

## B.7. REPEATED DOSE (28 DAYS) TOXICITY (ORAL)

### 1. **METHOD**

#### 1.1 **Introduction**

See General Introduction Part B.

#### 1.2 **Definitions**

See General Introduction Part B.

#### 1.3 **Principle of the test method**

The test substance is orally administered daily in graduated doses to several groups of experimental animals, one dose level per group for a period of 28 days. During the period of administration the animals are observed closely, each day for signs of toxicity. Animals which die or are killed during the test are necropsied and at the conclusion of the test surviving animals are killed and necropsied.

This method places more emphasis on neurological effects as a specific endpoint, and the need for careful clinical observations of the animals, so as to obtain as much information as possible, is stressed. The method should identify chemicals with neurotoxic potential, which may warrant further indepth investigation of this aspect. In addition, the method may give an indication of immunological effects and reproductive organ toxicity.

#### 1.4 **Description of the test method**

##### 1.4.1 *Preparations*

Healthy young adult animals are randomly assigned to the control and treatment groups. Cages should be arranged in such a way that possible effects due to cage placement are minimized. The animals are identified uniquely and kept in their cages for at least five days prior to the start of the study to allow for acclimatisation to the laboratory conditions.

The test substance is administered by gavage or via the diet or drinking water. The method of oral administration is dependent on the purpose of the study, and the physical/chemical properties of the substance.

Where necessary, the test substance is dissolved or suspended in a suitable vehicle. It is recommended that, wherever possible, the use of an aqueous solution/suspension be considered first, followed by consideration of a solution/emulsion in oil (e.g. corn oil) and then by possible solution in other vehicles. For vehicles other than water the toxic characteristics of the vehicle must be known. The stability of the test substance in the vehicle should be determined.

##### 1.4.2 *Test conditions*

###### 1.4.2.1 Test animals

The preferred rodent species is the rat, although other rodent species may be used. Commonly used laboratory strains of young healthy adult animals should be employed. The females should be nulliparous and non-pregnant. Dosing should begin as soon as possible after weaning and, in any case, before the animals are nine weeks old.

At the commencement of the study the weight variation of animals used should be minimal and not exceed  $\pm 20\%$  of the mean weight of each sex.

Where a repeated dose oral study is conducted as a preliminary to a long term study, preferably animals from the same strain and source should be used in both studies.

###### 1.4.2.2 Number and sex

At least 10 animals (five female and five male) should be used at each dose level. If interim kills are planned, the number should be increased by the number of animals scheduled to be killed before the completion of the study.

In addition, a satellite group of 10 animals (five animals per sex) may be treated with the high dose 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.

#### 1.4.2.3 Dose levels

Generally, at least three test groups and a control group should be used. Except for treatment with the test substance, animals in the control group should be handled in an identical manner to the test group subjects. If a vehicle is used in administering the test substance, the control group should receive the vehicle in the highest volume used.

If from assessment of other data, no effects would be expected at a dose of 1000 mg/kg bw/d, a limit test may be performed. If there are no suitable data available, a range finding study may be performed to aid the determination of the doses to be used.

Dose levels should be selected taking into account any existing toxicity and (toxico-) kinetic data available for the test substance or related materials. The highest dose level should be chosen with the aim of inducing toxic effects but not death or severe suffering. Thereafter, a descending sequence of dose levels should be selected with a view to demonstrating any dosage related response and no-observed-adverse effects at the lowest dose level (NOAEL). Two to four fold intervals are frequently optimal for setting the descending dose levels and addition of a fourth test group is often preferable to using very large intervals (e.g. more than a factor of 10) between dosages.

For substances administered via the diet or drinking water it is important to ensure that the quantities of the test substance involved do not interfere with normal nutrition or water balance. When the test substance is administered in the diet either a constant dietary concentration (ppm) or a constant dose level in terms of the animals' body weight may be used; the alternative used must be specified. For a substance administered by gavage, the dose should be given at similar times each day, and adjusted as necessary to maintain a constant dose level in terms of animal body weight.

Where a repeated dose study is used as a preliminary to a long term study, a similar diet should be used in both studies.

