Adopted:
21st September 1998

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Repeated Dose 90-day Oral Toxicity Study in Non-Rodents

INTRODUCTION

1. OECD Guidelines for the Testing of Chemicals are periodically reviewed in the light of scientific progress. The original guideline 409 was adopted in 1981. In this revised version changes have been made with the objective of obtaining additional information from the animals used in the study.

2. This revised version of Guideline 409 is to a large extent based on the outcome of an OECD Consultation Meeting of Experts on Sub-chronic and Chronic toxicity testing held in Rome on 2-3 November 1995 (1).

INITIAL CONSIDERATIONS

3. In the assessment and evaluation of the toxic characteristics of a chemical, the determination of sub-chronic oral toxicity using repeated doses may be carried out after initial information on toxicity has been obtained from acute or repeated dose 28-day toxicity tests. The 90-day study provides information on the possible health hazards likely to arise from repeated exposure over a period of rapid growth and into young adulthood. The study will provide information on the major toxic effects, indicate target organs and the possibility of accumulation, and can provide an estimate of a no-observed-adverse-effect level of exposure which can be used in selecting dose levels for chronic studies and for establishing safety criteria for human exposure.

4. The revised guideline allows for the identification in non-rodent species of adverse effects of chemical exposure and should only be used :

- where effects observed in other studies indicate a need for clarification/characterisation in a second, non-rodent species, or
- where toxicokinetic studies indicate that the use of a specific non-rodent species is the most relevant choice of laboratory animal, or
- where other specific reasons justify the use of a non-rodent species.

5. Definitions used are given in the Annex.

PRINCIPLE OF THE TEST

6. 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 90 days. During the period of

administration the animals are observed closely 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 also killed and necropsied.

DESCRIPTION OF THE METHOD

Selection of animal species

7. The commonly used non-rodent species is the dog, which should be of a defined breed; the beagle is frequently used. Other species, e.g., swine, mini-pigs, may also be used. Primates are not recommended and their use should be justified. Young, healthy animals should be employed, and in the case of the dog, dosing should begin preferably at 4-6 months and not later than 9 months of age. Where the study is conducted as a preliminary to a long-term chronic toxicity study, the same species/breed should be used in both studies.

Housing and feeding conditions

8. 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 the test substance when administered by this method. Caging should be appropriate to the species. Lighting should preferably be artificial, the sequence being 12 hours light, 12 hours dark. Housing and feeding conditions should meet specific requirements for the selected species as provided in authoritative legislation and guidance (2)(3)(4).

Preparation of animals

9. Healthy young animals, which have been acclimated to laboratory conditions and have not been subjected to previous experimental procedures, should be used. The duration of acclimatisation will depend upon the selected test species and their source. At least 5 days for dogs or purpose bred swine from a resident colony and at least 2 weeks for these animals if from external sources are recommended. The test animals should be characterised as to species, strain, source, sex, weight and/or age. Animals should be randomly assigned to the control and treatment groups. Cages should be arranged in such a way that possible effects due to cage placement are minimised. Each animal should be assigned a unique identification number.

Preparation of doses

10. The test compound may be administered in the diet or in the drinking water, by gavage or in capsules. The method of oral administration is dependent on the purpose of the study, and the physical-chemical properties of the test material.

11. 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 under the conditions of administration should be determined.

PROCEDURE

Number and sex of animals

12. At least 8 animals (four female and four 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. The number of animals at the termination of the study must be adequate for a meaningful evaluation of toxic effects. Based on previous knowledge of the chemical or a close analogue, consideration should be given to including an additional satellite group of 8 animals (four per sex) in control and in top dose group for observation after the treatment period of reversibility or persistence of any toxic effects. The duration of this post-treatment period should be fixed appropriately with regard to the effects observed.

Dosage

13. At least three dose levels and a concurrent control shall be used, except where a limit test is conducted (see paragraph 16). Dose levels may be based on the results of repeated dose or range finding studies and should take into account any existing toxicological and toxicokinetic data available for the test compound or related materials . Unless limited by the physical-chemical nature or biological effects of the test substance, the highest dose level should be chosen with the aim to induce toxicity but not death or severe suffering. A descending sequence of dose levels should be selected with a view to demonstrating any dosage related response and a no-observed-adverse-effect level (NOAEL) at the lowest dose level. 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 about 6 - 10) between dosages.

14. The control group shall be an untreated group or a vehicle-control group if a vehicle is used in administering the test substance. Except for treatment with the test substance, animals in the control group should be handled in an identical manner to those in the test groups. If a vehicle is used, the control group shall receive the vehicle in the highest volume used. If a test substance is administered in the diet, and causes reduced dietary intake, then a pair-fed control group may be useful in distinguishing between reductions due to palatability or toxicological alterations in the test model.

15. Consideration should be given to the following characteristics of the vehicle and other additives, as appropriate: Effects on the absorption, distribution, metabolism, or retention of the test substance; effects on the chemical properties of the test substance which may alter its toxic characteristics; and effects on the food or water consumption or the nutritional status of the animals.

Limit test

16. If a test at one dose level, equivalent to at least 1000 mg/kg body weight/day), using the procedures described for this study, produces no observed adverse 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. The limit test applies except when human exposure indicates the need for a higher dose level to be used.

Administration of doses

17. The animals are dosed with the test substance daily seven days each week for a period of 90 days. Any other dosing regime , e.g., five days per week, 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. Normally the volume should be kept as low as possible. 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.

