

14-day study)については、議論が行われなかった、

別添2(TG412、2008年1月28日修正案)と別添3(TG413、2008年1月23日修正案)に、配布された修正案を示した。なお、修正案ではTG412の名称が“Repeated dose inhalation toxicity: 28-day or 14-day study”から“Subacute Inhalation Toxicity”に変更されている。

2 TG412およびTG413の修正案への提案

TG412(2008年1月28日修正案)およびTG413(2008年1月23日修正案)の修正案について別添4の提案を行った。主な提案は、①全身暴露の推奨、②投与開始週齢、②病理検査の対象臓器の用語の統一である。

Comments for TG 412 and 413

1. Inhalation Chambers (TG 412 and 413)

The dynamic whole-body inhalation chamber should be preferred for subacute and subchronic inhalation toxicity studies to the dynamic nose-only chamber because of the following reasons.

1) In nose-only chamber, animals are held in restraining tubes/apparatus during exposure period. The restraint stress has long been known as a source of strong immunosuppression (Kanno J, et al. Effect of restraint stress on immune system and experimental B16 melanoma metastasis in aged mice. Mech Ageing Dev. 1997, 93:107-117) and possibly of alterations in basic metabolism due to strong stimuli to autonomous and sensory neural systems and starvation-linked pathways. Additional to this, our data shown in the Appendix animals restrained in the apparatus used for nose-only inhalation clearly showed reduction in thymus and spleen weight, These changes induced by standard procedure of nose-only inhalation can be a potent confounding factor to alter or bias the outcome of the toxicity of the test substance. The restraint stress is not a negligible factor for the evaluation of inhalation toxicity. Therefore, the nose-only inhalation study is biologically unfavorable for long term or repeated dosing inhalation studies, before a statement on strong restraint stress in terms of animal welfare is made.

2) Whole-body inhalation chamber has been widely used for inhalation toxicity studies, and many laboratories reserve the whole-body inhalation chambers to carry out inhalation toxicity study.

3) It is important to keep safe of the staffs working in the facility for inhalation study. To prevent leakage of test chemicals from the inhalation chamber into room, whole-body inhalation chamber is operated at negative pressure as compared to room atmosphere. On the other hand, nose-only chamber is operated generally at positive pressure to avoid influx of room air into inhalation chamber. We are concerned about leakage of test chemicals from nose-only inhalation chamber.

I would like to recommend correcting sentence 9 as follows:

The dynamic nose-only (the term “nose-only” includes head-only, nose-only, or snout-only) or dynamic whole-body chamber should be used for subacute (subchronic in TG 413) inhalation toxicity studies. The nature of the test article and the object of the test should be considered when selecting an inhalation chamber. Principles of the nose-only and whole-body exposure techniques and their particular advantages and disadvantages are addressed is DD 39 (2).

2. Preparation of Animals (TG 412 and 413)

I would like to recommend “6 to 9 weeks of age” instead of “7 to 9 weeks of age to assure full lung maturation”.

3. Clinical Pathology (TG 412 and 413)

Table 1. Standard Clinical Pathology Parameters

I would like to recommend “Heinz bodies or methaemoglobin” instead of “Heinz bodies”, because measurement of methaemoglobin is widely used as well as measurement of Heinz bodies for the same purpose.

4. Histopathology (TG 412 and 413)

Regarding the term of organs and tissues listed in Table 2 (Organs and Tissues Preserved During Gross Necropsy), there are some inconsistencies between TG 412 and 413 as follows:

