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OECD GUIDELINE FOR THE TESTING OF CHEMICALS

DRAFT PROPOSAL FOR A REVISED GUIDELINE 412

Subacute Inhalation Toxicity: 28-Day Study

SUMMARY

This revised Toxicity Guideline 412 (TG 412) has been designed to fully characterize test article toxicity by the inhalation route following repeated exposure for a limited period of time, and to provide data for quantitative inhalation risk assessments. Groups of 5 male and 5 female rodents are exposed 6 hours per day for 28 days to a) the test article at three or more concentration levels, b) filtered air (negative control), and/or c) the vehicle (vehicle control). Animals are generally exposed 5 days per week but exposure for 7 days per week is also allowed. Males and females are always tested, but they may be exposed at different concentration levels if it is known that one sex is more sensitive to a given test article. This guideline also allows the study director the flexibility to include satellite (reversibility) groups, bronchoalveolar lavage (BAL), neurologic tests, and additional clinical pathology and histopathological evaluations in order to better characterize the toxicity of a test article.

INTRODUCTION

1. OECD Guidelines are periodically reviewed in the light of scientific progress, animal welfare considerations, and changing regulatory needs. The original subacute inhalation Test Guideline 412 (TG 412) was adopted in 1981 (1). TG 412 has been revised to reflect the state of the science and to meet current and future regulatory needs.

2. This guideline enables the characterization of adverse effects following repeated daily inhalation exposure to a test article for 28 days. The data derived from 28-day subacute inhalation toxicity studies can be used for quantitative risk assessments (if not followed by a 90-day subchronic inhalation toxicity study (TG 413). The data can also provide information on the selection of concentrations for longer term studies such as the 90-day subchronic inhalation toxicity study. This Test Guideline is not specifically intended for the testing of nanomaterials. Definitions used in the context of this Guideline can be found in Annex 1 and GD 39 (2).

INITIAL CONSIDERATIONS

3. All available information on the test article should be considered by the testing laboratory prior to conducting the study in order to enhance the quality of the study and minimize animal usage. Information that will assist in the selection of appropriate test concentrations might include the identity, chemical structure, and physico-chemical properties of the test article; results of any *in vitro* or *in vivo* toxicity tests; anticipated use(s) and potential for human exposure; available (Q)SAR data and toxicological data on structurally related substances; and data derived from acute inhalation toxicity testing. If neurotoxicity is expected or is observed in the course of the study, the study director may choose to include appropriate evaluations such as a functional observational battery

(FOB) and measurement of motor activity. Although the timing of exposures relative to specific examinations may be critical, the performance of these additional activities should not interfere with the basic study design.

4. Dilutions of corrosive or irritating test articles may be tested at concentrations that will yield the desired degree of toxicity (refer to GD 39 (2)). When exposing animals to these materials, the targeted concentrations should be low enough to not cause marked pain and distress, yet sufficient to extend the concentration-response curve to levels that reach the regulatory and scientific objective of the test. These concentrations must be selected on a case-by-case basis, preferably based upon an adequately designed range-finding study, that provides information regarding the critical endpoint, the irritation threshold, and the time of onset (see paragraphs 11-13). The justification for concentration selection should be provided.

5. Moribund animals or animals obviously in pain or showing signs of severe and enduring distress should be humanely killed unless there are compelling reasons to do otherwise (these reasons must be described in the study report). Moribund animals are considered in the same way as animals that die 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 on Humane Endpoints (3).

DESCRIPTION OF THE METHOD

Selection of Animal Species

6. Healthy young adult rodents of commonly used laboratory strains should be employed. The preferred species is the rat. Justification should be provided if other species are used.

Preparation of Animals

7. Females should be nulliparous and non-pregnant. On the day of randomization, animals should be young adults 7 to 9 weeks of age to assure full lung maturation. Body weights should be within ±20% of the mean weight for each sex. The animals are randomly selected, marked for individual identification, and kept in their cages for at least 5 days prior to the start of the test to allow for acclimatization to laboratory conditions.

