

Commission Directive 2004/73/EC of 29 April 2004 adapting to technical progress for the twenty-ninth time Council Directive 67/548/EEC on the approximation of the laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances (Text with EEA relevance)

ANNEX 2B

B.1 bis.ACUTE ORAL TOXICITY - FIXED DOSE PROCEDURE

1. METHOD

This test method is equivalent to OECD TG 420 (2001)

1.1 INTRODUCTION

Traditional methods for assessing acute toxicity use death of animals as an endpoint. In 1984, a new approach to acute toxicity testing was suggested by the British Toxicology Society based on the administration at a series of fixed dose levels (1). The approach avoided using death of animals as an endpoint, and relied instead on the observation of clear signs of toxicity at one of a series of fixed dose levels. Following UK (2) and international (3) *in vivo* validation studies the procedure was adopted as a testing method in 1992. Subsequently, the statistical properties of the Fixed Dose Procedure have been evaluated using mathematical models in a series of studies (4)(5)(6). Together, the *in vivo* and modelling studies have demonstrated that the procedure is reproducible, uses fewer animals and causes less suffering than the traditional methods and is able to rank substances in a similar manner to the other acute toxicity testing methods.

Guidance on the selection of the most appropriate test method for a given purpose can be found in the Guidance Document on Acute Oral Toxicity Testing (7). This Guidance Document also contains additional information on the conduct and interpretation of Testing Method B.1 bis.

It is a principle of the method that in the main study only moderately toxic doses are used, and that administration of doses that are expected to be lethal should be avoided. Also, doses that are known to cause marked pain and distress, due to corrosive or severely irritant actions, need not be administered. Moribund animals, or animals obviously in pain or showing signs of severe and enduring distress shall be humanely killed, and are considered in the interpretation of the test results in the same way as animals that died on test. Criteria for making the decision to kill moribund or severely suffering animals, and guidance on the recognition of predictable or impending death, are the subject of a separate Guidance Document (8).

The method provides information on the hazardous properties and allows the substance to be ranked and classified according to the Globally Harmonised System (GHS) for the classification of chemicals which cause acute toxicity (9).

The testing laboratory should consider all available information on the test substance prior to conducting the study. Such information will include the identity and chemical structure of the substance; its physico-chemical properties; the results of any other *in vitro* or *in vivo* toxicity tests on the substance; toxicological data on structurally related substances; and the anticipated use(s) of the substance. This information is necessary to satisfy all concerned that the test is relevant for the protection of human health, and will help in the selection of an appropriate starting dose.

1.2 DEFINITIONS

Acute oral toxicity: refers to those adverse effects occurring following 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 48 hours but dies later during the 14-day observation period.

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

Evident toxicity: is a general term describing clear signs of toxicity following the administration of test substance (see (3) for examples) such that at the next highest fixed dose either severe pain and enduring signs of severe distress, moribund status (criteria are presented in the Humane Endpoints Guidance Document (8)), or probable mortality in most animals can be expected.

GHS: Globally Harmonised Classification System for Chemical Substances and Mixtures. A joint activity of OECD (human health and the environment), UN Committee of Experts on Transport of Dangerous Goods (physical-chemical properties) and ILO (hazard communication) and co-ordinated by the Interorganisation Programme for the Sound Management of Chemicals (IOMC).

Impending death: when moribund state or death is expected prior to the next planned time of observation. Signs indicative of this state in rodents could include convulsions, lateral position, recumbence, and tremor. (See the Humane Endpoint Guidance Document (8) for more details).

LD₅₀ (median lethal dose): 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).

Limit dose: refers to a dose at an upper limitation on testing (2000 or 5000 mg/kg).

Moribund status: being in a state of dying or inability to survive, even if treated. (See the Humane Endpoint Guidance Document (8) for more details).

Predictable death: presence of clinical signs indicative of death at a known time in the future before the planned end of the experiment, for example: inability to reach water or food. (See the Humane Endpoint Guidance Document (8) for more details).

1.3 PRINCIPLE OF THE TEST METHOD

Groups of animals of a single sex are dosed in a stepwise procedure using the fixed doses of 5, 50, 300 and 2000 mg/kg (exceptionally an additional fixed dose of 5000 mg/kg may be considered, see section 1.6.2). The initial dose level is selected on the basis of a sighting study as the dose expected to produce some signs of toxicity without causing severe toxic effects or mortality. Clinical signs and conditions associated with pain, suffering, and impending death, are described in detail in a separate OECD Guidance Document (8). Further groups of animals may be dosed at higher or lower fixed doses, depending on the presence or absence of signs of toxicity or mortality. This procedure continues until the dose causing evident toxicity or no more than one death is identified, or when no effects are seen at the highest dose or when deaths occur at the lowest dose.