- 1) [Eyes and eyelids (including retina)], [Optic nerve] in TG 412 vs [Eyes (retina, optic nerve) and eyelids] in TG 413
- 2) Nasopharyngeal tissues (at least 4 levels; 1 level to include the nasopharyngeal duct and including Nasal Associated Lymphoid Tissue (NALT)) in TG 412 vs Nasopharyngeal tissues (at least 4 levels; 1 level to include the nasopharyngeal duct), Nasal Associated Lymphoid Tissue (NALT), Nasal Turbinates (at least 4 levels) in TG 413
- 3) Trachea (at least 2 levels including 1 longitudinal section through the bifurcation and 1 transverse section) in TG 412 vs Trachea (at least 2 levels including 1 longitudinal section through the carina and 1 transverse section) in TG 413
- 4) Lymph nodes, draining upper (cervical/ submandibular) and lower (mediastinal/ tracheobronchial/ hilar) respiratory tract in TG 412 vs Upper (cervical/ submandibular) and lower (mediastinal/ tracheobronchial/ hilar) respiratory tract draining lymph nodes in TG 413

The trimming levels of nasopharyngeal tissues

Three levels should be acceptable.

Appendix (Jun Kanno and Yukio Ogawa)

Effect of Restraint Stress given by nose-only inhalation apparatus on Thymus and Spleen Weight of mice.

1. Methods

Animals: 12-week old male C57BL/6 mice. All mice were kept individually in wood-chip bedded polycarbonate plastic cages (including restrained mice with apparatus)

Treatment:

Group1 (Control group); No restraint (free access to food and drinking water)

Group2 (Starvation only group); No restraint (water and food were not supplied from 10:00 to 16:00 as same as Group3)

Group3 (Restraint Group); Restraint (water and food were not supplied during the restraint)

Restraintment apparatus: SoftRestraint (IX-XN1-SR: inExpose nose-only exposure SoftRestraint for mice, SCIREQ Scientific Respiratory Equipment Inc., (Montreal, Canada) was used. This type of soft restrainer is claimed to be less harmful/stressful than the hard tube-type restrainers.

The animals in the group 3 were held 6 hours (from 10:00 to 16:00)/day for two consecutive days in restraining tubes (room air).

Body weigh was measured at start and terminal of the restraint. All animals were necropsied at the terminal of the second day, and the thymus and spleen were weighed.

2. Result

	Group1 (Control) No restraint (free access to food and drinking water)	Group2 (Starvation only) No restraint (water and food were not supplied from 10:00 to 16:00 as same as Group3)	Group3 (Restraint) Restraint (water and food were not supplied during the restraint), 6 hours /day for 2 days
No. of animal	6	6	6
Body weight			

at start	27.68 ± 1.42 g	27.23 ± 1.63 g (98)	27.43 ± 1.82 g (99)
at terminal	27.18 ± 1.85 g	26.23 ± 1.97 g (97)	24.28 ± 1.18 g (89)**
Thymus weight			
absolute	38.38 ± 5.84 mg	35.38 ± 7.48 mg (92)	22.90 ± 9.37 mg (60)**
relative	0.142 ± 0.024 %	0.135 ± 0.025 % (95)	0.093 ± 0.036 % (66)*
Spleen weight			
absolute	74.25 ± 6.04 mg	66.88 ± 7.76 mg (90)	54.25 ± 8.17 mg (73)**
relative	0.275 ± 0.038 %	0.255 ± 0.025 % (93)	0.223 ± 0.030 % (81)*

Values were represented as mean ± SD

(): % of the control group.

** : T-test $p < 0.01$ between Control.

* : T-test $p < 0.05$ between Control.

3. Conclusion

A marked reduction in the thymus and spleen weight was indicated in the animals of Group3 which were held 6 hours /day for 2 days in the restraining tubes. Starvation had little contribution to this effect.

4. Acknowledgement: This study was humanely conducted under the regulation and permission of the Animal Care and Use Committee of the National Institute of Health Sciences (NIHS), Tokyo, Japan.

Figures:



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OECD GUIDELINE FOR THE TESTING OF CHEMICALS
DRAFT PROPOSAL FOR A REVISED GUIDELINE 412

Subacute Inhalation Toxicity

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. Although males and females are always tested, they may be exposed at different concentration levels to identify gender differences in sensitivity. This guideline also allows for the use of satellite groups.