Animal Husbandry

8. Animals should be individually identified, if possible with subcutaneous transponders, to facilitate observations and avoid confusion. 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 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 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. While animals are being exposed whole-body to an aerosol, they should be housed individually during exposure to prevent ingestion of the test article due to grooming of cage mates. Conventional 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

9. The nature of the test article and the object of the test should be considered when selecting an inhalation chamber. The default mode of exposure is nose-only (which term includes head-only, nose-only, or snout-only). While nose-only is the default mode of exposure, special objectives of the study may be better achieved by using the whole-body mode of exposure. The use of non-default modes of exposure should be based on the focus of the study and should be justified in the study report. Principles of the nose-only and whole-body exposure techniques and their particular advantages and disadvantages are addressed in GD 39 (2).

TOXICITY STUDIES

Limit Concentrations

10. Unlike with acute studies, there are no defined limit concentrations in 28-day subacute inhalation toxicity studies. The maximum concentration tested should instead be based on: 1) the maximum attainable concentration, 2) the "worst case" human exposure level, 3) the need to maintain an adequate oxygen supply, and/or 4) animal welfare considerations. In the absence of data-based limits, the acute GHS limits may be used. Justification must be provided if it is necessary to exceed these limits when testing gases or highly volatile test articles (e.g., refrigerants).

Range-Finding Study

11. Before commencing with the main study, it may be necessary to perform a range-finding study. A range-finding study is more comprehensive than a sighting study because it is not limited to concentration selection. Knowledge learned from a range-finding study can lead to a successful main study. A range-finding study may, for example, provide technical information regarding analytical methods, particle sizing, discovery of toxic mechanisms, clinical pathology and histopathological data, and estimations of what may be NOAEL and MTC concentrations in a main study. The study director may choose to use the range-finding study to identify the threshold of respiratory tract irritation (e.g., with histopathology of the respiratory tract, pulmonary function testing, or bronchoalveolar lavage), the upper concentration which is tolerated without undue stress to the animals, and the parameters that will best characterize a test article's toxicity.

12. A range-finding study may consist of one or more concentration levels. No more than three males and three females should be exposed at each concentration level. A range-finding study should last a minimum of 5 days and generally no more than 14 days. The rationale for the selection of concentrations for the main study should be provided in the study report. The objective of the main study is to demonstrate a concentration-response relationship based on what is anticipated to be the most sensitive endpoint. The low concentration should ideally be a no-observed-adverse effect concentration while the high concentration should elicit unequivocal toxicity without causing undue stress to the animals or affecting their longevity (3).

13. When selecting concentration levels for the range-finding study, all available information should be considered including structure-activity relationships and data for similar chemicals. A range-finding study may verify/refute what are considered to be the most sensitive mechanistically based endpoints, e.g., cholinesterase inhibition by organophosphates, methaemoglobin formation by erythrocytotoxic agents, thyroidal hormones (T_3, T_4) for thyrotoxicants, protein, LDH, or neutrophils in brochoalveolar lavage for innocuous poorly soluble particles or pulmonary irritant aerosols .

Main Study

14. The main subacute toxicity study generally consists of three concentration levels, and also concurrent negative (air) and/or vehicle controls as needed (see paragraph 17). Each test group contains five male and five female rodents that are exposed to the test article for 6 hours per day on a 5 day per week basis for a period of 4 weeks (28 days). Animals may also be exposed 7 days per week (e.g. when testing inhaled pharmaceuticals). Males and females are always tested, but they may be exposed at different concentration levels if it is known that one sex is more sensitive to a given test article. This will minimize the possibility of one sex having excessive death at the high concentration and/or no effect at the low concentration. If rodent species other than rats are exposed nose-only, maximum exposure durations may be adjusted to minimise species-specific distress. A rationale must be provided when using an exposure duration less than 6 hours/day, or when it is necessary to conduct a long duration (e.g., 22 hours/day) whole-body exposure study (refer to GD 39) (2). Feed should be withheld during the exposure period unless exposure exceeds 6 hours. Water may be provided throughout a whole-body exposure.

15. The target concentrations selected should identify the target organ(s) and demonstrate a clear concentration-response:

- The high concentration level should result in toxic effects but not cause lingering signs or lethality which would prevent a meaningful evaluation.

- The intermediate concentration level(s) should be spaced to produce a gradation of toxic effects between that of the low and high concentration.

- The low concentration level should produce little or no evidence of toxicity.

