Section 4
Health effects

Test Guideline No. 403
Acute Inhalation Toxicity

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OECD Guidelines for the Testing of Chemicals

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OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Acute Inhalation Toxicity

INTRODUCTION

1. OECD Guidelines are periodically reviewed in the light of scientific progress, changing regulatory needs, and animal welfare considerations. The original acute inhalation Test Guideline 403 was adopted in 1981. This revised Test Guideline 403 (TG 403) (1) has been designed to be more flexible, to reduce animal usage, and to fulfil regulatory needs. The revised TG 403 features two study types: a Traditional LC_{50} protocol and a Concentration x Time (C x t) protocol. Primary features of this Test Guideline are the ability to provide a concentration-response relationship ranging from non-lethal to lethal outcomes in order to derive a median lethal concentration (LC_{50}), non-lethal threshold concentration (*e.g.* LC_{01}), and slope, and to identify possible sex susceptibility. The C x t protocol should be used when there is a specific regulatory or scientific need that calls for the testing of animals over multiple time durations, such as for purposes of emergency response planning (*e.g.* deriving Acute Exposure Guideline Levels (AEGL), Emergency Response Planning Guidelines (ERPG), or Acute Exposure Threshold Levels (AETL) values), or for land-use planning.

2. Guidance on the conduct and interpretation of TG 403 studies can be found in the Guidance Document on Acute Inhalation Toxicity Testing (GD 39) (2). The TG 403 should be conducted only as a last resort after all the existing information has been considered (including in vitro, in chemico, and in silico studies). If in vivo testing is required, consideration should be given to using alternative acute inhalation studies that use fewer animals and more refined endpoints (TG 433 and TG 436). There are instances where the TG 403 may still be necessary or still required by some regulatory authorities. It should be noted that data generated from the earlier versions of this Test Guideline are equally valid.

3. Definitions used in the context of this Guideline are provided in GD 39 (2).

4. This Test Guideline enables test article characterization and quantitative risk assessment, and allows test articles to be ranked and classified according to the United Nations (UN) Globally Harmonized System of Classification and Labelling of Chemicals (GHS) (3). GD 39 (2) provides guidance in the selection of the appropriate Test Guideline for acute testing. When information on classification and labelling only is required, Test Guideline 436 (4) is generally recommended [see GD 39 (2)]. Test Guideline 403 is not specifically intended for the testing of specialized materials, such as poorly soluble isometric or fibrous materials or manufactured nanomaterials.

INITIAL CONSIDERATIONS

5. Before considering testing in accordance with this Test Guideline all available information on the test article, including existing studies (e.g. TG 436)(4) whose data would support not doing additional testing should be considered by the testing laboratory in order to minimize animal usage. Information that

may assist in the selection of the most appropriate species, strain, sex, mode of exposure and appropriate test concentrations include the identity, chemical structure, and physico-chemical properties of the test article; results of any in vitro or in vivo toxicity tests; anticipated uses and potential for human exposure; available (Q)SAR data and toxicological data on structurally related substances [see GD 39 (2)].

6. Testing corrosive and/or irritating test articles at concentrations that are expected to cause severe pain and/or distress should be avoided to the extent possible. The corrosive/irritating potential should be evaluated by expert judgment using such evidence as human and animal experience (e.g. from repeat dose studies performed at non-corrosive/irritant concentrations), existing in vitro data (e.g. from TGs 430 (5), 431 (6) or 435 (7)), pH values, information from similar substances or any other pertinent data, for the purpose of investigating whether further testing can be waived. For specific regulatory needs (e.g. for emergency planning purposes), the TG 403 may be used for exposing animals to these materials because it provides the study director or principal investigator with control over the selection of target concentrations. However, the targeted concentrations should not induce severe irritation/corrosive effects, yet sufficient to extend the concentration-response curve to levels that reach the regulatory and scientific objective of the test. These concentrations should be selected on a case-by-case basis and justification for concentration selection should be provided [see GD 39 (2)].

PRINCIPLE OF THE TEST

7. This revised TG 403 has been designed to obtain sufficient information on the acute toxicity of a test article to enable its classification and to provide lethality data (*e.g.* $LC_{50,}$ LC_{01} and slope) for one or both sexes as needed for quantitative risk assessments. This Guideline offers two test methods. The first method is a Traditional protocol in which groups of animals are exposed to a limit concentration (limit test) or a series of concentrations in a stepwise procedure for a predetermined duration of usually 4 hours. Other durations of exposure may apply to serve specific regulatory purposes. The second method is a (C x t) protocol in which groups of animals are exposed to one (limit concentration) or a series of multiple concentrations over multiple durations.

8. Moribund animals or animals obviously in pain or showing signs of severe and enduring distress should be humanely killed and are considered in the interpretation of the test result in the same way as animals that died on test. Criteria for making the decision to kill moribund or severely suffering animals, and guidance on the recognition of predictable or impending death, are the subject of an OECD Guidance Document No. 19 on Humane Endpoints (8).

DESCRIPTION OF THE METHOD

Selection of animal species

9. Healthy young adult animals of commonly used laboratory strains should be used. The preferred species is the rat and justification should be provided if other species are used.