#### 1.4.2.4 Limit test

If a test at one dose level of at least 1000 mg/kg body weight/day or, for dietary or drinking water administration, an equivalent percentage in the diet or drinking water (based upon body weight determinations), using the procedures described for this study, produces no observable toxic effects and if toxicity would not be expected based upon data from structurally related substances, then a full study using three dose levels may not be considered necessary. The limit test applies except when human exposure indicates the need for a higher dose level to be used.

#### 1.4.2.5 Observation period

The observation period should be 28 days. Animals in a satellite group scheduled for follow-up observations should be kept for at least a further 14 days without treatment to detect delayed occurrence, or persistence of, or recovery from toxic effects.

### 1.4.3 Procedure

The animals are dosed with the test substance daily seven days each week for a period of 28 days; use of a five-day per week dosing regime needs to be justified. When the test substance is administered by gavage, this should be done in a single dose to the animals using a stomach tube or a suitable intubation cannula. The maximum volume of liquid that can be administered at one time depends on the size of the test animal. The volume should not exceed 1 ml/100 g body weight, except in the case of aqueous solutions where 2 ml/100 g body weight may be used. Except for irritating or corrosive substances which will normally reveal exacerbated effects with higher concentrations, variability in test volume should be minimised by adjusting the concentration to ensure a constant volume at all dose levels.

#### 1.4.3.1 General observations

General clinical observations should be made at least once a day, preferably at the same time(s) each day and considering the peak period of anticipated effects after dosing. The health condition of the animals should be recorded. At least twice daily, all animals are observed for morbidity and mortality. Moribund animals and animals in severe distress or pain should be removed when noticed, humanely killed and necropsied.

Once before the first exposure (to allow for within-subject comparisons), and at least once a week thereafter, detailed clinical observations should be made in all animals. These observations should be made outside the home cage in a standard arena and preferably at the same time, each 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 the 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, stereotypes (e.g. excessive grooming, repetitive circling) or bizarre behaviour (e.g. self-mutilation, walking backwards) should also be recorded.

In the fourth exposure week sensory reactivity to stimuli of different types (e.g. auditory, visual and proprioceptive stimuli), assessment of grip strength and motor activity assessment should be conducted. Further details of the procedures that could be followed are given in the literature (see General Introduction Part B).

Functional observations conducted in the fourth exposure week 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 dose study may enhance the ability to select dose 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 significantly interfere with the functional test performance.

#### 1.4.3.2 Body weight and food/water consumption

All animals should be weighed at least once a week. Measurements of food and water consumption should be made at least weekly. If the test substance is administered via the drinking water, water consumption should also be measured at least weekly.

#### 1.4.3.3 Haematology

The following haematological examinations should be made at the end of the test period: haematocrit, haemoglobin concentration, erythrocyte count, total and differential leucocyte count, platelet count and a measure of blood clotting time/potential.

Blood samples should be taken from a named site just prior to or as part of the procedure for killing the animals, and stored under appropriate conditions.

#### 1.4.3.4 Clinical biochemistry

Clinical biochemistry determinations to investigate major toxic effects in tissues and, specifically, effects on kidney and liver, should be performed on blood samples obtained of all animals just prior to or as part of the procedure for killing the animals (apart from those found moribund and/or intercurrently killed). Overnight fasting of the animals prior to blood sampling is recommended<sup>1</sup>. Investigations of plasma or serum shall include sodium, potassium, glucose, total cholesterol, urea, creatinine, total protein and albumin, at least two enzymes indicative of hepatocellular effects (such as alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, gamma glutamyl transpeptidase, and sorbitol dehydrogenase). Measurements of additional enzymes (of hepatic or other origin) and bile acids may provide useful information under certain circumstances.

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<sup>1</sup> For a number of measurements in serum and plasma, most notably for glucose, overnight fasting would be preferable. The major reason for this preference is that the increased variability which would inevitably result from non-fasting, would tend to mask more subtle effects and make interpretation difficult. On the other hand, however, overnight fasting may interfere with the general metabolism of the animals and, particularly in feeding studies, may disturb the daily exposure to the test substance. If overnight fasting is adopted, clinical biochemical determinations should be performed after the conduct of functional observations in week 4 of the study.