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 study). The data can also provide information on the selection of concentrations for longer term studies. Definitions used in the context of this Guideline can be found in Annex 1.

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.
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). 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, ideally by adequately designed sighting studies, and justification for concentration selection should be provided.

5. Unless there are compelling reasons to do otherwise (these reasons must be described in the study report), moribund animals or animals obviously in pain or showing signs of severe and enduring distress should be humanely killed. These 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 (2).

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.

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 $\pm 20\%$ 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 $22 \pm 3^\circ$ C. The relative humidity should ideally be maintained in the range of 30 to 70%. 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 is recommended that they 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 dynamic nose-only chamber (the term "nose-only" includes head-only, nose-only, or snout-only) is generally preferred for subacute inhalation toxicity studies. The study director must provide justification for using other dynamic systems (*e.g.*, whole-body inhalation chambers). Principles of the nose-only and whole-body exposure techniques and their particular advantages and disadvantages have been published elsewhere (3), and are addressed in GD 39.

TOXICITY STUDIES

Limit Concentrations

10. In principle, the limit concentrations for sighting and main studies are 20 mg/L for gases, 20 mg/L for vapours, and 2 mg/L for liquid and solid aerosols (or the maximum obtainable concentration). Justification should be provided when higher concentrations are used.

Sighting Study

11. If appropriate, the selection of test concentrations for the main study should be based on an adequately designed and conducted sighting study. The sighting study should clearly identify the threshold of acute respiratory tract irritation, the upper concentration which is tolerated without undue stress to the animals, and the parameters that will best characterize the test article's toxicity. A sighting study may consist of one or more concentration levels. No more than 3 males and 3 females should be exposed at each concentration level. A sighting study should last a minimum of 5 days and generally lasts no more than 14 days. The rationale for the selection of concentrations for the main study should be provided in the study report.

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

Main Study

13. The main subacute toxicity study consists of three or more concentration levels, and also concurrent negative and vehicle controls as needed (see paragraph 16). Animals are exposed for 28 days (4 weeks). Although males and females are always tested, they may be exposed at different concentration levels to identify gender differences in sensitivity. 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 Study

14. A satellite 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 groups consist of 5 males and 5 females treated for 28 days with the test article at the high concentration level and also concurrent negative and vehicle controls as needed (see paragraph 16).

Control Animals

15. Concurrent negative 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 should be included in the study.

Water should be used as the vehicle whenever possible. The selection of a suitable vehicle should be based on an appropriately conducted pre-study or historical data.

EXPOSURE CONDITIONS

Administration of Concentrations

16. Animals are exposed to the test article for 6 hours per day on a 5 day per week basis for a period of at least 28 days (4 weeks). Animals may also be exposed 7 days per week (e.g. when testing inhaled pharmaceuticals). If mice are exposed nose-only, exposures must not exceed 4 hours/day. Justification should be provided if it is necessary to conduct a longer daily duration (e.g. 22 hours/day whole-body exposure study). Although males and females are always tested, they may be exposed at different concentration levels to yield optimal data and to identify gender differences in sensitivity. Feed should be withheld during the exposure period unless exposure exceeds 6 hours (4 hours for mice). Water may be provided throughout a whole-body exposure.

17. 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.

Particle-Size Distribution

18. 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 (σ_g) 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, fibers, 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

19. 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, stability and homogeneity of the tested solution must be demonstrated.

MONITORING OF EXPOSURE CONDITIONS

Chamber Airflow

20. The flow of air through the exposure chamber should be carefully controlled, continuously monitored, and recorded at least hourly during each exposure. The monitoring of the test atmosphere concentration (or stability) is an integral measurement of all dynamic parameters and provides an indirect mean to control all relevant dynamic inhalation parameters. Therefore, the frequency of measurement of air flows may be reduced to one single measurement per exposure per day. Oxygen concentration should be at least 19% and carbon dioxide concentration should not exceed 1%.

