# B.8. REPEATED DOSE (28 DAYS) TOXICITY (INHALATION)

## 1. METHOD

### 1.1. INTRODUCTION

It is useful to have preliminary information on the particle size distribution, the vapour pressure, the melting point, the boiling point, the flash point and explosivity (if applicable) of the substance.

See also General Introduction Part B (A).

### 1.2. DEFINITION

See General Introduction Part B (B).

### 1.3. REFERENCE SUBSTANCES

None.

### 1.4. PRINCIPLE OF THE TEST METHOD

Several groups of experimental animals are exposed daily for a defined period to the test substance in graduated concentrations, one concentration being used per group, for a period of 28 days. Where a vehicle is used to help generate an appropriate concentration of the test 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 necropsied.

### 1.5. QUALITY CRITERIA

None.

### 1.6. DESCRIPTION OF THE TEST METHOD

#### 1.6.1. Preparations

The animals are kept under the experimental housing and feeding conditions for at.least five days prior to the experiment. Before the test, healthy young animals are randomized 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 or other additive is used to facilitate dosing, it should be known not to produce toxic effects. Historical data can be used if appropriate.

#### 1.6.2. Test Conditions

##### 1.6.2.1. Experimental Animals

Unless there are contra-indications, the rat is the preferred species. Commonly used laboratory strains of young healthy animals should be employed.

At the commencement of the study the range of weight variation in the animals used should not exceed ± 20 % of the appropriate mean value.

1.6.2.2. Number and Sex

At least 10 animals (five female and five male) should be used for each test group. The females should be nulliparous and non-pregnant. If interim sacrifices are planned, the numbers should be increased by the number of animals scheduled to be sacrificed before the completion of the study. In addition, a satellite group of 10 animals (five animals per sex) may be treated with the high concentration level for 28 days and observed for reversibility, persistence, or delayed occurrence of toxic effects for 14 days post-treatment. A satellite group of 10 control animals (five animals per sex) is also used.

1.6.2.3. Exposure Concentration

At least three concentrations are required, with a control or a vehicle control (corresponding to the concentration of vehicle at the highest level) if a vehicle is used. Except for treatment with 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 no, or few, fatalities. The lowest concentration should not produce any evidence of toxicity .Where there is a usable estimation of human exposure, 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 to permit a meaningful evaluation of the results.

1.6.2.4. Exposure Time

The duration of daily exposure should be six hours but other periods may be needed to meet specific requirements.

1.6.2.5. Equipment

The animals should be tested in inhalation equipment designed to sustain a dynamic airflow of at least 12 air changes per hour to ensure an adequate oxygen content and an evenly distributed exposure atmosphere. Where a chamber is used its design should minimize crowding of the test animals and maximize their exposure by inhalation of 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% of the volume of the test chamber. Oro-nasal, head only, or individual whole body chamber exposure may be used; the first two will minimize uptake by other routes.

1.6.2.6. Observation Period

The experimental animals should be observed daily for signs of toxicity during the entire treatment and recovery period. The time of death and the time at which signs of toxicity appear and disappear should be recorded.

1.6.3. Procedure

The animals are exposed to the test substance daily, five to seven days per week, for a period of 28 days. Animals in any satellite groups scheduled for follow-up observations should be kept for a further 14 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 ± 3 °C.

Ideally, the relative humidity should be maintained between 30 and 70 %, but in certain instances (e.g. tests of some aerosols) this may not be practicable. Maintenance of a slight negative pressure inside the chamber (≤ 5 mm of water) will prevent leakage of the test substance into the surrounding area. Food and water should be withheld during exposure.

A dynamic inhalation system with a suitable analytical concentration control system should be used. To establish suitable exposure concentrations a trial test is recommended. The airflow should be adjusted to ensure that conditions throughout the exposure chamber are homogeneous. The system should ensure that stable exposure conditions are achieved as rapidly as possible.