Optionally, the following urine analysis determinations could be performed during the last week of the study using timed urine volume collection; appearance, volume, osmolality or specific gravity, pH, protein, glucose and blood/blood cells.

In addition, studies to investigate serum markers of general tissue damage should be considered. Other determinations that should be carried out if the known properties of the test substance may, or are suspected to, affect related metabolic profiles include calcium, phosphate, fasting triglycerides, specific hormones, methaemoglobin and cholinesterase. These need to be identified for substances in certain classes or on a case-by-case basis.

Overall, there is a need for a flexible approach, depending on the species and the observed and/or expected effect with a given substance.

If historical baseline data are inadequate, consideration should be given to determination of haematological and clinical biochemistry variables before dosing commences.

#### 1.4.3.5 Gross necropsy

All animals in the study shall be subjected to a full, detailed gross necropsy which includes careful examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents. The liver, kidneys, adrenals, testes, epididymides, thymus, spleen, brain and heart of all animals should be trimmed of any adherent tissue, as appropriate, and their wet weight taken as soon as possible after dissection to avoid drying.

The following tissues should be preserved in the most appropriate fixation medium for both the type of tissue and the intended subsequent histopathological examination: all gross lesions, brain (representative regions including cerebrum, cerebellum and pons), spinal cord, stomach, small and large intestines (including Peyer's patches), liver, kidneys, adrenals, spleen, heart, thymus, thyroid, trachea and lungs (preserved by inflation with fixative and then immersion), gonads, accessory sex organs (e.g. uterus, prostate), urinary bladder, lymph nodes (preferably one lymph node covering the route of administration and another one distant from the route of administration to cover systemic effects), peripheral nerve (sciatic or tibial) preferably in close proximity to the muscle, and a section of bone marrow (or, alternatively, a fresh mounted bone marrow aspirate). The clinical and other findings may suggest the need to examine additional tissues. Also any organs considered likely to be target organs based on the known properties of the test substance should be preserved.

#### 1.4.3.6 Histopathological examination

Full histopathology should be carried out on the preserved organs and tissues of all animals in the control and high dose groups. These examinations should be extended to animals of all other dosage groups, if treatment-related changes are observed in the high dose group.

All gross lesions shall be examined.

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

## 2. DATA

Individual data should be provided. Additionally, all data should be summarised in tabular form showing for each test group the number of animals at the start of the test, the number of animals found dead during the test or killed for humane reasons and the time of any death or humane kill, the number showing signs of toxicity, a description of the signs of toxicity observed, including time of onset, duration, and severity of any toxic effects, the number of animals showing lesions, the type of lesions and the percentage of animals displaying each type of lesion.

When possible, numerical results should be evaluated by an appropriate and generally acceptable statistical method. The statistical methods should be selected during the design of the study.

### 3. **REPORTING**

#### **Test report**

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

##### *Test animals :*

- species/strain used;
- number, age and sex of animals;
- source, housing conditions, diet, etc.;
- individual weights of animals at the start of the test in weekly intervals thereafter and at the end of the test

##### *Test conditions :*

- justification for choice of vehicle, if other than water;
- rationale for dose level selection;
- details of test substance formulation/diet preparation, achieved concentration, stability and homogeneity of the preparation;
- details of the administration of the test substance;
- conversion from diet/drinking water test substance concentration (ppm) to the actual dose (mg/kg body weight/day), if applicable;
- details of food and water quality

##### *Results :*

- body weight/body weight changes;
- food consumption, and water consumption, if applicable;
- toxic response data by sex and dose level, including signs of toxicity;
- nature, severity and duration of clinic observations (whether reversible or not);
- sensory activity, grip strength and motor activity assessments;
- haematological tests with relevant base-line values;
- clinical biochemistry tests with relevant base-line values;
- body weight at killing and organ weight data;
- necropsy findings;
- a detailed description of all histopathological findings;
- absorption data if available;
- statistical treatment of results, where appropriate.

##### *Discussion of results.*

##### *Conclusions.*

### 4. **REFERENCES**

This method is analogous to OECD TG 407.