Chamber Temperature and Relative Humidity

21. Chamber temperature should be maintained at $22 \pm 3^\circ$ 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 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

22. 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. The nominal concentration is not used to characterize the animals' exposure, although it can be used to characterize gas exposure if an analytical method is not available (assuming 100% exposure efficiency with pressurized gases; not applicable to generated gases). The determination of nominal concentrations for solid test articles may require the dust generation system to be dismantled. If this results in day-to-day variability in actual test concentrations, nominal concentrations need not be measured.

Test Article: Actual Concentration

23. The actual concentration is the test article concentration at the animals' breathing zone in an inhalation chamber; samples are therefore taken in the breathing zone. 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. When using non-specific methods, stability of the test compound over the duration of the study must be known. If this information is not available, a reanalysis of the test material at regular intervals during the course of the study may be necessary. For aerosolised agents that may evaporate/sublimate, it must be shown that all phases were collected by the method chosen.

24. One lot of the test article should be used, if possible, throughout the duration of the study, and the research sample should be stored under conditions that maintain its purity and stability. Preferably prior to the start of the study, there should be a characterization of the test article including its purity and, if technically feasible, the name and quantities of unknown contaminants and impurities.

25. The exposure atmosphere should be held as constant as practicable. A 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. Individual chamber concentration samples should deviate from the mean chamber concentration by no more than $\pm 10\%$ for gases and vapours, and by no more than $\pm 20\%$ for liquid or solid aerosols. Time to chamber equilibration and decay (t_{95}) should be recorded for whole-body exposures, but is unnecessary for nose-only exposures due to low chamber volume.

26. 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), both phases may behave differently in an inhalation chamber. Therefore, at least one indicator substance (analyte) 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.

Test Article: Particle Size Distribution

27. The particle size distribution of aerosols should be determined at least weekly for each concentration level by using a cascade impactor or aerodynamic particle sizer (APS). A second device, such as a gravimetric filter or an impinger, should be used to confirm the accuracy 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). If equivalence of APS and impactor methods can be shown, then APS methods may be used throughout the study. Particle sizing should be performed for vapours if there is any possibility that vapour condensation will result in the formation of an aerosol.

OBSERVATIONS

28. The animals should be observed frequently during the exposure period. Careful clinical observations should be made during and following exposure, or more frequently when indicated by the response of the animals to treatment. When animal observation is hindered by the use of animal tubes or poorly lit whole body chambers, animals should be carefully observed after exposure and before the next exposure day in order to assess reversibility or exacerbation of toxic effects.

29. All observations are systematically 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. When animals are killed for humane reasons or found dead, the time of death should be recorded as precisely as possible. It is important to note that poor appearance immediately following exposure is generally not a treatment-related clinical sign.

30. 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.

31. Additional assessments may be included in the study protocol such as kinetics, biomonitoring, lung lavage, lung function, retention of poorly soluble materials that accumulate in lung tissue, and behavioural changes.

BODY WEIGHTS

32 Individual animal weights should be recorded shortly before the first exposure (day 0), twice weekly thereafter (preferably on Friday and Monday to demonstrate recovery over an exposure-free weekend), at the time of death or euthanasia, and at study termination. Satellite animals (if used) should continue to be weighed twice weekly throughout the recovery period and at study termination.

FOOD AND WATER CONSUMPTION

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

CLINICAL PATHOLOGY

34. Clinical pathology assessments should be made for all animals, including control and satellite animals, when they are sacrificed. If historical baseline data are inadequate, determination of clinical pathology

parameters before exposure commences should be considered. Periodic testing throughout the study is recommended for large animals. The time interval between exposure and blood collection should be defined. Rapid sampling after exposure is indicated for parameters with a short plasma half-time (e.g., COHb, CHE, and MetHb).