Preparation of animals

10. Females should be nulliparous and nonpregnant. On the exposure day, animals should be young adults 8 to 12 weeks of age, and body weights should be within ±20% of the mean weight for each sex of any previously exposed animals of the same age. The animals are randomly selected and marked for individual identification. The animals are kept in their cages for at least 5 days prior to the start of the test to allow for acclimatization to laboratory conditions. Animals should also be acclimatised to the test

apparatus for a short period prior to testing, as this will lessen the stress caused by introduction to the new environment.

Animal husbandry

11. The temperature of the experimental animal maintenance room should be 22±3°C. The relative humidity should ideally be maintained in the range of 30 to 70%, though this may not be possible when using water as a vehicle. Before and after exposures, animals generally should be caged in groups by sex and concentration, but the number of animals per cage should not interfere with clear observation of each animal and should minimize losses due to cannibalism and fighting. When animals are to be exposed nose-only, it may be necessary for them to be acclimated to the restraining tubes. The restraining tubes should not impose undue physical, thermal, or immobilization stress on the animals. Restraint may affect physiological endpoints such as body temperature (hyperthermia) and/or respiratory minute volume. If generic data are available to show that no such changes occur to any appreciable extent, then pre-adaptation to the restraining tubes is not necessary. Animals exposed whole-body to an aerosol should be housed individually during exposure to prevent them from filtering the test aerosol through the fur of their cage mates. Conventional and certified laboratory diets may be used, except during exposure, accompanied with an unlimited supply of municipal drinking water. Lighting should be artificial, the sequence being 12 hours light/12 hours dark.

Inhalation chambers

12. The nature of the test article and the objective of the test should be considered when selecting an inhalation chamber. The preferred mode of exposure is nose-only (which term includes head-only, nose-only or snout-only). Nose-only exposure is generally preferred for studies of liquid or solid aerosols and for vapours that may condense to form aerosols. Special objectives of the study may be better achieved by using a whole-body mode of exposure, but this should be justified in the study report. To ensure atmosphere stability when using a whole-body chamber, the total volume of the test animals should not exceed 5% of the chamber volume. Principles of the nose-only and whole body exposure techniques and their particular advantages and disadvantages are described in GD 39 (2).

EXPOSURE CONDITIONS

Administration of concentrations

13. Nose-only exposures may be any duration up to 6 hours in rats. If mice are exposed nose-only, exposures generally should not exceed 4 hours. Justification should be provided if longer duration studies are needed [see GD 39 (2)]. Animals exposed to aerosols in whole-body chambers should be housed individually to prevent ingestion of test article due to grooming of cage mates. Feed should be withheld during the exposure period. Water may be provided throughout a whole-body exposure.

14. Animals are exposed to the test article as a gas, vapour, aerosol, or a mixture thereof. The physical state to be tested depends on the physico-chemical properties of the test article, the selected concentration, and/or the physical form most likely present during the handling and use of the test article. Hygroscopic and chemically reactive test articles should be tested under dry air conditions. Care should be taken to avoid generating explosive concentrations.

Particle-size distribution

15. Particle sizing should be performed for all aerosols and for vapours that may condense to form aerosols. To allow for exposure of all relevant regions of the respiratory tract, aerosols with mass median aerodynamic diameters (MMAD) ranging from 1 to 4 μm with a geometric standard deviation (σg) in the range of 1.5 to 3.0 are recommended (2) (9) (10). Although a reasonable effort should be made to meet this standard, expert judgment should be provided if it cannot be achieved. For example, metal fumes may be smaller than this standard, and charged particles, fibres, and hygroscopic materials (which increase in size in the moist environment of the respiratory tract) may exceed this standard.

Test article preparation in a vehicle

16. A vehicle may be used to generate an appropriate concentration and particle size of the test article in the atmosphere. As a rule, water should be given preference. Particulate material may be subjected to mechanical processes to achieve the required particle size distribution, however, care should be taken to not decompose or alter the test article. In cases where mechanical processes are believed to have altered test article composition (*e.g.* extreme temperatures from excessive milling due to friction), the composition of the test article should be verified analytically. Adequate care should be taken to not contaminate the test material. It is not necessary to test non-friable granular materials which are purposefully formulated to be un-inhalable. An attrition test should be used to demonstrate that respirable particles are not produced when the granular material is handled. If an attrition test produces respirable articles, an inhalation toxicity test should be performed.

Control animals

17. A concurrent negative (air) control group is not necessary. When a vehicle other than water is used to assist in generating the test atmosphere, a vehicle control group should only be used when historical inhalation toxicity data are not available. If a toxicity study of a test article formulated in a vehicle reveals no toxicity, it follows that the vehicle is non-toxic at the concentration tested; thus, there is no need for a vehicle control.

MONITORING OF EXPOSURE CONDITIONS

Chamber airflow

18. The flow of air through the chamber should be carefully controlled, continuously monitored, and recorded at least hourly during each exposure. The monitoring of test atmosphere concentration (or stability) is an integral measurement of all dynamic parameters and provides an indirect means to control all relevant dynamic atmosphere generation parameters. Special consideration should be given to avoiding re-breathing in nose-only chambers in cases where airflow through the exposure system are inadequate to provide dynamic flow of test article atmosphere. There are prescribed methodologies that can be used to demonstrate that re-breathing does not occur under the selected operation conditions (2) (11). Oxygen concentration should be at least 19% and carbon dioxide concentration should not exceed 1%. If there is reason to believe that these standards cannot be met, oxygen and carbon dioxide concentrations should be measured.

