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

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

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

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

36. The following examinations should be made:

a) Haematology, including haematocrit, haemoglobin concentration, erythrocyte count, total and differential leucocyte count, and a measure of clotting potential, such as clotting time, prothrombin time, thromboplastin time, or platelet count should be investigated at the end of the test period.

b) Clinical biochemistry determinations in blood should also be carried out at the end of the study. The selection of specific tests will be influenced by observations on the mode of action of the substance. Suggested determinations are calcium, phosphorus, chloride, sodium, potassium, fasting glucose (with period of fasting appropriate to the species), serum alanine aminotransferase, serum aspartate aminotransferase, ornithine decarboxylase, gamma glutamyl transpeptidase, urea nitrogen, albumen, blood creatinine, total bilirubin and total serum protein measurements. Other determinations which may be necessary for an adequate toxicological evaluation include analyses of lipids, hormones, acid/base balance, methaemoglobin, cholinesterase activity, etc. Additional clinical biochemistry may be employed where necessary to extend the investigation of observed toxic effects.

c) Urinalysis is not required on a routine basis but only when there is an indication based on expected or observed toxicity.

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

Pathology

Gross necropsy

38. All animals in the study should be subjected to a full gross necropsy which includes examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents. The liver, kidneys, adrenals, heart, spleen, thymus, brain, and testes should be weighed wet as soon as possible after dissection to avoid drying. The following organs and tissues should be preserved in a suitable medium for possible future histopathological examination: nasal passages (including nasal-associated lymphoid tissue-NALT), larynx, trachea, lungs; which should be removed intact, weighed and treated with a suitable fixative to ensure that lung structure is maintained (perfusion with the fixative is considered to be an effective procedure), lymph nodes draining the respiratory tissues (cervical/mandibular and mediastinal/ tracheobronchial/hilar nodes), liver, kidneys, spleen, thymus, adrenals, heart, testes and any target organs, that is, those showing gross lesions or changes in size.

Histopathology

39. Histological examination should be performed on the preserved organs and tissues of the high concentration group and the control group(s). These examinations may be extended to animals of other concentration groups, if considered necessary to investigate the changes observed in the high concentration group. Animals in a satellite group should be examined histologically with particular emphasis on those organs and tissues identified as showing effects in the other treated groups.

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

EVALUATION OF RESULTS

41. The findings of a repeated dose inhalation study should be considered in terms of the toxic effects and the necropsy and histopathological findings. The evaluation will include the relationship between the concentration of the test substance and the duration of exposure, and the presence or absence, the incidence and severity, of abnormalities, including behavioural and clinical abnormalities, gross lesions, identified target organs, body weight changes, effects on mortality and any other general or specific toxic effects. A properly conducted 28-day or 14-day study should provide information on the effects of repeated inhalation exposure and can indicate the need for further longer term studies. It can also provide information on the selection of concentrations for longer term studies.

DATA AND REPORTING

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

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

Test Report

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

Test substance:

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

Vehicle:

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

Test animals

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

Test conditions

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

Test atmosphere data

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

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

Results:

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

Discussion and interpretation of results:

- discussion and interpretation of results, including technical aspects of difficulties arising from generation/characterization of the test atmospheres and discussion as to whether changes observed are caused by systemic or local effects
- concentration-response relationships and No-Observed-Adverse-Effect-Level (NOAEL)(if applicable)
- a repeated dose inhalation study will provide information on the effects of repeated inhalation exposure to a substance. Extrapolation from the results of the study to man is valid to a limited degree, but it can provide useful information on the toxicity and mode of action of the substance by the inhalation route.

Conclusions.

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ANNEX 1

DEFINITIONS

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

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

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

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

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

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

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

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

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

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