Distributions (10) and the Guidance Document on Acute Inhalation (11).

Concentration of Test Substance

Physical measurements

25. Monitoring of measurements should be made for the following:

a) The rate of air flow should preferably be monitored continuously.

b) During the exposure period, the actual concentrations of the test substances should be held as constant as practicable (generally within ±10% of the mean concentration in case of gases/vapours and within ±20% of the mean concentration in case of aerosols).

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 at least once per week at each concentration level.

d) Temperature and humidity (preferably continuously).

26. The consistency of the concentration of the compound in the test atmospheres should be monitored at regular intervals. Ideally, a monitoring device *e.g.,* an aerosol photometer for particulates or a total hydrocarbon analyser for volatile materials may be used to demonstrate the stability of the exposure conditions prevailed and that the inhalation chamber equilibrium concentration can be maintained within the requirements of this guideline. The monitoring of the test atmosphere is an integral measurement of all dynamic parameters and provide an indirect mean to control all relevant dynamic inhalation parameters (8). Therefore, the frequency of measurement of air flows (see below) may be reduced to one single measurement per exposure per day. However, instruments may not be adequately used when their sensoring unit is exposed to excessive quantities of test material. Therefore, for high concentrations of particulate materials, an assessment should be made whether the monitoring of the physical chamber parameters generates relevant data. If necessary, the rate of airflows should be adjusted regularly to ensure that all relevant conditions throughout the equipment are essentially the same. Assessments concerning possible artifacts, collection efficiency, stability and recovery of the test material sampled should be made.

27. The exposure technique, generation and characterization of the test atmospheres both for vapors and liquid aerosols should allow for determination of the analytical (actual) concentration during the course of an exposure at a frequency of at least 3-times during each exposure day, and for calculation of the nominal concentration(s). For liquid aerosols, the characterization of test atmospheres should include discussion of procedures used to improve the respirability of particles, the generation of high test exposure concentrations, the recovery of the test compound (nominal versus analytical (actual) concentration), and the characterisation of particle size and distribution. The frequency of these determinations should be performed at least once per week during the exposure period.

28. Characterisation of test atmospheres for dry aerosols should include determination of the analytical concentration during the course of exposure at a frequency of at least three times during each exposure period (unless precluded by testing of low concentrations). For solid non-volatile test substances (powders and liquids) gravimetric and chemical-analytical procedures are considered to be equivalent. Characterisation of particle-size distribution should be analysed at least once per week during the exposure period.

29. When the test substance is a mixture, the analytical (actual) concentration should include the total formulation, and not just the ingredient determined. It is not necessary to analyse inert components provided that the mixture at the animals breathing zone is analogous to the formulation. If there is some difficulty in measuring chambers analytical (actual) concentrations due to precipitation, non-homogeneous mixtures, volatile components, or other factors, additional analyses of any inert components may be necessary.

30. Measurements of the inhalation chamber temperature and humidity should be performed in the breathing area of the animals. The temperature during the exposures should be maintained at 22±3C°. The relative humidity should be maintained between 30 and 70% with the aim of 50-60%. When water is used as a vehicle, or when high concentrations of dry particulate matter are aerosolized, values outside of this range may be obtained.

Sampling of test chamber atmosphere

31. The samples are taken in the breathing zone of the test animals. During sampling the airflow should be monitored at regular intervals to detect changes in the flow rate caused by an increased resistance in the adsorbent used. If impingers containing vapours (except water) are used during sampling of test atmospheres, substantial evaporation (precluding appropriate subsequent analysis) of the solvent should be prevented. Sampling ports should be designed in such a way that potential sampling errors as a result of anisokinetic sampling or by size-sampling are minimized. The tolerance limits for the radius of the sample probes are calculated according to published formulas (12, 13). Further details are provided in handbooks of aerosol physics (9, 14).

Experimental animals

Clinical examinations

32. 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. 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. Moribund

animals should be removed and humanely killed when noticed. When animals are killed for humane reasons or found dead, the time of death should be recorded.