1.4 DESCRIPTION OF THE TEST METHOD

1.4.1 Selection of animal species

The preferred rodent species is the rat, although other rodent species may be used. Normally females are used (7). This is because literature surveys of conventional LD₅₀ tests show that usually there is little difference in sensitivity between the sexes, but in those cases where differences are observed, females are generally slightly more sensitive (10). However, if knowledge of the toxicological or toxicokinetic properties of structurally related chemicals indicates that males are likely to be more sensitive then this sex should be used. When the test is conducted in males, adequate justification should be provided.

Healthy young adult animals of commonly used laboratory strains should be employed. Females should be nulliparous and non-pregnant. Each animal, at the commencement of its dosing,

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should be between 8 and 12 weeks old and its weight should fall in an interval within $\pm 20\%$ of the mean weight of any previously dosed animals.

1.4.2 Housing and feeding conditions

The temperature of the experimental animal room should be 22°C ($\pm 3^{\circ}\text{C}$). Although the relative humidity should be at least 30% and preferably not exceed 70% other than during room cleaning the aim should be 50-60%. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. Animals may be group-caged by dose, but the number of animals per cage must not interfere with clear observations of each animal.

1.4.3 Preparation of animals

The animals are randomly selected, marked to permit individual identification, and kept in their cages for at least 5 days prior to the start of dosing to allow for acclimatisation to the laboratory conditions.

1.4.4 Preparation of doses

In general test substances should be administered in a constant volume over the range of doses to be tested by varying the concentration of the dosing preparation. Where a liquid end product or mixture is to be tested however, the use of the undiluted test substance, i.e. at a constant concentration, may be more relevant to the subsequent risk assessment of that substance, and is a requirement of some regulatory authorities. In either case, the maximum dose volume for administration must not be exceeded. The maximum volume of liquid that can be administered at one time depends on the size of the test animal. In rodents, the volume should not normally exceed 1ml/100g of body weight: however in the case of aqueous solutions 2 ml/100g body weight can be considered. With respect to the formulation of the dosing preparation, the use of an aqueous solution/suspension/emulsion is recommended wherever possible, followed in order of preference by a solution/suspension/emulsion in oil (e.g. com oil) and then possibly solution in other vehicles. For vehicles other than water the toxicological characteristics of the vehicle should be known. Doses must be prepared shortly prior to administration unless the stability of the preparation over the period during which it will be used is known and shown to be acceptable.

1.5 PROCEDURE

1.5.1 Administration of doses

The test substance is administered in a single dose by gavage using a stomach tube or a suitable intubation canula. In the unusual circumstance that a single dose is not possible, the dose may be given in smaller fractions over a period not exceeding 24 hours.

Animals should be fasted prior to dosing (e.g. with the rat, food but not water should be withheld over-night; with the mouse, food but not water should be withheld for 3-4 hours). Following the period of fasting, the animals should be weighed and the test substance administered. After the substance has been administered, food may be withheld for a further 3-4 hours in rats or 1-2 hours in mice. Where a dose is administered in fractions over a period of time, it may be necessary to provide the animals with food and water depending on the length of the period.

1.5.2 Sighting study

The purpose of the sighting study is to allow selection of the appropriate starting dose for the main study. The test substance is administered to single animals in a sequential manner following the flow charts in Annex 1. The sighting study is completed when a decision on the starting dose for the main study can be made (or if a death is seen at the lowest fixed dose).

The starting dose for the sighting study is selected from the fixed dose levels of 5, 50, 300 and 2000 mg/kg as a dose expected to produce evident toxicity based, when possible, on evidence from *in vivo* and *in vitro* data from the same chemical and from structurally related chemicals. In the absence of such information, the starting dose will be 300 mg/kg.

A period of at least 24 hours will be allowed between the dosing of each animal. All animals should be observed for at least 14 days.

Exceptionally, and only when justified by specific regulatory needs, the use of an additional upper fixed dose level of 5000 mg/kg may be considered (see Annex 3). For reasons of animal welfare concern, testing of animals in GHS Category 5 ranges (2000-5000 mg/kg is discouraged and should only be considered when there is a strong likelihood that the results of such a test have a direct relevance for protecting human or animal health or the environment.