Satellite (Reversibility) Study

16. A satellite (reversibility) study may be used to observe reversibility, persistence, or delayed occurrence of toxicity for a post-treatment period of an appropriate length, but no less than 14 days. Satellite (reversibility) groups consist of five males and five females exposed contemporaneously with the experimental animals in the main study. Satellite (reversibility) study groups should be exposed to the test article at the highest concentration level and there should be concurrent air and/or vehicle controls as needed (see paragraph 17).

Control Animals

17. Concurrent negative (air) control animals should be handled in a manner identical to the test group animals except that they are exposed to filtered air rather than test article. When water or another substance is used to assist in generating the test atmosphere, a vehicle control group, instead of a negative (air) control group, should be included in the study. Water should be used as the vehicle whenever possible. When water is used as the vehicle, the control animals should be exposed to air with the same relative humidity as the exposed groups. The selection of a suitable vehicle should be based on an appropriately conducted pre-study or historical data. If a vehicle's toxicity is not well known, the study director may choose to use both a negative (air) control and a vehicle control, but this is strongly discouraged. If historical data reveal that a vehicle is non-toxic, then there is no need for a negative (air) control group and only a vehicle control should be used. If a pre-study of a test article formulated in a vehicle reveals no toxicity, it follows that the vehicle is non-toxic at the concentration tested and this vehicle control should be used.

EXPOSURE CONDITIONS

Administration of Concentrations

18. 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. Particulate material may be subjected to mechanical processes to decrease the particle size. Further guidance is provided in GD 39 (2).

Particle-Size Distribution

19. 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 3 µm with a geometric standard deviation (σg) in the range of 1.5 to 3.0 are recommended (4). Although a reasonable effort should be made to meet this standard, expert judgement should be provided if it cannot be achieved. For example, metal fume particles may be smaller than this standard, and charged particles and fibers may exceed it.

Test Article Preparation in a Vehicle

20. Ideally, the test article should be tested without a vehicle. If it is necessary to use a vehicle to generate an appropriate test article concentration and particle size, water should be given preference. Whenever a test article is dissolved in a vehicle, its stability must be demonstrated.

MONITORING OF EXPOSURE CONDITIONS

Chamber Airflow

21. The flow of air through the exposure chamber should be carefully controlled, continuously monitored, and recorded at least hourly during each exposure. The real-time monitoring of the test atmosphere concentration (or temporal stability) is an integral measurement of all dynamic parameters and provides an indirect means to control all relevant dynamic inhalation parameters. If the concentration is monitored real-time, the frequency of measurement of air flows may be reduced to one single measurement per exposure per day. Special consideration should be given to avoiding rebreathing in nose-only chambers. Oxygen concentration should be at least 19% and carbon dioxide concentration should not exceed 1%. If there is reason to believe that this standard cannot be met, oxygen and carbon dioxide concentrations should be measured. If measurements on the first day of exposure show that these gases are at proper levels, no further measurements should be necessary.

Chamber Temperature and Relative Humidity

22. 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 continuously and recorded hourly during each exposure where possible. The relative humidity should preferably 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

23. 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 inhalation 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

24. The actual concentration is the test article concentration as sampled at the animals' breathing zone in an inhalation chamber. Actual concentrations can be obtained either 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 must be shown that all phases were collected by the method chosen.

25. One lot of the test article should be used throughout the duration of the study, 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 but is not limited by 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. color, physical nature, etc.).

26. The exposure atmosphere should be held as constant as practicable. A real-time monitoring device, such as an aerosol photometer for aerosols or a total hydrocarbon analyser for vapours may be used to demonstrate the stability of the exposure conditions. Actual chamber concentration should be measured at least 3 times during each exposure day for each exposure level. If not feasible due to limited air flow rates or low concentrations, one sample per exposure period is acceptable. Ideally, this sample should then be collected over the entire exposure period. Individual chamber concentration samples should deviate from the mean chamber concentration by no more than ±10% for gases and vapours, and by no more than ±20% for liquid or solid aerosols. Time to attain chamber equilibration (t_{95}) should be calculated and and reported. The duration of an exposure spans the time that the test article is generated. This takes into account the times required to attain chamber equilibration (t_{95}) and decay. Guidance for estimating t_{95} can be found in GD 39. (2)