33. Before the first exposure, to allow for within subjects comparisons, detailed clinical observations should be made in all animals. Based on clinical signs observed during the course of the study and/or based on anticipated neurotoxic potential, detailed clinical observations should also be made in all animals in the fourth week of exposure (because of practical reasons weekly testing before each daily exposure may interfere with the 6-hour exposure duration). These observations should be made outside the home cage in a standard arena and preferably at the same time. They should be carefully recorded, preferably using scoring systems, explicitly defined by the testing laboratory. Effort should be made to ensure that variations in the test conditions are minimal and that observations are preferably conducted by observers unaware of treatment. Signs noted should include, but not be limited to, changes in skin, fur eyes, mucous membranes, occurrence of secretions and excretions and autonomic activity (*e.g.*, lacrimation, piloerection, pupil size, unusual respiratory pattern). Changes in gait, posture and response to handling as well as the presence of clonic or tonic movements, stereotypies (*e.g.*, excessive grooming, repetitive circling) or bizarre behaviour (*e.g.*, self-mutilation, walking backwards) should also be recorded.

34. In the last week of exposure sensory reactivity to stimuli to different types (*e.g.*, auditory, visual and proprioceptive stimuli), assessment of grip strength and motor activity assessment should be conducted. These observations may be omitted when the study is conducted as a preliminary study to a subsequent subchronic (90-day) study. In that case, the functional observations should be included in this follow-up study. On the other hand, the availability of data on functional observations from the repeated inhalations study may enhance the ability to select concentration levels for a subsequent subchronic study. Exceptionally, functional observations may also be omitted for groups that otherwise reveal signs of toxicity to an extent that would siginificantly interfere with the functional test performance.

35. Measurements should be made of food consumption weekly and the animals weighed weekly. At the end of the treatment period animals are weighed and humanely killed.

36. 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.

b) Clinical biochemistry determinations in blood should also 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 alanine aminotransferase, serum aspartate aminotransferase, 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.

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

Pathology

Gross necropsy

38. 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, heart, spleen, thymus, brain, 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: nasal passages (including nasal-associated lymphoid tissue-NALT), larynx, trachea, 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), lymph nodes draining the respiratory tissues (cervical/mandibular and mediastinal/ tracheobronchial/hilar nodes), liver, kidneys, spleen, thymus, adrenals, heart, testes and any target organs, that is, those showing gross lesions or changes in size.

Histopathology

39. 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.

40. The nasal tissues should be examined at different, standardized levels (15, 16, 17, 18) to allow adequate examination of the squamous, transitional (non-ciliated respiratory), respiratory (ciliated respiratory) and olfactory epithelium, and the nasal-associated lymphoid tissue or NALT (19, 20). The larynx should include the ventral base of the epiglottis (21, 22, 23). The trachea should include the carina of the bifurcation and the lungs should include the main bronchi.

EVALUATION OF RESULTS

41. 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.

DATA AND REPORTING

42. Individual animal data should be provided. Additionally, all data should be summarized in tabular form, showing for each test group the number of animals used, the number of animals displaying signs of toxicity, the number of animals found dead during the test or killed for humane reasons, time of death of individual animals, a description and the time course of effects and reversibility, and necropsy findings.

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

Test Report

44. The test report should include the following information, as appropriate:

Test substance:

- physical nature, purity and, where relevant, physico-chemical properties (including isomerisation);
- identification data and Chemical Abstract Services Registry Number, if known.

Vehicle:

- justification for use of vehicle and justification for choice of vehicle (if other than water).

Test animals

- species/strain used;
- microbiological status of the animals, when known;
- acclimatization period;
- number, age and sex of animals (including, where appropriate, a rationale for use of males instead of females);
- source, housing conditions, historical data, diet, etc.

Test conditions

- inhalation chambers
- source and description of equipment
- calibration of equipment
- dimensions and volumes
- pressure difference (positive or negative)
- exposure ports per chamber, location of animals in the chambers
- stability of test atmospheres
- location of temperature and humidity sensors in the chambers
- location of sampling of test atmospheres
- description of methods used for calibration/validation
- treatment of air supplied/extracted

Test atmosphere data

- airflow rates through generation device(s)
- by pass air (dilution air)
- total airflow rates supplied/extracted
- airflow rate/exposure port (nose-only) or animal load/chamber (whole body)
- time required to reach inhalation equilibrium, t90 or t99 (k x chamber volume/flow; for t90 k = 2.303, and for t99 k = 4.605)
- number of volume changes per hour
- description of methods used for calibration/validation B generation of the test atmospheres
- metering devices (if applicable)
- description of the system(s) used to generate the test atmospheres
- physical nature of vehicle used
- concentration of test compound in vehicle

