No-effect level/No-toxic-effect level/No-adverse-effect level is the maximum dose used in a test which produces no adverse effects. A no-effect level is expressed in terms of the weight of a substance given daily per unit weight of test animal (mg/kg). When administered to animals in food or drinking water the no-effect level is expressed as mg/kg of food or mg/ml of water.

- Principle of the test method

The test substance is administered orally in graduated daily doses to several groups of experimental animals, one dose per group, for a period of 90 days. During the period of administration the animals are observed daily to detect signs of toxicity. Animals which die during the period of administration are necropsied, and at the conclusion of the test all surviving animals are sacrificed and necropsied and appropriate histopathological examinations carried out.

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 randomised and assigned to the treatment groups. The test substance may be administered in the diet, by gavage, in capsules or in the drinking water. All animals should be dosed by the same method during the entire experimental period. If a vehicle or other additives are used to facilitate dosing they should not interfere with absorption of the test substance or produce toxic effects.

- Experimental animals

Selection of species

A variety of test species may be used, although this Guideline is intended for rodents. When a rodent is required the preferred species is the rat. (For a non-rodent subchronic oral toxicity study, see Test Guideline 409.) Commonly used laboratory strains of young, healthy animals should be employed and dosing should begin as soon as possible after weaning, ideally before the rats are 6, and in any case not more than 8, weeks old. At the commencement of the

study the weight variation of animals used should not exceed ± 20 per cent of the mean weight. Where a subchronic oral 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 at each dose level. It is, however, considered that in view of the importance of the subchronic study, it would be advantageous to increase the number of animals per sex per 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. The number of animals at the termination of the study must be adequate for a meaningful evaluation of toxic effects. In addition, a satellite group of 20 animals (10 of each sex) may be treated with the high dose 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

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. The choice of diet may be influenced by the need to ensure a suitable admixture of a test substance when administered by this method. Animals may be caged in groups by sex or individually; for group caging not more than five animals should be housed per cage.

- Test conditions

Dose levels

At least three dose levels 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. The highest dose level should result in toxic effects but not produce an

incidence of fatalities which would prevent a meaningful evaluation. The lowest dose level should not produce any evidence of toxicity. Where there is a usable estimation of human exposure the lowest level should exceed this. Ideally, the intermediate dose level(s) should produce minimal observable toxic effects. If more than one intermediate dose is used the dose levels 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.

For substances of low toxicity it is important to ensure that when administered in the diet quantities of the test substance involved do not interfere with normal nutrition. 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 at intervals (weekly or bi-weekly) to maintain a constant dose level in terms of animal body weight. Where a subchronic study is used as a preliminary to a long term study, a similar diet should usually be used in both studies.

Limit test

If a test at one dose level of at least 1000 mg/kg body weight (but expected human exposure may indicate the need for a higher dose level), 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 compounds, then a full study using three dose levels may not be considered necessary.

Observations

The observation period should be for at least 90 days. Animals in a satellite group scheduled for follow-up observations should be kept for a further 28 days without treatment to detect recovery from, or persistence of, toxic effects.

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.

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- Procedure

The animals are dosed with the test substance ideally on 7 days per week, over a period of 90 days. However, based primarily on practical considerations, dosing in gavage or capsule studies on a 5 day per week basis is considered to be acceptable. Signs of toxicity should be recorded as they are observed including the time of onset, degree and duration. Cage-side observations should include, but not be limited to, changes in skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous system, somatomotor activity and behaviour pattern. Measurements should be made of food consumption (or water consumption when the test substance is administered in the drinking water) 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 90-day period all surviving animals are sacrificed. Any moribund animals should be removed and sacrificed when noticed.

- Clinical examinations

The following examinations should be made:

(a) Ophthalmological examination, using an ophthalmoscope or equivalent suitable equipment, should be made prior to the administration of 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 test period.

(c) Clinical biochemistry determination on 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 apropriate 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 measurements. Other determinations which may be necessary for an adequate toxicological evaluation include analyses of lipids, hormones, acid/base balance, methaemoglobin, 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, consideration should be given to determination of haematological and clinical biochemistry parameters before dosing commences.

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

*Now known as serum alanine aminotransferase.

**Now known as serum aspartate aminotransferase.

Histopathology

(a) Full histopathology should be carried out on the 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) The lungs of animals in the low and intermediate dose groups should be subjected to histopathological examination for evidence of infection, since this provides a convenient assessment of the state of health of the animals. Consideration should also be given to histopathological examination of the liver and kidneys in these groups. Further histopathological examination may not be required routinely on the animals in these groups but must always be carried out in 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 the 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 methods may be used; the statistical methods should be selected during the design of the study.

- Evaluation of results

The findings of a subchronic oral toxicity study should be evaluated in conjunction with the findings of preceding studies and considered in terms of the toxic effects and the necropsy and histopathological findings. The evaluation will include the relationship between the dose of

the test substance 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 subchronic test should provide a satisfactory estimation of a no-effect level.

In any study which demonstrates an absence of toxic effects, further investigation to establish absorption and bioavailability of the test substance should be considered.

• Test report

The test report must include the following information:

- species/strain used;
- toxic response data by sex and dose;
- 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;
- results of ophthalmological examination;
- haematological tests employed and results with relevant baseline data;
- clinical biochemistry tests employed and results with relevant baseline data;
- necropsy findings;
- detailed description of all histopathological findings; and
- statistical treatment of results where appropriate.

- Interpretation of the results

A subchronic oral toxicity study will provide information on the effects of repeated oral exposure to a substance. Extrapolation of the results of the study to man is valid to a limited degree but it can provide useful information on no-effect levels and permissible human exposure.

4. LITERATURE

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