18. 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 animal's body weight may be used; any alternative used must be specified. For a substance administered by gavage or by capsule, 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 the 90 day study is used as a preliminary to a long term chronic toxicity study, a similar diet should be used in both studies.

Observations

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

20. General clinical observations should be made at least once a day, preferably at the same time(s) each day, taking into consideration the peak period of anticipated effects after dosing. The clinical condition of the animals should be recorded. At least twice daily, usually at the beginning and end of each day, all animals should be inspected for signs of morbidity and mortality.

21. At least once prior to the first exposure (to allow for within-subject comparisons), and once a week thereafter, detailed clinical observations should be made in all animals. These observations should be made, where practical outside the home cage in a standard arena and preferably at similar times on each occasion. Effort should be made to ensure that variations in the observation conditions are minimal. Signs of toxicity should be carefully recorded, including time of onset, degree and duration. Observations 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, pilo-erection, 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 any bizarre behaviour should also be recorded

22. 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 treatment related changes in the eyes are detected all animals should be examined.

Body weight and food/water consumption

23. All animals should be weighed at least once a week. Measurements of food 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. Water consumption may also be considered for dietary or gavage studies during which drinking activity may be altered.

Haematology and Clinical Biochemistry

24. Blood samples should be taken from a named site and stored, if applicable, under appropriate conditions. At the end of the test period, samples are collected just prior to or as part of the procedure for killing the animals.

25. Haematology, including haematocrit, haemoglobin concentration, erythrocyte count, total and differential leukocyte count, platelet count and a measure of clotting potential such as clotting time, prothrombin time, or thromboplastin time should be investigated at the start of the study, then either at monthly intervals or midway through the test period and finally at the end of the test period.

26. Clinical biochemistry determinations to investigate major toxic effects in tissues and, specifically, effects on kidney and liver, should be performed on blood samples obtained from all animals at the start, then either at monthly intervals or midway through the test and finally at the end of the test period. Test areas which should be considered are electrolyte balance, carbohydrate metabolism, and liver and kidney function. The selection of specific tests will be influenced by observations on the mode of action of the test substance. Animals should be fasted for a period appropriate to the species prior to blood sampling. Suggested determinations include calcium, phosphorus, chloride, sodium, potassium, fasting glucose, alanine aminotransferase, aspartate aminotransferase, ornithine decarboxylase, gamma glutamyl transpeptidase, urea nitrogen, albumin, blood creatinine, total bilirubin and total serum protein measurements.

27. Urinalysis determinations should be performed at least at the start, then midway and finally at the end of the study using timed urine volume collection. Urinalysis determinations include appearance, volume, osmolality or specific gravity, pH, protein, glucose and blood/blood cells. Additional parameters may be employed where necessary to extend the investigation of observed effect(s).

28. In addition, studies to investigate markers of general tissue damage should be considered. Other determinations which may be necessary for an adequate toxicological evaluation include analyses of lipids, hormones, acid/base balance, methaemoglobin, and cholinesterase inhibition. Additional clinical biochemistry may be employed where necessary to extend the investigation of observed effects. These need to be identified for chemicals in certain classes or on a case-by-case basis.

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

Pathology

Gross necropsy

30. 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 with gall bladder, kidneys, adrenals, testes, epididymides, ovaries, uterus, thyroid (with parathyroids), thymus, spleen, brain and heart of all

animals (apart from those found moribund and/or inter-currently killed) should be trimmed of any adherent tissue, as appropriate, and their wet weight taken as soon as possible after dissection to avoid drying.

31. 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 medulla/pons), spinal cord (at three levels: cervical, mid-thoracic and lumbar), pituitary, eyes, thyroid, parathyroid, thymus, oesophagus, salivary glands, stomach, small and large intestines (including Peyer's patches), liver, gall bladder, pancreas, kidneys, adrenals, spleen, heart, trachea and lungs, aorta, gonads, uterus, accessory sex organs, female mammary gland, 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, a section of bone marrow (and/or a fresh bone marrow aspirate) and skin. 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.

Histopathology

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

33. All gross lesions should be examined.

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

DATA AND REPORTING

Data

35. 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.

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

Test report

37. The test report must include the following information:

Test substance:

- physical nature, purity and physicochemical properties;
- identification data.

Vehicle (if appropriate):

- justification for choice of vehicle, if other than water.

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.

Test conditions:

- 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;
- actual doses (mg/kg body weight/day), and conversion factor from diet/drinking water test substance concentration (ppm) to the actual dose, 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 clinical observations (whether reversible or not);
- ophthalmological examination;
- haematological tests with relevant base-line values;
- clinical biochemistry tests with relevant base-line values;
- terminal body weight, organ weights and organ/body weight ratios;
- necropsy findings;
- a detailed description of all histopathological findings;
- absorption data if available;
- statistical treatment of results, where appropriate.

Discussion of results.

Conclusions.

LITERATURE

(1) OECD (Rome, 1995). Report of the Consultation Meeting on Sub-chronic and Chronic Toxicity/Carcinogenicity Testing.

(2) EEC Council Directive 86/609/EEC on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes. Official Journal, 29, L358, 18th December 1986.

(3) National Research Council, 1985. Guide for the care and use of laboratory animals. NIH Publication No. 86-23. Washington D.C., US. Dept. of Health and Human Services.

(4) GV-SOLAS (Society for Laboratory Animal Science, Gesellschaft für Versuchstierkunde, December, 1989). Publication on the Planning and Structure of Animal Facilities for Institutes Performing Animal Experiments. ISBN 3-906255-06-9.