- calculation of nominal concentration (may not be applicable to aerosols)
- description of methodology used to optimize the respirability of particles
- characterisation of the test atmospheres
- sampling conditions used for analytical (actual) and particle-size measurements
- concentration of test compound in the test atmospheres using specific or non-specific techniques (*e.g.*, gravimetrical versus chromatographic techniques)
- particle-size distribution: MMAD, GSD, per cent respirable mass < 3 µm
- comparison of nominal and analytical (actual) chamber concentrations (if applicable)
- temperature and humidity of air

Results:

- tabulation of chamber temperature, humidity and airflow;
- tabulation of chamber nominal and actual concentration data;
- tabulation of particle size data including analytical sample collection data, particle size distribution and calculations of MMAD and GSD;
- tabulation of toxic response data by sex and concentration
- tabulation of time of death during the study or whether animals survived to termination
- toxic or other effects
- time of observation of each abnormal sign and its subsequent course
- tabulation of food and body weight data
- haematological tests employed and results
- clinical biochemistry tests employed and results
- urinalysis and results
- necropsy findings
- a detailed description of all histopathological findings; and
- statistical treatment of results where appropriate.

Discussion and interpretation of results:

- discussion and interpretation of results, including technical aspects of difficulties arising from generation/characterization of the test atmospheres and discussion as to whether changes observed are caused by systemic or local effects
- concentration-response relationships and No-Observed-Adverse-Effect-Level (NOAEL)(if applicable)
- 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.

Conclusions.

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ANNEX 1

DEFINITIONS

Aerosol: A suspension of solid or liquid particles in a suspension in a gas, as a foam, paste or powder or in a liquid state or in a gaseous state.

Dust: Solid particles formed from a substance or mixture, capable of being suspended in air. These particles may have irregular shapes with sizes ranging from sub-micrometer up to over 100 µm.

Evident toxicity is a general term describing clear signs of toxicity following the administration of a test substance, (22) such that at the next highest fixed concentration either severe pain and enduring signs of severe distress, moribund condition (criteria are presented in the Humane Endpoints Guidance Document (5)) or probable mortality in most animals can be expected. Body weight is recognized as a critical indicator of evident toxicity, and animals exhibiting a 20% decrement should be closely monitored.

GHS (Globally Harmonized System of Classification and Labelling of Chemicals): a system proposing the classification of chemicals according to standardised types and levels of physical, health and environmental hazards, and addressing corresponding communication elements, such as pictograms, signal words, hazard statements, precautionary statements and safety data sheets, so that to convey information on their adverse effects with a view to protect people and the environment. A joint activity of OECD (human health and the environment), UN Committee of Experts on Transport of Dangerous Goods (physical-chemical properties) and ILO (hazard communication) and co-ordinated by the Interorganisation Programme for the Sound Management of Chemicals (IOMC).

Humane endpoint: A humane endpoint can be defined as the earliest indicator in an animal experiment of severe pain, severe distress, suffering or impending death. See OECD Guidance Document No. 19 (5).

Impending death: when moribund state or death is expected prior to the next planned time of observation. Signs indicative of this state in rodents could include convulsions, lateral position, recumbence, and tremor. (See the Humane Endpoint Guidance Document (5) for more details).

Mass median aerodynamic diameter (MMAD): The median aerodynamic diameter and, along with the geometric standard deviation, is used to describe the particle size distribution of any aerosol statistically, based on the weight and size of the particles. Fifty percent of the particles by weight will be smaller than the median diameter and 50 percent of the particles will be larger.

Mist: Finely divided liquid droplets of a substance or mixture suspended in air with sizes generally ranging from 2 to 100 µm. A mist can be formed by condensation of supersaturated vapours or by physical shearing of liquids, such as nebulization, atomisation, spraying or bubbling.

Moribund condition: being in a state of dying or inability to survive, even if treated. (See the Humane Endpoint Guidance Document (5) for more details).

Vapour: The gaseous form of a substance or mixture which is normally in liquid or solid state at ambient conditions of temperature and pressure.