PROCEDURE

Full test

13. Individual animals are dosed in sequence at 24 h intervals, one at a time, and then observed for a minimum of 24 hours. However, the time intervals between dosing should not be fixed rigidly and may be adjusted as appropriate, in case of delayed mortality. The first animal is dosed at the toxicologist's best estimate of the LD50. If the animal survives, the second animal receives a higher dose, unless the limit dose was used as the starting dose. If the first animal dies or appears moribund the second animal receives a lower dose. Moribund state is characterised by symptoms such as shallow, laboured or irregular respiration, muscular weakness or tremors, absence of voluntary response to external stimuli, cyanosis and coma. Criteria for making the decision to humanely kill moribund and severely suffering animals are the subject of a separate Guidance Document. Animals killed for humane reasons are considered in the same way as animals that died on test.

14. For selecting the starting dose, all available information should be used, including information on structure-activity relationships. When the information suggests that mortality is unlikely then a limit test should be conducted (see paragraph 16). When there is no information on the substance to be tested, for animal welfare reasons it is recommended to use the starting dose of 200 or 500 mg/kg body weight.

15. The dose for each successive animal is adjusted up or down, depending on the outcome of the previous animal. If feasible, a dose progression factor of 1.3 is used. Other factors may be used, if justified. After reaching the reversal of the initial direction (the point where a decreasing dose pattern requires an increase due to a tested animal's survival or an increasing dose pattern results in a decrease due to lethality), four additional animals are dosed using the same UDP. This is the end of the normal test.

Limit test

16. Doses should not exceed 2000 mg/kg which is considered the upper limit dose. When the first animal is dosed with the upper limit dose and survives, the second animal receives the same dose. When a total of three animals have been dosed with the limit dose and no deaths have occurred, then three animals of the other sex should be tested at the limit dose level. If there is again no lethality, the test can be terminated.

Optional testing

17. Information from one sex may be adequate to assess acute toxicity. However, if found desirable, comparability of response in the other sex can be evaluated by administering to generally not more than 3 animals, doses above and below the estimated LD50. The point intermediate between doses where responses change can be taken as an approximate estimate of the lethal dose.

Administration of doses

18. The test substance is administered in a single dose by gavage, using an oral dosing needle or rubberised tubing.

19. The animals should be fasted prior to dosing by withholding food overnight. Fasted body weight of each rat is determined and the dose is calculated according to the body weight. After dosing food may be withheld for a further 3-4 hours. The volume should not exceed 1 ml/100g body weight, except in the case of aqueous solutions where 2 ml/100g body may be used.

Observations

20. Animals are observed individually after dosing at least once during the first 30 minutes, periodically during the first 24 hours, with special attention given during the first 4 hours, and daily thereafter for a total of 14 days. However, the duration of the observation period should not be fixed rigidly. It should be determined by the toxic reactions, time of onset and length of recovery period, and may thus be extended when considered necessary.

21. Observations include mortality and clinical signs. These include changes in skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous systems, and somatomotor activity and behaviour pattern. Attentions should be directed to observations of tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma.

Body weight

22. Individual weights of animals should be determined shortly before the test substance is administered, at least weekly thereafter, at the time of death or at day 14 in the case of survival. Weight changes should be calculated and recorded.

Pathology

23. All animals, including those which die during the test or are killed for animal welfare reasons during the test and those that survive at day 14, are subjected to gross necropsy. The necropsy should entail a macroscopic inspection of the visceral organs. As deemed appropriate, microscopic analysis of target organs and clinical chemistry may be included to gain further information on the nature of the toxicity of the test material.

DATA AND REPORTING

Data

24. Individual animal data should be provided. Additionally, all data should be summarised in tabular form, showing for each test concentration the number of animals used, the number of animals displaying signs of toxicity, the number of animals found dead during the test or killed for humane reasons, time of death of individual animals, a description and the time course of toxic effects and reversibility, and necropsy findings.

Calculation of LD50

25. The LD50 is calculated using the maximum likelihood method (8)(9). The following statistical details may be helpful in implementing the maximum likelihood calculations suggested.

All deaths, whether immediate or delayed or humane kills, are incorporated for the purpose of the maximum likelihood analysis. Following Dixon (8), the likelihood function is written as follows:

$$L = L_1 L_2 ....L_n ,$$

where

L is likelihood of the experimental outcome, given μ and σ, and n the number of animals tested.

$L_i = 1 - F(Z_i)$ if the i^{th} animal survived, or
$L_i = F(Z_i)$ if the i^{th} animal died ,

where

F = cumulative, standard normal density,
$Z_i = [\log(d_i) - \mu] / \sigma$
d_i = dose given to the i^{th} animal
μ = log LD50, and
σ = standard deviation

An estimate of σ of 0.12 is used unless a better generic or case-specific value is available.

26. The calculation can be performed using either SAS (10) or BMDP (11) computer program packages. Other computer programs may also be used. Typical instructions for these packages are given in appendices to the ASTM Standard E 1163-87 (4). The program output is an estimate of log LD50 and its standard error.

Report

27. The test report must include the following information:

Test substance:

- physical nature, purity and physicochemical properties (including isomerisation);
- identification data.

Vehicle (if appropriate):

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

Test animals:

- species/strain used;
- microbiological status of the animals, when known;
- number, age and sex of animals;
- rationale for use of males instead of females;
- source, housing conditions, diet, etc.;
- individual weights of animals at the start of the test, at day 7, and at day 14.

Test conditions:

- rationale for initial dose level selection and for follow-up dose levels;
- details of test substance formulation;
- details of the administration of the test substance;
- details of food and water quality (including diet type/source, water source).

Results:

- body weight/body weight changes;
- tabulation of response data by sex and dose level for each animal (i.e. animals showing signs of toxicity including nature, severity, duration of effects, and mortality);
- time course of onset of signs of toxicity and whether these were reversible for each animal;
- necropsy findings and any histopathological findings for each animal, if available.
- LD_{50} data;
- statistical treatment of results.

Discussion and interpretation of results.

Conclusions.

LITERATURE

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ANNEX

DEFINITIONS

Acute oral toxicity is the adverse effects occurring within a short time of oral administration of a single dose of a substance or multiple doses given within 24 hours.

Delayed death means that an animal does not die or appear moribund within 24 hours but dies later during the observation period.

Dosage is a general term comprising the dose, its frequency and the duration of dosing.

Dose is the amount of test substance administered. Dose is expressed as weight (g, mg) or as weight of test substance per unit weight of test animal (e.g. mg/kg).

Moribund status of an animal is the result of the toxic properties of a test substance where death is anticipated. For making decisions as to the next step in this test, animals killed for humane reasons are considered in the same way as animals that died.

LD50 (median lethal dose), oral, is a statistically derived single dose of a substance that can be expected to cause death in 50 per cent of animals when administered by the oral route. The LD50 value is expressed in terms of weight of test substance per unit weight of test animal (mg/kg).