35. 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, and blood gases).

Table 1. Standard Clinical Pathology Parameters

Haematology	
Erythrocyte count (RBC)	Heinz bodies
Haematocrit (Hct)	Total leukocyte count (WBC)
Haemoglobin concentration (Hb)	Differential leukocyte count (Diffs)
Mean corpuscular haemoglobin (MCH)	Platelet count (Plate)
Mean corpuscular volume (MCV)	Clotting potential (select one):
Mean corpuscular haemoglobin concentration (MCHC)	Prothrombin time (PT)
Reticulocytes (Retics)	Clotting time (CT)
	Partial thromboplastin time (PTT)
Clinical Chemistry	
Fasting glucose (GLU)*	Alanine aminotransferase (ALT)
Total cholesterol (Chol)	Aspartate aminotransferase (AST)
Triglycerides (TRIG)	Alkaline phosphatase (ALP) or
Blood urea nitrogen (BUN)	Sorbitol dehydrogenase (SDH)
Total bilirubin (T. Bili)	Potassium (K)
Creatinine (Creat)	Sodium (Na)
Lactate dehydrogenase (LDH)	Calcium (Ca)
Total protein (T. Prot)	Phosphorus (P)
Albumin (Alb)	Chloride (Cl)
Globulin (Glob)	
Urinalysis (optional)	
Appearance (colour and turbidity)	pH
Volume	Total protein
Specific gravity or osmolality	Glucose

* Fasting period 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).

36. If bronchoalveolar lavage (BAL) is performed, it should be carried out in the same animals used for histopathological examination of the lungs in order to minimize animal usage. This is accomplished by using the right lung lobes for lavage and the left unlavaged lung for weighing and microscopic examination. The BAL fluid is analysed for total and differential cell 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. The targeted lavage volume (per cycle) should be 20 mL/kg-rat. If the focus of the study is structural lung changes such as emphysema and fibrosis, then lavaged lungs may be suitable for histopathology.

GROSS PATHOLOGY AND ORGAN WEIGHTS

37. 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 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.

38. Table 2 lists the organs and tissues that should be preserved in a suitable medium during gross necropsy for histopathological examination. The preservation of 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. 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. The organs within square brackets are optional.

Table 2. Organs and Tissues Preserved During Gross Necropsy

Adrenals	Seminal vesicles
Bone marrow (and/or fresh aspirate)	Spinal cord (cervical, mid-thoracic, and lumbar)
Brain (including sections of cerebrum, cerebellum, and medulla/pons)	Spleen
[Eyes and eyelids (including retina)]	Stomach
Heart	Testes
Kidneys	Thymus
Larynx (3 levels, 1 level to include the base of the ...epiglottis)	Thyroid
Liver	Trachea (at least 2 levels including 1 ...longitudinal section through the ...bifurcation and 1 transverse section)
Lung (all lobes at one level, including main bronchi)	[Urinary bladder]
Lymph nodes, draining upper (cervical/...submandibular) and lower (mediastinal/...tracheobronchial/hilar) respiratory tract	Uterus (including cervix)
Nasopharyngeal tissues (at least 4 levels; 1 level to include the nasopharyngeal duct and including Nasal Associated Lymphoid Tissue(NALT))	All gross lesions
Oesophagus	
Olfactory bulb	
[Optic nerve]	
Ovaries	

39. 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).

40. 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

41. 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. When a satellite 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

42. 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

43. 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, including source and historical data, and justification for using a species other than the rat.
- 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

- 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 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 and decay (t_{95}) for whole-body exposures.
- 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/m³, etc.) rather than in units of volume (ppm, ppb, etc.).
- Particle size distribution, mass median aerodynamic diameter (MMAD), and geometric

standard deviation (σ g), 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).

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 σ g.
- 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.

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.
- The target organ(s) should be identified.
- The NOAEL and LOAEL should be determined.