Measurements or monitoring should be made:

(a) of the rate of airflow (continuously);

(b) of the actual concentration of the test substance measured in the breathing zone. During the daily exposure period the concentration should not vary by more than ± 15% of the mean value. However, in the case of some aerosols, this level of control may not be achievable and a wider range would then be acceptable. During the total duration of the study, the day-to-day concentrations should be held as constant as practicable. For aerosols, at least one particle size analysis should be performed per test group weekly;

(c) of temperature and humidity, continuously if possible.

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, their degree and duration. Observations should include changes in the skin and fur, eyes, mucous membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behaviour pattern. Measurements should be made weekly of the animals' weight. It is also recommended that food consumption is measured 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 necropsied. Moribund animals and animals in severe distress or pain should be removed when noticed, humanely killed and necropsied.

The following examinations shall be made at the end of the test on all animals including the controls:

(i) haematology, including at least haematocrit, haemoglobin concentration, erythrocyte count, total and differential leucocyte count and a measure of clotting potential;

(ii) clinical blood biochemistry including at least one parameter of liver and kidney function: serum alanine aminotransferase (formerly known as glutamic pyruvic transaminase), serum aspartate aminotransferase (formerly known as glutamic oxaloacetic transaminase), urea nitrogen, albumin, blood creatinine, total bilirubin and total serum protein measurements;

Other determinations which may be necessary for an adequate toxicological evaluation include calcium, phosphorus, chloride, sodium, potassium, fasting glucose analysis of lipids, hormones, acid/base balance, methaemoglobin and cholinesterase activity.

Additional clinical biochemistry may be employed, where necessary, to extend the investigation of observed toxic effects.

1.6.3.1. Gross Necropsy

All animals in the study should be subjected to a full gross necropsy. At least the liver, kidneys, adrenals, lungs, and testes should be weighed wet as soon as possible after dissection to avoid drying. Organs and tissues (the respiratory tract, liver, kidneys, spleen, testes, adrenals, heart, and any organs showing gross lesions or changes in size) should be preserved in a suitable medium for possible future histopathological examination. The lungs should be removed intact, weighed and treated with a suitable fixative to ensure that lung structure is maintained.

1.6.3.2. Histopathological Examination

In the high-concentration group and in the control(s), histological examination should be performed on preserved organs and tissues. Organs and tissues showing defects attributable to the test substance at the highest dosage level should be examined in alllower-dosage groups. Animals in any satellite groups should be examined histologically with particular emphasis on those organs and tissues identified as showing effects in the other treated groups.

2. **DATA**

Data should be summarized in tabular form, showing for each test group the number of animals at the start of the test and the number of animals displaying each type of lesion.

All observed results should be evaluated by an appropriate statistical method. Any recognized statistical method may be used.

3. **REPORTING**

3.1. TEST REPORT

The test report shall, if possible, include the following information:

-species, strain, source, environmental conditions, diet, etc.;

-test conditions:

Description of exposure apparatus including design, type, dimensions, source of air, system for generating 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, where appropriate, stability of aerosol concentrations or particle size distribution, should be described.

Exposure data:

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

a) airflow rates through the inhalation equipment;
b) temperature and humidity of air;
c) nominal concentrations (total amount of test substance fed into the inhalation equipment divided by the volume of air);
d) nature of vehicle, if used;
e) actual concentrations in test breathing zone;
f) the mass median aerodynamic diameter (MMAD) and the geometric standard deviation (GSD);

-toxic response data by sex and concentration;

-time of death during the study or whether animals survived to termination;

-description of toxic or other effects; no-effect level;

-the time of observation of each abnormal sign and its subsequent course;

-food and body-weight data;

-haematological tests employed and results;

-clinical biochemistry tests employed and results;

-necropsy findings;

-a detailed description of all histopathological findings;

-a statistical treatment of results where possible;

-discussion of the results;

-interpretation of results.

3.2. EVALUATION AND INTERPRETATION

See General Introduction Part B (D).

4. **REFERENCES**

See General Introduction Part B (E).