Chamber temperature and relative humidity

19. Chamber temperature should be maintained at 22±3°C. Relative humidity in the animals' breathing zone, for both nose-only and whole-body exposures, should be monitored and recorded at least

three times for durations of up to 4 hrs, and hourly for shorter durations. The relative humidity should ideally be maintained in the range of 30 to 70%, but this may either be unattainable (*e.g.* when testing water based formulations) or not measurable due to test article interference with the test method.

Test article: Nominal concentration

20. Whenever feasible, the nominal exposure chamber concentration should be calculated and recorded. The nominal concentration is the mass of generated test article divided by the total volume of air passed through the chamber system. The nominal concentration is not used to characterize the animals' exposure, but a comparison of the nominal concentration and the actual concentration gives an indication of the generation efficiency of the test system, and thus may be used to discover generation problems.

Test article: Actual concentration

21. The actual concentration is the test article concentration at the animals' breathing zone in an inhalation chamber. Actual concentrations can be obtained by specific methods (*e.g.* direct sampling, adsorptive or chemical reactive methods, and subsequent analytical characterisation) or by non-specific methods such as gravimetric filter analysis. The use of gravimetric analysis is acceptable only for single component powder aerosols or aerosols of low volatility liquids and should be supported by appropriate pre-study test article-specific characterisations. Multi-component powder aerosol concentration may also be determined by gravimetric analysis. However, this requires analytical data which demonstrate that the composition of airborne material is similar to the starting material. If this information is not available, a reanalysis of the test material (ideally in its airborne state) at regular intervals during the course of the study may be necessary. For aerosolised agents that may evaporate or sublimate, it should be shown that all phases were collected by the method chosen. The target, nominal, and actual concentrations should be provided in the study report, but only actual concentrations are used in statistical analyses to calculate lethal concentration values.

22. One lot of the test article should be used, if possible, and the test sample should be stored under conditions that maintain its purity, homogeneity, and stability. Prior to the start of the study, there should be a characterization of the test article, including its purity and, if technically feasible, the identity, and quantities of identified contaminants and impurities. This can be demonstrated by, but is not limited to, the following data: retention time and relative peak area, molecular weight from mass spectroscopy or gas chromatography analyses, or other estimates. Although the test sample's identity is not the responsibility of the test laboratory, it may be prudent for the test laboratory to confirm the sponsor's characterization at least in a limited way (*e.g.* colour, physical nature, etc.).

23. The exposure atmosphere shall be held as constant as practicable and monitored continuously and/or intermittently depending on the method of analysis. When intermittent sampling is used, chamber atmosphere samples should be taken at least twice in a four hour study. If not feasible due to limited air flow rates or low concentrations, one sample may be collected over the entire exposure period. If marked sample-to-sample fluctuations occur, the next concentrations tested should use four samples per exposure. Individual chamber concentration samples should not deviate from the mean concentration by more than ±10% for gases and vapours or ±20% for liquid or solid aerosols. Time to chamber equilibration (t_{95}) should be calculated and recorded. The duration of an exposure spans the time that the test article is generated and this takes into account the times required to attain t_{95}. Guidance for estimating t_{95} can be found in GD 39 (2).

24. For very complex mixtures consisting of gases/vapours, and aerosols (*e.g.* combustion atmospheres and test articles propelled from purpose-driven end-use products/devices), each phase may behave differently in an inhalation chamber so at least one indicator substance (analyte), normally the principal active substance in the tested product formulation, of each phase (gas/vapour and aerosol) should

be selected. When the test article is a mixture (*e.g.* a formulation), the analytical concentration should be reported for the total formulation and not just for the active ingredient or the component (analyte). Additional information regarding actual concentrations can be found in GD 39 (2).

Test article: Particle size distribution

25. The particle size distribution of aerosols should be determined at least twice during each 4 hour exposure by using a cascade impactor or an alternative instrument such as an aerodynamic particle sizer. If equivalence of the results obtained by a cascade impactor or an alternative instrument can be shown, then the alternative instrument may be used throughout the study. A second device, such as a gravimetric filter or an impinger/gas bubbler, should be used in parallel to the primary instrument to confirm the collection efficiency of the primary instrument. The mass concentration obtained by particle size analysis should be within reasonable limits of the mass concentration obtained by filter analysis [see GD 39 (2)]. If equivalence can be demonstrated in the early phase of the study, then further confirmatory measurements may be omitted. For animal welfare reasons, measures should be taken to minimize inconclusive data which may lead to a need to repeat an exposure. Particle sizing should be performed for vapours if there is any possibility that vapour condensation may result in the formation of an aerosol, or if particles are detected in a vapour atmosphere with potential for mixed phases (see paragraph 15).

PROCEDURE

26. Two study types are described below: the Traditional protocol, and the C x t protocol. Both protocols may include a sighting study, a main study, and/or a limit test (Traditional protocol) or testing at a limit concentration (C x t). If one sex is known to be more susceptible, the study director may choose to perform these studies using only the susceptible sex. If rodent species other than rats are exposed nose-only, maximum exposure durations may be adjusted to minimise species-specific distress. Before commencing, all available data should be considered in order to minimize animal usage. For example, data generated using TG 436 (4) may eliminate the need for a sighting study, and may also demonstrate whether one sex is more susceptible [see GD 39 (2)].