27. The exposure atmosphere should be held as constant as practicable. A real-time monitoring device, such as an aerosol photometer for aerosols or a total hydrocarbon analyser for vapours, may be used to demonstrate the stability of the exposure conditions. Actual chamber concentration should be measured at least 3 times during each exposure day for each exposure level. If not feasible due to limited air flow rates or low concentrations, one sample per exposure period is acceptable. Ideally, this sample should then be collected over the entire exposure period. Individual chamber concentration samples should deviate from the mean chamber concentration by no more than ±10% for gases and vapours, and by no more than ±20% for liquid or solid aerosols. Time to attain chamber equilibration (t_{95}) should be calculated and reported for whole-body exposures, but is unnecessary for

nose-only exposures due to low chamber volume and rapid attainment of t_{95} relative to the exposure durtion. The duration of a whole-body exposure spans the time that the test article is generated, which takes into account the times to attain chamber equilibration and decay (t_{95}). Guidance for estimating t_{95} can be found in GD 39. (2)

28. 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. Therefore, at least one indicator substance (analyte), normally the principal active in the tested product formulation, of each phase (gas/vapour and aerosol) must 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

29. The particle size distribution of aerosols should be determined at least weekly for each concentration level by using a cascade impactor or an alternative instrument.such as an aerodynamic particle sizer (APS). A second device, such as a gravimetric filter or an impinger/gas bubbler, should be used 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 of the results obtained by a cascade impactor and the alternative instrument can be shown, then the alternative instrument may be used throughout the study. 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.

OBSERVATIONS

30. The animals should be clinically observed, before, during and after the exposure period. More frequent observations may be indicated depending on he response of the animals during exposure. When animal observation is hindered by the use of animal restraint tubes, poorly lit whole body chambers, or opaque atmospheres, animals should be carefully observed after exposure. Observations before the next day's exposure can assess any reversibility or exacerbation of toxic effects. The time of death or the time when an animal is found dead should be recorded.

31. All observations are recorded with individual records being maintained for each animal. Unless there are compelling reasons to do otherwise, animals found in a moribund condition and animals showing severe pain and/or enduring signs of severe distress should be humanely killed without delay for animal welfare reasons (3). When animals are killed for humane reasons or found dead, the time of death should be recorded as precisely as possible.

32. Cage-side observations should include changes in the skin and fur, eyes, and mucous membranes; changes in the respiratory and circulatory systems, changes in the autonomic and central nervous systems; and changes in somatomotor activity and behaviour patterns. Attention should be directed to observations of tremors, convulsions, salivation, diarrhoea, lethargy, sleep, and coma. The measurement of rectal temperatures may provide supportive evidence of reflex bradypnea or hypo/hyperthermia related to treatment or confinement. Additional assessments may be included in the study protocol such as kinetics, biomonitoring, lung function, and behavioural changes.

BODY WEIGHTS

33. Individual animal weights should be recorded shortly before the first exposure (day 0), twice weekly thereafter (generally on Fridays and Mondays to demonstrate recovery over an exposure-free

weekend), and at the time of death or euthanasia. If there are no effects in the first 2 weeks, body weights may be measured weekly for the remainder of the study. Satellite (reversibility) animals (if used) should continue to be weighed weekly throughout the recovery period. At study termination, all animals should be weighed shortly before sacrifice to allow for an unbiased calculated of organ to body weight ratios.

FOOD AND WATER CONSUMPTION

34. Food consumption should be measured weekly. Water consumption may also be measured.

CLINICAL PATHOLOGY

35. Clinical pathology assessments should be made for all animals, including control and satellite (reversibility) animals, when they are sacrificed. The time interval between the end of exposure and blood collection should be recorded, particularly when the reconstitution of the addressed endpoint is rapid. Sampling following the end of exposure is indicated for those parameters with a short plasma half-time (e.g., COHb, CHE, and MetHb).

36. Table 1 lists the clinical pathology parameters that are generally required for all toxicology studies. Urinalysis is not required on a routine basis, but may be performed when deemed useful based on expected or observed toxicity. The study director may choose to assess additional parameters in order to better characterize a test article's toxicity (e.g., cholinesterase, lipids, hormones, acid/base balance, methaemoglobin, creatine kinase, myeloid/erythroid ratio, troponins, and arterial blood gases).