In cases where an animal tested at the lowest fixed dose level (5mg/kg) in the sighting study dies, the normal procedure is to terminate the study and assign the substance to GHS Category 1 (as shown in Annex 1). However, if further confirmation of the classification is required, an optional supplementary procedure may be conducted, as follows. A second animal is dosed at 5mg/kg. If this second animal dies, then GHS Category 1 will be confirmed and the study will be immediately terminated. If the second animal survives, then a maximum of three additional animals will be dosed at 5mg/kg. Because there will be a high risk of mortality, these animals should be dosed in a sequential manner to protect animal welfare. The time interval between dosing each animal should be sufficient to establish that the previous animal is likely to survive. If a second death occurs, the dosing sequence will be immediately terminated and no further animals will be dosed. Because the occurrence of a second death (irrespective of the number of animals tested at the time of termination) falls into outcome A (2 or more deaths), the classification rule of Annex 2 at the 5mg/kg fixed dose is followed (Category 1 if there are 2 or more deaths or Category 2 if there is no more than 1 death). In addition, Annex 4 gives guidance on the classification in the EU system until the new GHS is implemented.

1.5.3 Main study

1.5.3.1 Numbers of animals and dose levels

The action to be taken following testing at the starting dose level is indicated by the flow charts in Annex 2. One of three actions will be required; either stop testing and assign the appropriate hazard classification class, test at a higher fixed dose or test at a lower fixed dose. However, to protect animals, a dose level that caused death in the sighting study will not be revisited in the main study (see Annex 2). Experience has shown that the most likely outcome at the starting dose level will be that the substance can be classified and no further testing will be necessary.

A total of five animals of one sex will normally be used for each dose level investigated. The five animals will be made up of one animal from the sighting study dosed at the selected dose level together with an additional four animals (except, unusually, if a dose level used on the main study was not included in the sighting study).

The time interval between dosing at each level is determined by the onset, duration, and severity of toxic signs. Treatment of animals at the next dose should be delayed until one is confident of survival of the previously dosed animals. A period of 3 or 4 days between dosing at each dose level is recommended, if needed, to allow for the observation of delayed toxicity. The time interval may be adjusted as appropriate, e.g., in case of inconclusive response.

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When the use of an upper fixed dose of 5000 mg/kg is considered, the procedure outlined in Annex 3 should be followed (see also section 1.6.2).

1.5.3.2 *Limit test*

The limit test is primarily used in situations where the experimenter has information indicating that the test material is likely to be nontoxic, i.e., having toxicity only above regulatory limit doses. Information about the toxicity of the test material can be gained from knowledge about similar tested compounds or similar tested mixtures or products, taking into consideration the identity and percentage of components known to be of toxicological significance. In those situations where there is little or no information about its toxicity, or in which the test material is expected to be toxic, the main test should be performed.

Using the normal procedure, a sighting study starting dose of 2000 mg/kg (or exceptionally 5000 mg/kg) followed by dosing of a further four animals at this level serves as a limit test for this guideline.

1.6 OBSERVATIONS

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, except where they need to be removed from the study and humanely killed for animal welfare reasons or are found dead. However, the duration of observation 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. The times at which signs of toxicity appear and disappear are important, especially if there is a tendency for toxic signs to be delayed (11). All observations are systematically recorded, with individual records being maintained for each animal.

Additional observations will be necessary if the animals continue to display signs of toxicity. Observations should 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. Attention should be directed to observations of tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma. The principles and criteria summarised in the Humane Endpoints Guidance Document should be taken into consideration (8). Animals found in a moribund condition and animals showing severe pain or enduring signs of severe distress should be humanely killed. When animals are killed for humane reasons or found dead, the time of death should be recorded as precisely as possible.

1.6.1 **Body weight**

Individual weights of animals should be determined shortly before the test substance is administered and at least weekly thereafter. Weight changes should be calculated and recorded. At the end of the test surviving animals are weighed and then humanely killed.

1.6.2 **Pathology**

All test animals (including those that die during the test or are removed from the study for animal welfare reasons) should be subjected to gross necropsy. All gross pathological changes should be recorded for each animal. Microscopic examination of organs showing evidence of gross pathology in animals surviving 24 or more hours after the initial dosing may also be considered because it may yield useful information.

2 **DATA**

Individual animal data should be provided. Additionally, all data should be summarised in tabular form, showing for each test group 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.

3 REPORTING

3.1 Test report

The test report must include the following information, as appropriate:

Test substance:

- physical nature, purity, and, where relevant, physico-chemical properties (including isomerisation);
- identification data, including CAS number.