TRADITIONAL PROTOCOL:

General considerations: Traditional protocol

27. In a Traditional study, groups of animals are exposed to a test article for a fixed period of time (generally 4 hours) in either a nose-only or whole-body exposure chamber. Animals are exposed to either a limit concentration (limit test), or to at least three concentrations in a stepwise procedure (main study). A sighting study may precede a main study unless some information about the test article already exists, such as a previously performed TG 436 study [see GD 39 (2)].

Sighting study: Traditional protocol

28. A sighting study is used to estimate test article potency, identify sex differences in susceptibility, and assist in selecting exposure concentration levels for the main study or limit test. When selecting concentration levels for the sighting study, all available information should be used including available (Q)SAR data and data for similar chemicals. No more than three males and three females should be exposed at each concentration (3 animals/sex may be needed to establish a sex difference). A sighting study may consist of a single concentration, but more concentrations may be tested if necessary. A sighting

study should not test so many animals and concentrations that it resembles a main study. A previously performed TG 436 study (4) may be used instead of a sighting study [see GD 39 (2)].

Limit test: Traditional protocol

29. A limit test is used when the test article is known or expected to be virtually non-toxic, i.e. eliciting a toxic response only above the regulatory limit concentration. In a limit test, a single group of three males and three females is exposed to the test article at a limit concentration. Information about the toxicity of the test material can be gained from knowledge about similar tested compounds or similar tested mixtures or products, taking into consideration the identity and percentage of components known to be of toxicological significance. In those situations where there is little or no information about its toxicity, or the test material is expected to be toxic, the main test should be performed.

30. The selection of limit concentrations usually depends on regulatory requirements. When the GHS Classification System is used, the limit concentrations for gases, vapours, and aerosols are 20000 ppm, 20 mg/L, and 5 mg/L, respectively (or the maximum attainable concentration) (3). It can be technically challenging to generate limit concentrations of some test articles, especially as vapours and aerosols. When testing aerosols, the primary goal should be to achieve a respirable particle size (MMAD of 1-4 µm). This is possible with most test articles at a concentration of 2 mg/L. Aerosol testing at greater than 2 mg/L should only be attempted if a respirable particle size can be achieved [see GD 39 (2)]. GHS discourages testing in excess of a limit concentration for animal welfare reasons (3). The limit concentration should only be considered when there is a strong likelihood that results of such a test would have direct relevance for protecting human health (3), and justification provided in the study report. In the case of potentially explosive test articles, care should be taken to avoid conditions favourable for an explosion. To avoid an unnecessary use of animals, a test run without animals should be conducted prior to the limit test to ensure that the chamber conditions for a limit test can be achieved.

31. If mortality or moribundity is observed at the limit concentration, the results of the limit test can serve as a sighting study for further testing at other concentrations (see main study). If a test article's physical or chemical properties make it impossible to attain a limit concentration, the maximum attainable concentration should be tested. If less than 50% lethality occurs at the maximum attainable concentration, no further testing is necessary. If the limit concentration could not be attained, the study report should provide an explanation and supportive data. If the maximum attainable concentration of a vapour does not elicit toxicity, it may be necessary to generate the test article as a liquid aerosol.

Main study: Traditional protocol

32. A main study is typically performed using five males and five females (or 5 animals of the susceptible sex, if known) per concentration level, with at least three concentration levels. Sufficient concentration levels should be used to obtain a robust statistical analysis. The time interval between exposure groups is determined by the onset, duration, and severity of toxic signs. Exposure of animals at the next concentration level should be delayed until there is reasonable confidence of survival for previously tested animals. This allows the study director to adjust the target concentration for the next exposure group. Due to the dependence on sophisticated technologies, this may not always be practical in inhalation studies, so the exposure of animals at the next concentration level should be based on previous experience and scientific judgement. GD 39 (2) should be consulted when testing mixtures.

CONCENTRATION X TIME (C X T) PROTOCOL

General considerations: C x t protocol

33. A step-wise C x t study may be considered as an alternative to a Traditional protocol when assessing inhalation toxicity (12) (13) (14). This approach allows animals to be exposed to a test article at several concentration levels and for multiple time durations. All testing is performed in a nose-only chamber (whole-body chambers are not practical for this protocol). A flow diagram in Annex 1 illustrates this protocol. A simulation analysis has shown that the Traditional protocol and the C x t protocol are both capable of yielding robust LC_{50} values, but the C x t protocol is generally better at yielding robust LC_{01} and LC_{10} values (15).

34. A simulation analysis has demonstrated that using two animals per C x t interval (one per sex using both sexes, or two of the more susceptible sex) may generally be adequate when testing 4 concentrations and 5 exposure durations in a main study. Under some circumstances, the study director may elect to use two rats per sex per C x t interval (15). Using 2 animals per sex per concentration and time point may reduce bias and variability of the estimates, increase the estimation success rate, and improve confidence interval coverage. However, in case of an insufficient close fit to the data for estimation (when using one animal per sex or two animals of the more susceptible sex) a 5^{th} exposure concentration may also suffice. Further guidance on the number of animals and concentrations to be used in a C x t study can be found in GD 39 (2).