Table 1. Standard Clinical Pathology Parameters

Haematology	
Erythrocyte count	Heinz bodies
Haematocrit	Total leukocyte count
Haemoglobin concentration	Differential leukocyte count
Mean corpuscular haemoglobin	Platelet count
Mean corpuscular volume	Clotting potential (select one):
Mean corpuscular haemoglobin concentration	Prothrombin time
	Clotting time
Reticulocytes	Partial thromboplastin time
Clinical Chemistry	
Glucose*	Alanine aminotransferase
Total cholesterol	Aspartate aminotransferase
Triglycerides	Alkaline phosphatase or Sorbitol dehydrogenase
Blood urea nitrogen	
Total bilirubin	Potassium
Creatinine	Sodium
Lactate dehydrogenase	Calcium
Total protein	Phosphorus
Albumin	Chloride
Globulin	
Urinalysis (optional)	
Appearance (colour and turbidity)	PH
Volume	Total protein
Specific gravity or osmolality	Glucose

* Because a lengthy fasting period can introduce bias in glucose measurements for the treated versus control animals, the study director should determine whether it is appropriate to fast the animals. If a fasting period is used, it should be appropriate to the species used; for the rat this may be 16 h (overnight fasting). Determination of fasting glucose may be carried out after overnight fasting during the last exposure week, or after overnight fasting prior to necropsy (in the latter case together with all other clinical pathology parameters).

37. When there is evidence that the lower respiratory tract (i.e., the alveoli) is the primary site of deposition and retention, then bronchoalveolar lavage (BAL) may be the technique of choice to quantitatively analyse hypothesis-based dose-effect parameters focusing on alveolitis, pulmonary inflammation, and phospholipidosis. This allows for dose-response and time-course changes of alveolar injury to be suitably probed. The BAL fluid may be analysed for total and differential leukocyte counts, total protein, and lactate dehydrogenase. Other parameters that may be considered are those indicative of lysosomal injury, phospholipidosis, fibrosis, and irritant or allergic inflammation which may include the determination of pro-inflammatory cytokines/chemokines. BAL measurements generally complement the results from histopathology examinations but cannot replace them. Guidance on how to perform lung lavage can be found in GD 39. (2)

GROSS PATHOLOGY AND ORGAN WEIGHTS

38. All test animals, including those which die during the test or are removed from the study for animal welfare reasons, should be subjected to complete exsanguination (if feasible) and gross necropsy. The time between the end of each animal’s last exposure and their sacrifice should be recorded. If a necropsy cannot be performed immediately after a dead animal is discovered, the animal should be refrigerated (not frozen) at a temperature 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.

39. Table 2 lists the organs and tissues that should be preserved in a suitable medium during gross necropsy for histopathological examination. The preservation of the [bracketed] organs and tissues and any other organs and tissues is at the discretion of the study director. The **bolded** organs should be trimmed and weighed wet as soon as possible after dissection to avoid drying. The thyroid and epididymides should only be weighed if needed because trimming artefacts may hinder histopathological evaluation. Tissues and organs should be fixed in 10% buffered formalin or another suitable fixative as soon as necropsy is performed, and no less than 48 hours prior to trimming.

Table 2. Organs and Tissues Preserved During Gross Necropsy

Adrenals
Bone marrow (and/or fresh aspirate)
Brain (including sections of cerebrum, cerebellum, and medulla/pons)
[Eyes (retina, optic nerve) and eyelids]

Heart
Kidneys
Larynx (3 levels, 1 level to include the base of the epiglottis)
Liver
Lung (all lobes at one level, including main bronchi)
Lymph nodes from the hilar region of the lung, especially for poorly soluble particulate test materials. For more in depth examinations and/or studies with immunological focus, additional lymph nodes may be considered, e.g. those from the mediastinal, cervical/submandibular and/or auricular regions.
Nasopharyngeal tissues (at least 4 levels; 1 level to include the nasopharyngeal duct and including Nasal Associated Lymphoid Tissue(NALT)
Oesophagus
[Olfactory bulb]
Ovaries
Seminal vesicles
Spinal cord (cervical, mid-thoracic, and lumbar)
Spleen
Stomach
Testes
Thymus
Thyroid
Trachea (at least 2 levels including 1 longitudinal section through the carina and 1 transverse section)
[Urinary bladder]
Uterus (including cervix)
All gross lesions

40. The lungs should be removed intact, weighed, and instilled with a suitable fixative at a pressure of 20-30 cm of water to approximately 80-90% of total lung capacity to ensure that lung structure is maintained (5). Sections should be collected for all lobes at one level, including main bronchi, but if lung lavage is performed, the unlavaged lobe should be sectioned at three levels (not serial sections).

