90 days. Where a vehicle is used to help generate an appropriate concentration of the substance in the atmosphere, a vehicle control group should be used. During the period of administration the animals are observed daily to detect signs of toxicity. Animals which die during the test are necropsied, and at the conclusion of the test surviving animals are sacrificed and necropsied.

B. DESCRIPTION OF THE TEST PROCEDURE

• Preparations

Healthy young adult animals are acclimatised to the laboratory conditions for at least 5 days prior to the test. Before the test, animals are randomised and assigned to the required number of groups. Where necessary, a suitable vehicle may be added to the test substance to help generate an appropriate concentration of the substance in the atmosphere. If a vehicle is used it should be shown not to influence absorption of the test substance or produce toxic effects.

• Experimental animals

Selection of species

A variety of test species may be used. This Guideline is intented primarily for use with rodents. Where a rodent is required, the preferred species is the rat. Commonly used laboratory strains of young healthy animals should be employed. At the commencement of the study the weight variation of animals should not exceed ± 20 per cent of the mean weight. Where a subchronic inhalation study is conducted as a preliminary to a long-term study, the same species and strain should be used in both studies.

Number and sex

At least 20 animals (10 female and 10 male) should be used for each test group. The females should be nulliparous and non-pregnant. If interim sacrifices are planned the number should be increased by the number of animals scheduled to be sacrificed before the completion of the study. In addition, a satellite group of 20 animals (10 animals per sex) may be treated with the high concentration level for 90 days and observed for reversibility, persistence, or delayed occurrence of toxic effects for a post-treatment period of appropriate length, normally not less than 28 days.

Housing and feeding conditions (before and after exposure)

The temperature in the experimental animal room should be 22°C (± 3°) and the relative humidity 30-70 per cent. When the lighting is artificial the sequence should be 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. Animals may be caged in groups by sex or individually; the number of animals per cage should not interfere with clear observation of each animal.

- Equipment

The animals should be tested in inhalation equipment designed to sustain a dynamic air flow of 12 to 15 air changes per hour and ensure an adequate oxygen content of 19 per cent and an evenly distributed exposure atmosphere. Where a chamber is used its design should minimise crowding of the test animals and maximise their exposure to the test substance. As a general rule to ensure stability of a chamber atmosphere, the total "volume" of the test animals should not exceed 5 per cent of the volume of the test chamber. Oro-nasal or head only exposure may be used if it is desirable to avoid concurrent exposure by the dermal or oral routes.

A dynamic inhalation system with a suitable analytical concentration control system should be used. The rate of air flow should be adjusted to ensure that conditions throughout the equipment are essentially the same. Maintenance of slight negative pressure inside the chamber will prevent leakage of the test substance into the surrounding areas.

- Test conditions

Exposure concentrations

At least three concentrations with a control and, where appropriate, a vehicle control (corresponding to the concentration of vehicle at the highest exposure level) should be used. Except for exposure to the test substance, animals in the control group should be handled in an identical manner to the test group animals. The highest concentration should result in toxic effects but not produce an incidence of fatalities which would prevent a meaningful evaluation. The lowest concentration should not produce any evidence of toxicity. Where there is a usable estimation of human exposures, the lowest concentration should exceed this. Ideally, the

intermediate concentration should produce minimal observable toxic effects. If more than one intermediate concentration is used, the concentrations should be spaced to produce a gradation of toxic effects. In the low and intermediate groups and in the controls the incidence of fatalities should be low, in order to permit a meaningful evaluation of the results.

In the case of potentially explosive test susbstances, care should be taken to avoid generating explosive concentrations.

Exposure time

The duration of daily exposure should be 6 hours after equilibration of the chamber concentrations. Other durations may be used to meet specific requirements.

Observation period

A careful clinical examination should be made at least once each day. Additional observations should be made daily with appropriate actions taken to minimise loss of animals to the study, e.g. necropsy or refrigeration of those animals found dead and isolation or sacrifice of weak or moribund animals.

- Procedure

The animals are exposed to the test substance ideally on a 7-day per week basis for a period of 90 days. However, based primarily on practical considerations, exposure on a 5-day per week basis is considered to be acceptable. Animals in a satellite group scheduled for follow-up observations should be kept for at least a further 28 days without treatment to detect recovery from, or persistence of, toxic effects. The temperature at which the test is performed should be maintained at 22°C (± 2°). Ideally, the relative humidity should be maintained between 30 and 70 per cent, but in certain instances (e.g. tests of aerosols) this may not be practicable. Food and water should be withheld during exposure.

- Physical measurements

Measurements or monitoring should be made of the following:

(a) The rate of air flow, preferably, should be monitored continuously.

(b) During the exposure period the actual concentrations of the test substance should be held as constant as practicable.

(c) During the development of the generating system, particle size analysis should be performed to establish the stability of aerosol concentrations. During exposure, analysis should be conducted as often as necessary to determine the consistency of particle size distribution.

(d) Temperature and humidity (preferably continuously).