Sighting study: C x t protocol

35. A sighting study is used to estimate test article potency and to assist in selecting exposure concentration levels for the main study. A sighting study using up to three animals/sex/concentration [for details see Appendix III of GD 39 (2)] may be needed to choose an appropriate starting concentration for the main study and to minimize the number of animals used. It may be necessary to use three animals per sex to establish a sex difference. These animals should be exposed for a single duration, generally 240 min. The feasibility of generating adequate test atmospheres should be assessed during technical pre-tests without animals. It is generally not necessary to perform a sighting study if mortality data are available from a TG 436 study (4). When selecting the initial target concentration in a TG 403 study, the study director should consider the mortality patterns observed in any available TG 436 studies (4) for both sexes and for all concentrations tested [see GD 39 (2)].

Initial Concentration: C x t protocol

36. The initial concentration (Exposure Session I) (Annex 1) will either be a limit concentration or a concentration selected by the study director based on the sighting study. Groups of 1 animal/sex are exposed to this concentration for multiple durations (e.g. 15, 30, 60, 120, or 240 minutes), resulting in a total number of 10 animals (called Exposure Session I) (Annex 1).

37. The selection of limit concentrations usually depends on regulatory requirements. When the GHS Classification System is used, the limit concentrations for gases, vapours, and aerosols are 20000 ppm, 20 mg/L and 5 mg/L, respectively (or the maximum attainable concentration) (3). It can be technically challenging to generate limit concentrations of some test articles, especially as vapours and aerosols. When testing aerosols, the goal should be to achieve a respirable particle size (i.e. an MMAD of 1-4 µm) at a limit concentration of 2 mg/L. This is possible with most test articles. Aerosol testing at greater than 2 mg/L should only be attempted if a respirable particle size can be achieved [see GD 39 (2)]. GHS

discourages testing in excess of a limit concentration for animal welfare reasons (3). Testing in excess of the limit concentration should only be considered when there is a strong likelihood that results of such a test would have direct relevance for protecting human health (3), justification should be provided in the study report. In the case of potentially explosive test articles, care should be taken to avoid conditions favourable for an explosion. To avoid an unnecessary use of animals, a test run without animals should be conducted prior to testing at the initial concentration to ensure that the chamber conditions for this concentration can be achieved.

38. If mortality or moribundity is observed at the initial concentration, the results at this concentration can serve as a starting point for further testing at other concentrations (see main study). When a test article's physical or chemical properties make it impossible to attain a limit concentration, the maximum attainable concentration should be tested. If less than 50% lethality occurs at the maximum attainable concentration, no further testing is necessary. If the limit concentration could not be attained, the study report should provide an explanation and supportive data. If the maximum attainable concentration of a vapour does not elicit toxicity, it may be necessary to generate the test article as a liquid aerosol.

Main study: C x t protocol

39. The initial concentration (Exposure Session I) (Annex 1) tested in the main study will either be a limit concentration or a concentration selected by the study director based on the sighting study. If mortality has been observed during or following Exposure Session I, the minimum exposure (C x t) which results in mortality will be taken as a guide to establish the concentration and periods of exposure for Exposure Session II. Each subsequent exposure session will depend on the previous session (see Annex 1).

40. For many test articles the results obtained at the initial concentration, together with three additional exposure sessions with a smaller time grid (i.e. the geometric spacing of exposure periods as indicated by the factor between successive periods, generally $\sqrt{2}$), will be sufficient to establish the C x t mortality relationship (16), but there may be some benefit to using a 5th exposure concentration [see Annex 1 and GD 39 (2)]. For mathematical treatment of results for the C x t protocol, see Annex 1.

OBSERVATIONS

41. The animals should be clinically observed frequently during the exposure period. Following exposure, clinical observations should be made at least twice on the day of exposure, or more frequently when indicated by the response of the animals to treatment, and at least once daily thereafter for a total of 14 days. The length of the observation period is not fixed, but should be determined by the nature and time of onset of clinical signs and length of the recovery period. The times at which signs of toxicity appear and disappear are important, especially if there is a tendency for signs of toxicity to be delayed. All observations are systematically recorded with individual records being maintained for each animal. Animals found in a moribund condition and animals showing severe pain and/or enduring signs of severe distress should be humanely killed for animal welfare reasons. Care should be taken when conducting examinations for clinical signs of toxicity that initial poor appearance and transient respiratory changes, resulting from the exposure procedure, are not mistaken for test article-related toxicity that would require premature killing of the animals. The principles and criteria summarised in the Guidance Document on Humane Endpoints (GD 19) should be taken into consideration (8). When animals are killed for humane reasons or found dead, the time of death should be recorded as precisely as possible.

42. Cage-side observations should include changes in the skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous systems, and somatomotor activity and

behaviour patterns. When possible, any differentiation between local and systemic effects should be noted. Attention should be directed to observations of tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma. The measurement of rectal temperature may provide supportive evidence of reflex bradypnea or hypo/hyperthermia related to treatment or confinement.

Body weights

43 Individual animal weights should be recorded once during the acclimatization period, on the day of exposure prior to exposure (day 0), and at least on days 1, 3 and 7 (and weekly thereafter), and at the time of death or euthanasia if exceeding day 1. Body weight is recognized as a critical indicator of toxicity so animals exhibiting a sustained decrement of $\geq$20%, compared to pre-study values, should be closely monitored. Surviving animals are weighed and humanely killed at the end of the post-exposure period.