41. At least 4 levels of the nasopharyngeal tissues should be examined, one of which should include the nasopharyngeal duct, (5, 6, 7, 8, 9) to allow adequate examination of the squamous, transitional (non-ciliated respiratory), respiratory (ciliated respiratory) and olfactory epithelium, and the draining lymphatic tissue (NALT; 10, 11). Three levels of the larynx should be examined, and one of these levels should include the base of the epiglottis (12). At least two levels of the trachea should be examined including one longitudinal section through the carina of the bifurcation of the extrapulmonary bronchi and one transverse section.

HISTOPATHOLOGY

42. A histopathological evaluation of all the organs and tissues listed in Table 2 should be performed for the control and high concentration groups, and for all animals which die or are sacrificed during the study. Particular attention should be paid to the respiratory tract, target organs, and gross lesions. The organs and tissues that have lesions in the high concentration group should be examined in all groups. The study director may choose to perform histopathological evaluations for additional groups

to demonstrate a clear concentration response. When a satellite (reversibility) group is used, histopathological evaluation should be performed for all tissues and organs identified as showing effects in the treated groups. If there are excessive early deaths or other problems in the high exposure group that compromise the significance of the data, the next lower concentration should be examined histopathologically. An attempt should be made to correlate gross observations with microscopic findings.

DATA AND REPORTING

Data

43. Individual animal data on body weights, food consumption, clinical pathology, gross pathology, organ weights, and histopathology 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. 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 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. Source and historical data may be provided, if they are from animals exposed under similar exposure, housing, and fasting conditions.
- Number, age, and sex of animals.
- Method of randomization.
- 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

- Detailed description of the inhalation chamber including volume and a diagram.
- Source and description of equipment used for the exposure of animals as well as generation of the atmosphere.
- Equipment for measuring temperature, humidity, particle-size, and actual concentration.
- Source of air 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 chamber (whole-body).
- Temporal homogeneity/stability of test atmosphere.
- Location of temperature and humidity sensors and sampling of test atmosphere in the chamber.
- Treatment of air supplied/extracted.
- Air flow rates, air flow rate/exposure port (nose-only), or animal load/chamber (whole-body).
- Time to inhalation chamber equilibrium (t_{95}).
- 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 (mg/L, mg/m^3, etc.) rather than in units of volume (ppm, ppb, etc.).
- Particle size distribution, mass median aerodynamic diameter (MMAD), and geometric standard deviation (σg), including their methods of calculation. Individual particle size analyses must 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.
- 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 equipment used to monitor chamber temperature, humidity, and chamber airflow (i.e. development of a calibration curve).
- Details of the equipment used to collect samples for determination of chamber concentration and particle size distribution.
- Details of the chemical analytical method used and method validation (including efficiency of recovery of test article from the sampling medium).

- Method of randomization in assigning animals to test and control groups.
- Details of food and water quality (including diet type/source, water source).
- 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).
- Tabulation of individual animal weights.
- Tabulation of food consumption
- Tabulation of clinical pathology data
- Necropsy findings and histopathological findings for each animal, if available.
- Tabulation of any other parameters measured

Discussion and interpretation of results

- Particular emphasis should be made to the description of methods used to meet the criteria of this test guideline, e.g., the limit concentration or the particle size.
- The respirability of particles in light of the overall findings must be addressed, especially if the particle-size criteria could not be met.
- The consistency of methods used to determine nominal and actual concentrations, and the relation of actual concentration to nominal concentration must 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.
- 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 (3).
- The target organ(s) should be identified.
- The NOAEL and LOAEL should be determined.

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ANNEX 1

Definitions

A selection of definitions will be added at a future date.