• Clinical examinations

Animals should be observed during and following exposure. Observations are made and recorded systematically; individual records should be maintained for each animal. All the animals should be observed daily and signs of toxicity recorded including the time of onset, the degree and duration. Cage-side observations should include, but not be limited to, changes in the skin and fur, eyes and mucous membranes and also respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behaviour pattern. Measurements should be made of food consumption weekly and the animals weighed weekly. Regular observation of the animals is necessary to ensure that animals are not lost from the study due to causes such as cannibalism, autolysis of tissues or misplacement. At the end of the study period all survivors in the non-satellite treatment groups are sacrificed. Moribund animals should be removed and sacrificed when noticed.

The following examinations should be made:

(a) Ophthalmological examination, using an ophthalmoscope or equivalent suitable equipment, should be made prior to exposure to the test substance and at the termination of the study, preferably in all animals but at least in the high dose and control groups. If changes in the eyes are detected, all animals should be examined.

(b) 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 study.

(c) Clinical biochemistry determination in blood should be carried out at the end of the test period. Test areas which are considered appropriate to all studies are electrolyte balance, carbohydrate metabolism, liver and kidney function. 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 glutamic-pyruvic transaminase*, serum glutamic-oxaloacetic transaminase**, ornithine decarboxylase, gamma glutamyl transpeptidase, urea nitrogen, albumen, blood creatinine, total bilirubin and total serum protein measurement. Other determinations which may be necessary for an adequate toxicological evaluation include analyses of lipids, hormones, acid/base balance, methaemoglobin, and cholinesterase activity. Additional clinical biochemistry may be employed where necessary to extend the investigation of observed effects.

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

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

- Pathology

Gross necropsy

All animals 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 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: all gross lesions, 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), naso-pharyngeal tissues, brain - including sections of medulla/pons, cerebellar cortex and

* Now known as serum alanine aminotransferase.

** Now known as serum aspartate aminotransferase.

cerebral cortex, pituitary, thyroid/parathyroid, thymus, trachea, heart, aorta, salivary glands, liver, spleen, kidneys, adrenals, pancreas, gonads, uterus, (accessory genital organs), (skin), gall bladder (if present), oesophagus, stomach, duodenum, jejunum, ileum, caecum, colon, rectum, urinary bladder, representative lymph node, (female mammary gland), (thigh musculature), peripheral nerve, (eyes), sternum with bone marrow, (femur - including articular surface), (spinal cord at three levels - cervical, midthoracic and lumbar), and (exorbital lachrymal glands). (The tissues between brackets need only be examined if indicated by signs of toxicity or target organ involvement.)

Histopathology

(a) Full histopathology should be carried out on the respiratory tract and other organs and tissues of all animals in the control and high dose groups.

(b) All gross lesions should be examined.

(c) Target organs in other dose groups should be examined.

(d) Lungs of animals in the low and intermediate dose groups should also be subjected to histopathological examination, since this can provide a convenient assessment of the state of health of the animals. Further histopathological examination may not be required routinely on the animals in these groups but must always be carried out on organs which showed evidence of lesions in the high dose group.

(e) When a satellite group is used, histopathology should be performed on tissues and organs identified as showing effects in other treated groups.

3. DATA AND REPORTING

- Treatment of results

Data may be summarised in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing lesions, the types of lesions, and the percentage of animals displaying each type of lesion.

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

- Evaluation of results

The findings of a subchronic inhalation toxicity study should be evaluated in conjunction with the findings of preceding studies and considered in terms of the observed toxic effects and the necropsy and histopathological findings. The evaluation will include the relationship between the concentration of the test substance and 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 90-day subchronic test should provide a satisfactory estimation of a no-effect level.

- Test report

The test report should include the following information:

(a) Test conditions

Description of exposure apparatus, including design, type, dimensions, source of air, system for generating particulates and aerosols, method of conditioning air, treatment of exhaust air and the method of housing animals in a test chamber when this is used.

The equipment for measuring temperature, humidity and particulate/aerosol concentrations and size should be described.

(b) Exposure data

These should be tabulated and presented with mean values and a measure of variability (e.g. standard deviation) and should include:

- airflow rates through the inhalation equipment;
- temperature and humidity of air;
- nominal concentration (total amount of test substance fed into the inhalation equipment divided by volume of air);
- actual concentration in test breathing zone; and
- particle size distribution (e.g. median aerodynamic diameter of particles with standard deviation from the mean).

(c) Animal data

- species/strain used;
- toxic response data by sex and concentration;
- time of death during the study or whether animals survived to termination;
- toxic or other effects;
- the time of observation of each abnormal sign and its subsequent course;
- food and body weight data;
- haematological tests employed and results with relevant baseline data;
- clinical biochemistry tests employed and results with relevant baseline data;
- necropsy findings;
- a detailed description of all histopathological findings; and
- statistical treatment of results where appropriate.

• Interpretation of the results

A subchronic 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 a substance by the inhalation route, on no-effect levels and on permissible human exposure.

4. LITERATURE

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