Pathology

44. All test animals, including those which die during the test or are euthanized and removed from the study for animal welfare reasons, should be subjected to gross necropsy. If necropsy cannot be performed immediately after a dead animal is discovered, the animal should be refrigerated (not frozen) at temperatures low enough to minimize autolysis. Necropsies should be performed as soon as possible, normally within a day or two. All gross pathological changes should be recorded for each animal with particular attention to any changes in the respiratory tract.

45. Additional examinations included *a priori* by design may be considered to extend the interpretive value of the study, such as measuring lung weight of surviving rats, and/or providing evidence of irritation by microscopic examination of the respiratory tract. Examined organs may also include those showing evidence of gross pathology in animals surviving 24 or more hours, and organs known or expected to be affected. Microscopic examination of the entire respiratory tract may provide useful information for test articles that are reactive with water, such as acids and hygroscopic test articles.

DATA AND REPORTING

Data

46. Individual animal data on body weights and necropsy findings should be provided. Clinical observation data should be summarized in tabular form, showing for each test group the number of animals used, the number of animals displaying specific 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 time course of toxic effects and reversibility, and necropsy findings.

Test report

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

Test animals and husbandry

- Description of caging conditions, including: number (or change in number) of animals per cage, bedding material, ambient temperature and relative humidity, photoperiod, and identification of diet;
- Species/strain used and justification for using a species other than the rat;

- Number, age and sex of animals;
- Method of randomization;
- Details of food and water quality (including diet type/source, water source);
- Description of any pre-test conditioning including diet, quarantine, and treatment for disease;

Test article

- Physical nature, purity and, where relevant, physico-chemical properties (including isomerization);
- Identification data and Chemical Abstract Services (CAS) Registry Number, if known;

Vehicle

- Justification for use of vehicle and justification for choice of vehicle (if other than water);
- Historical or concurrent data demonstrating that the vehicle does not interfere with the outcome of the study;

Inhalation chamber

- Description of the inhalation chamber including dimensions and volume;
- Source and description of equipment used for the exposure of animals as well as generation of atmosphere;
- Equipment for measuring temperature, humidity, particle-size, and actual concentration;
- Source of air and treatment of air supplied/extracted and system used for conditioning;
- Methods used for calibration of equipment to ensure a homogeneous test atmosphere;
- Pressure difference (positive or negative);
- Exposure ports per chamber (nose-only); location of animals in the system (whole-body);
- Temporal homogeneity/stability of test atmosphere;
- Location of temperature and humidity sensors and sampling of test atmosphere in the chamber;
- Air flow rates, air flow rate/exposure port (nose-only), or animal load/chamber (whole-body);
- Information about the equipment used to measure oxygen and carbon dioxide, if applicable;
- Time required to reach inhalation chamber equilibrium (t95)
- Number of volume changes per hour;
- Metering devices (if applicable);

Exposure data

- Rationale for target concentration selection in the main study;
- Nominal concentrations (total mass of test article generated into the inhalation chamber divided by the volume of air passed through the chamber);
- Actual test article concentrations collected from the animals' breathing zone; for test mixtures that produce heterogeneous physical forms (gases, vapours, aerosols), each may be analysed separately;
- All air concentrations should be reported in units of mass (e.g. mg/L, mg/m3, etc.); units of volume (e.g. ppm, ppb, etc.) may also be reported parenthetically;

- Particle size distribution, mass median aerodynamic diameter (MMAD), and geometric standard deviation (σg), including their methods of calculation. Individual particle size analyses should be reported;

Test conditions

- Details of test article preparation, including details of any procedures used to reduce the particle size of solid materials or to prepare solutions of the test article. In cases where mechanical processes may have altered test article composition, include the results of analyses to verify the composition of the test article;
- A description (preferably including a diagram) of the equipment used to generate the test atmosphere and to expose the animals to the test atmosphere;
- Details of the chemical analytical method used and method validation (including efficiency of recovery of test article from the sampling medium);
- The rationale for the selection of test concentrations;

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 the MMAD and σg;
- Tabulation of response data and concentration level for each animal (i.e. animals showing signs of toxicity including mortality, nature, severity, time of onset and duration of effects);
- Individual body weights of animals collected on study; date and time of death if prior to scheduled euthanasia, time course of onset of signs of toxicity and whether these were reversible for each animal;
- Necropsy findings and histopathological findings for each animal, if available;
- Lethality estimates (e.g. LC50, LD01) including 95% confidence limits, and slope (if provided by the evaluation method);
- Statistical relation, including estimate for the exponent n (C x t protocol). The name of the statistical software used should be provided;

Discussion and interpretation of results

- Particular emphasis should be made to the description of methods used to meet this Test Guideline's criteria, e.g. the limit concentration or the particle size;
- The respirability of particles in light of the overall findings should be addressed, especially if the particle-size criteria could not be met;
- An explanation should be provided if there was a need to humanely sacrifice animals in pain or showing signs of severe and enduring distress, based on the criteria in the OECD Guidance Document on Humane Endpoints (8);
- If testing with TG 436 (4) was discontinued in favour of TG 403, justifications should be provided;
- The consistency of methods used to determine nominal and actual concentrations, and the relation of actual concentration to nominal concentration should be included in the overall assessment of the study;

- The likely cause of death and predominant mode of action (systemic versus local) should be addressed.