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OECD GUIDELINE FOR THE TESTING OF CHEMICALS
DRAFT PROPOSAL FOR A REVISED GUIDELINE 413

Subchronic Inhalation Toxicity

SUMMARY

This revised Toxicity Guideline 413 (TG 413) has been designed to fully characterize test article toxicity by the inhalation route for a subchronic duration, and to provide robust data for quantitative inhalation risk assessments. Groups of 10 male and 10 female rodents are exposed 6 hours per day for 90 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. Although males and females are always tested, they may be exposed at different concentration levels to identify gender differences in sensitivity. This guideline also allows for the use of satellite groups and interim sacrifices.

INTRODUCTION

1. OECD Guidelines are periodically reviewed in the light of scientific progress, animal welfare considerations, and changing regulatory needs. The original subchronic inhalation Test Guideline 413 (TG 413) was adopted in 1981 (1). TG 413 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 90 days (approximately 10% of the lifespan of a rat). The data derived from subchronic inhalation toxicity studies can be used for quantitative risk assessments. Definitions used in the context of this Guideline can be found in Annex 1.

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 other repeated exposure studies. 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.
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, ideally by adequately designed sighting studies, and justification for concentration selection should be provided.

5. Unless there are compelling reasons to do otherwise (these reasons must be described in the study report), moribund animals or animals obviously in pain or showing signs of severe and enduring distress should be humanely killed. These 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.

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 $\pm 20\%$ 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 identified, preferably with subcutaneous transponders, to facilitate observations and avoid confusion. The temperature of the experimental animal maintenance room should be $22 \pm 3^\circ$ C. The relative humidity should ideally be maintained in the range of 30 to 70%. 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 is recommended that they be acclimated to the restraining tubes. While animals are being exposed whole-body to an aerosol, they should be housed individually to prevent ingestion of 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 dynamic nose-only chamber (the term "nose-only" includes head-only, nose-only, or snout-only) is generally preferred for subchronic inhalation toxicity studies. The study director must provide justification for using other dynamic systems (*e.g.*, whole-body inhalation chambers). 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. The limit concentrations for sighting and main studies are 20 mg/L for gases, 20 mg/L for vapours (or the maximum obtainable concentration), and 2 mg/L for liquid and solid aerosols.

Sighting Study

11. The selection of test concentrations for the main study should be based on an adequately designed and conducted sighting study. The sighting study should clearly identify the threshold of acute respiratory tract irritation, the upper concentration which is tolerated without undue stress to the animals, and the parameters that will best characterize the test article's toxicity. A sighting 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 sighting study should last a minimum of 5 days. The rationale for the selection of concentrations for the main study should be provided in the study report.

12. When selecting concentration levels for the sighting study, all available information should be considered including structure-activity relationships and data for similar chemicals. A sighting study should verify/refute what are considered to be the most sensitive mechanistically based endpoints, e.g., CHE inhibition by organophosphates, methaemoglobin formation by erythrocytotoxic agents, thyroidal hormones (T₃, T₄) for thyrotoxicants, protein, neutrophils, and innocuous poorly soluble particles in bronchoalveolar lavage for pulmonary irritants.

Main Study

13. The main subchronic toxicity study consists of three or more concentration levels, and also concurrent negative and vehicle controls as needed (see paragraph 16). Animals are exposed for 90 days (13 weeks). Although males and females are always tested, they may be exposed at different concentration levels to identify gender differences in sensitivity. 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.

Interim Sacrifices

14. If interim sacrifices are planned, the number of animals at each exposure level should be increased by the number to be sacrificed before study completion.

Satellite Study

15. A satellite 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 groups consist of 10 males and 10 females treated for 90 days with the test article at the high concentration level and also concurrent negative and vehicle controls as needed (see paragraph 16).

Control Animals

16. Concurrent negative 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 should be included in the study. Water should be used as the vehicle whenever possible. The selection of a suitable vehicle should be