

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 corrosive¹ 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 $\pm 20\%$ 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 $22\pm 3^{\circ}\text{C}$. 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 $\pm 10\%$ of the mean concentration in case of gases/vapours and within $\pm 20\%$ 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 sensing 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 $22\pm3^{\circ}\text{C}$. 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