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ANNEX 1

C x t PROTOCOL

1. A step-wise Concentration x Time (C x t) study may be considered as an alternative to the Traditional protocol for assessing inhalation toxicity (12) (13) (14). It should be performed preferentially when there is a specific regulatory or scientific need that calls for the testing of animals over multiple time durations such as for emergency response planning or land use planning. This approach usually begins with testing at a limit concentration (Exposure Session I) in which animals are exposed to a test article for five time durations (e.g. 15, 30, 60, 120 and 240 min) so that multiple durations of time will be obtained within one exposure session (see Figure 1). When the GHS Classification System is used, the limit concentrations for gases, vapours, and aerosols are 20000 ppm, 20 mg/L, and 5 mg/L, respectively. These levels may only be exceeded if there is a regulatory or scientific need for testing at these levels (see paragraph 37 in the TG 403 main text).

2. In situations where there is little or no information about the toxicity of a test article, a sighting study should be performed in which groups of no more than 3 animals per sex are exposed to target concentrations selected by the study director, generally for 240 min.

3. If a limit concentration is tested during Exposure Session I and less than 50% mortality is observed, no additional testing is needed. If there is a regulatory or scientific need to establish the concentration/time/response relationship at higher levels than the indicated limit concentration, the next exposure should be carried out at a higher level such as at two times the limit concentration (i.e. 2L in figure 1).

4. If toxicity is observed at the limit concentration, additional testing (main study) is necessary. These additional exposures are carried out either at lower concentrations (in Figure 1: Exposure Sessions II, III or IV) or at higher concentrations using shorter durations (in Figure 1: Exposure Session IV) using durations that are adapted and not as widely spaced.

5. The test (initial concentration and additional concentrations) is carried out using 1 animal/sex per concentration/time point or with 2 animals of the more susceptible sex per concentration/time point. Under some circumstances, the study director may elect to utilize 2 rats per sex per concentration/time point (or 4 animals of the susceptible sex per concentration/time point) (15). Using 2 animals per sex per concentration/time point generally reduces bias and variability of the estimates, increases the estimation success rate, and improves confidence interval coverage relative to the protocol as described here. Further details are provided in GD 39 (2).

6. Ideally, each exposure session is carried out on one day. This gives the opportunity to delay the next exposure until there is reasonable confidence of survival, and it allows the study director to adjust the target concentration and durations for the next exposure session. It is advised to start each exposure session with the group that will be exposed the longest, e.g. the 240-min exposure group, followed by the 120 minute exposure group, and so on. If, for example, animals in the 240 minute group are dying after 90 minutes or showing severe signs of toxicity (e.g. extreme changes in breathing pattern such as laboured breathing), it would not make sense to expose a group for 120 minutes because mortality would likely be 100%. Thus the study director should select shorter exposure durations for that concentration (e.g. 90, 65, 45, 33 and 25 minutes).

7. The chamber concentration should be measured frequently to determine the time-weighted-average concentration for each exposure duration. Whenever possible, the time of death for each animal (rather than the exposure duration) should be used in the statistical analysis.

8. The results of the first four exposure sessions should be examined to identify a data gap in the concentration-time curve (see Figure 1). In case of an insufficient fit, an additional exposure (5th concentration) may be performed. Concentration and exposure durations for the 5th exposure should be chosen to cover this gap.

9. All exposure sessions (including the first Exposure Session) will be used to calculate the concentration-time-response relationship using Statistical Analysis (16). If possible, for each C x t interval, the time-weighted average concentration and the duration of exposure until death (if death occurs during the exposure) should be used.

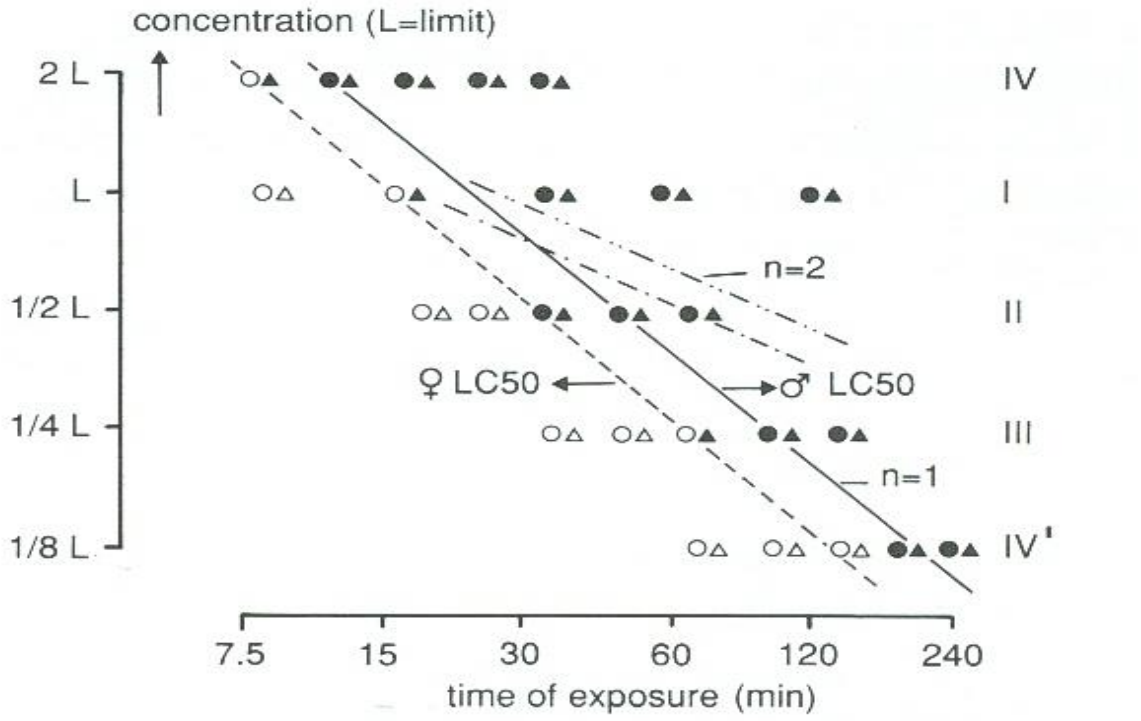

Figure 1: Hypothetical illustration of a concentration-time-mortality relationship in rats.

Open symbols = survivors; closed symbols = dead animals
Triangles = females; circles = males
Solid line = LC50 values (range 7.5-240 min) for males with n=1
Dashed line = LC50 values (range 7.5-240 min) for females with n=1
Dotted lines = hypothetical LC50 values line for males and females if n had been equal to 2 (12).

10. Below is an example of the stepwise procedure:

Exposure Session I –Testing at the limit concentration (see Figure 1)
- 1 animal/sex per concentration/time point; 10 animals in total a
- Target concentrationb = limit concentration.
- Expose five groups of animals at this target concentration for durations of 15, 30, 60, 120 and 240 minutes, respectively.

↓

Exposure Session IIc – Main Study
- 1 animal/sex per concentration/time point; 10 animals in total.
- Expose five groups of animals at a lower concentration d (1/2L) with slightly longer exposure durations (factor √2 spaced; see Figure 1).

↓

Exposure Session III – Main Study
- 1 animal/sex per concentration/time point; 10 animals total.
- Expose five groups of animals at a lower concentration d (1/4L) with slightly longer exposure durations (factor √2 spaced; see Figure 1).

↓

Exposure Session IV′ – Main Study
- 1 animal/sex per concentration/time point; 10 animals total.
- Expose five groups of animals at a lower concentration d (1/8L) with slightly longer exposure durations (factor √2 spaced; see Figure 1).

↓ or

Exposure Session IV – Main Study
- 1 animal/sex per concentration/time point; 10 animals total.
- Expose five groups of animals at a higher concentration e (2L) with slightly shorter exposure durations (factor √2 spaced; see Figure 1).

a If no sex susceptibility information is available, rats of both sexes will be used, i.e. 1 animal/sex per concentration. Based on existing information, or if it becomes apparent during this exposure session that one sex is more susceptible, 10 animals of the susceptible sex will be used (2 animals per concentration/time point) at each concentration level during subsequent testing.

b When the GHS Classification System is used, the limit concentrations for gases, vapours, and aerosols are 20000 ppm, 20 mg/L, and 5 mg/L, respectively. In case of expected toxicity or based on the results of the sighting study, lower starting concentrations should be chosen. In case of regulatory or scientific needs, higher concentrations may be used.

c Ideally, exposure of animals at the next concentration level should be delayed until there is reasonable confidence of survival for previously treated animals. This allows the study director to adjust the target concentration and durations for the next exposure session.

d The minimum dose (concentration x time) which resulted in mortality during testing

at initial concentration (first exposure session) will be taken as a guide to establish the next combination of concentration and exposure durations. Typically, the concentration will be decreased two-fold (1/2L) and animals will be exposed over a new time range with a finer grid using a geometric division of exposure periods with a factor 1.4 ($\sqrt{2}$; see reference 12) around the time according to the minimum lethal dose level (time x concentration) observed during the first exposure. In this figure (figure 1), mortality in Exposure session I was first observed at 15 min; the durations during session II are therefore centred around 30 min, and are 15, 21 30, 42 and 60 min. After the first two exposures, it is strongly advised to plot the data in a similar figure as indicated above, and to check whether the relationship between concentration and time has an angle of 45 degrees (n=1) or if the concentration-time-response relationship is less steep (e.g. n=2) or steeper (e.g. n =0.8). In the latter cases it is strongly advised to adapt the next concentrations and durations accordingly.

e In certain cases it may be necessary to increase the concentration (2L) over a new time range with a still finer grid using a geometric division of exposure periods with a factor 1.4 ($\sqrt{2}$) around the time according to the minimum lethal concentration level observed during the first exposure. The minimum exposure duration should preferably exceed 5 minutes; the maximum exposure duration should not exceed 8 hours.

Mathematical treatment of results for the C x t protocol

11. A C x t procedure with 4 or 5 exposure concentrations and five durations will yield 20 or 25 data points, respectively. With these data points, the C x t relationship can be calculated using statistical analysis (16):

$$Probit\ (P) = b_0 + b_1\ ln\ C + b_2\ ln\ t \qquad \text{Equation 1}$$

where C = concentration; t = exposure duration, or

$$Response = f(C^n t) \qquad \text{Equation 2}$$

where $n = b_1 / b_2$.

Using equation 1, the LC50 value can be calculated for a given time period (e.g. 4 hour, 1 hour, 30 minutes, or any time period within the range of time periods tested) using P = 5 (50% response). Note that Haber's rule is only applicable when n = 1. The LC01 can be calculated using P = 2.67.