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 (including, where appropriate, a rationale for use of males instead of females);
- source, housing conditions, diet etc.;

Test conditions:

- details of test substance formulation, including details of the physical form of the material administered;
- details of the administration of the test substance including dosing volumes and time of dosing;
- details of food and water quality (including diet type/source, water source);
- the rationale for the selection of the starting dose.

Results:

- tabulation of response data and dose level for each animal (i.e. animals showing signs of toxicity including mortality, nature, severity and duration of effects);
- tabulation of body weight and body weight changes;
- individual weights of animals at the day of dosing, in weekly intervals thereafter, and at time of death or sacrifice;
- date and time of death if prior to scheduled sacrifice.
- time course of onset of signs of toxicity and whether these were reversible for each animal;
- necropsy findings and histopathological findings for each animal; if available.

Discussion and interpretation of results.

Conclusions.

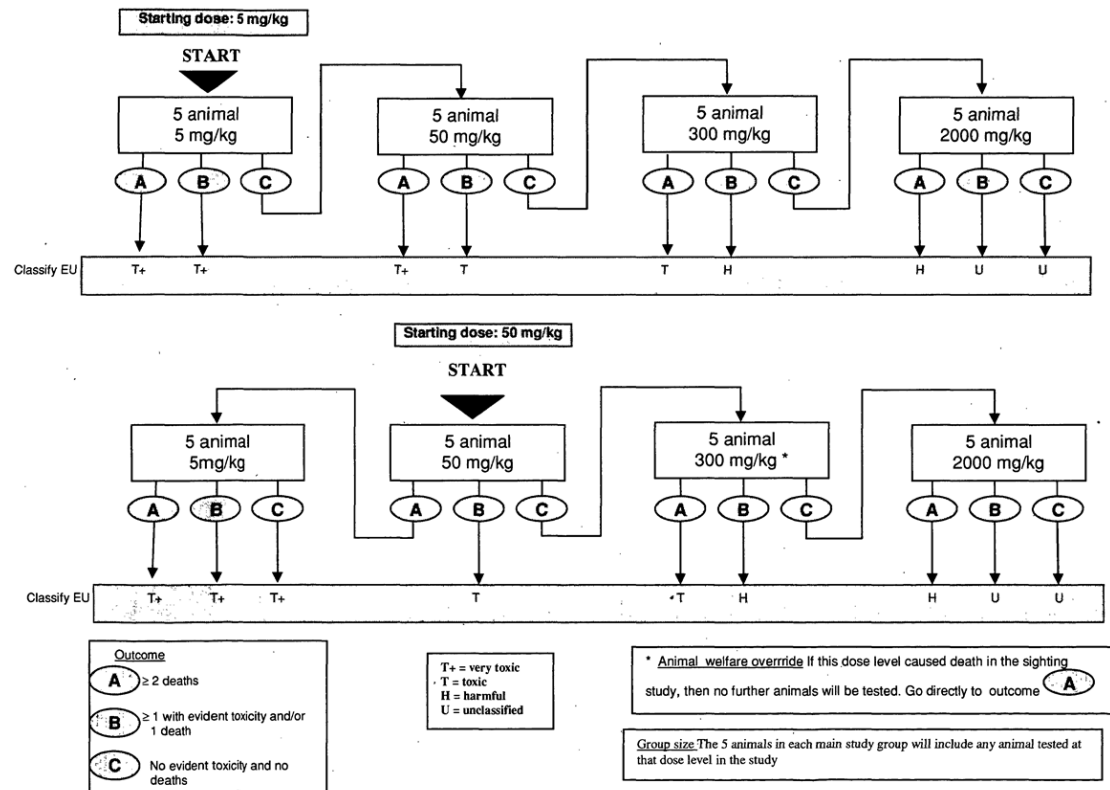
4 REFERENCES

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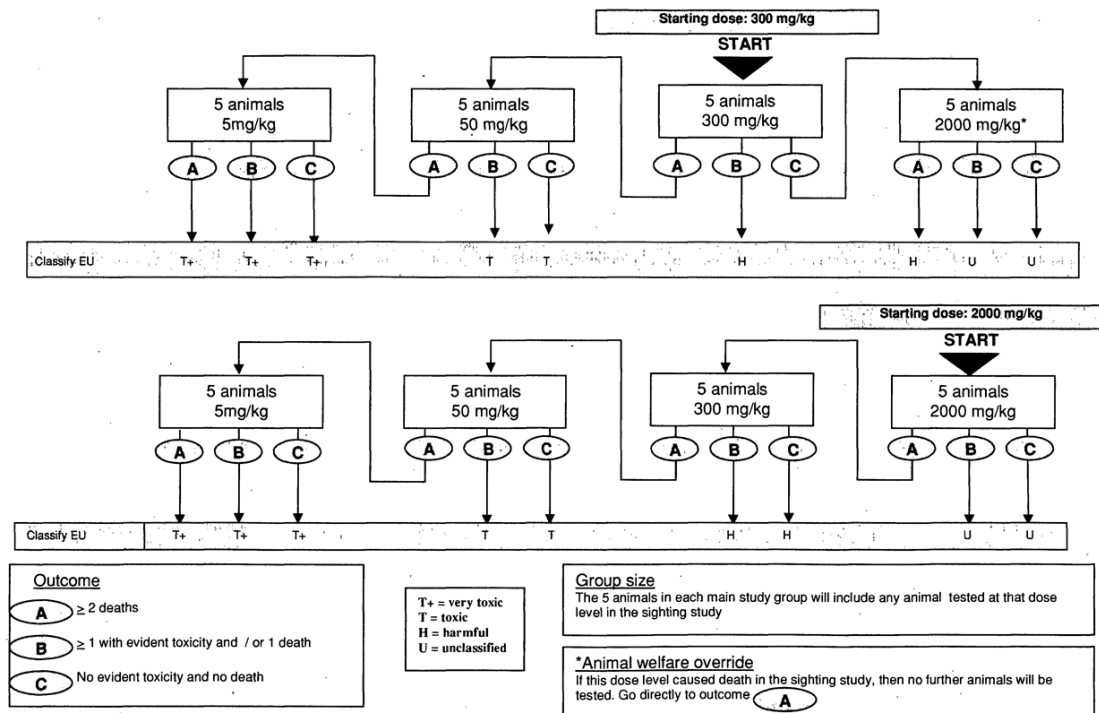
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- (8) OECD (2000). Guidance Document on the Recognition, Assessment and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation. Environmental Health and Safety Monograph Series on Testing and Assessment N. 19.
- (9) OECD (1998). Harmonised Integrated Hazard Classification for Human Health and Environmental Effects of Chemical Substances as endorsed by the 28th Joint Meeting of the Chemicals Committee and the Working Party on Chemicals in November 1998, Part 2, p. 11 [<http://webnet1.oecd.org/oecd/pages/home/displaygeneral/0,3380,EN-documents-521-14-no-24-no-0,FF.html>].
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ANNEX 1: FLOW CHART FOR THE SIGHTING STUDY

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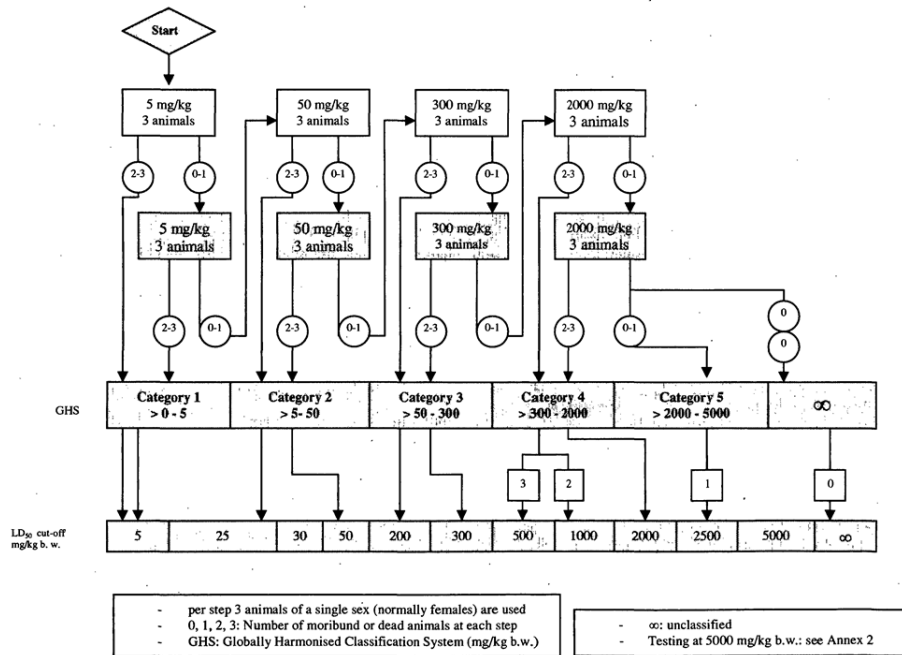


ANNEX 1: FLOW CHART FOR THE SIGHTING STUDY

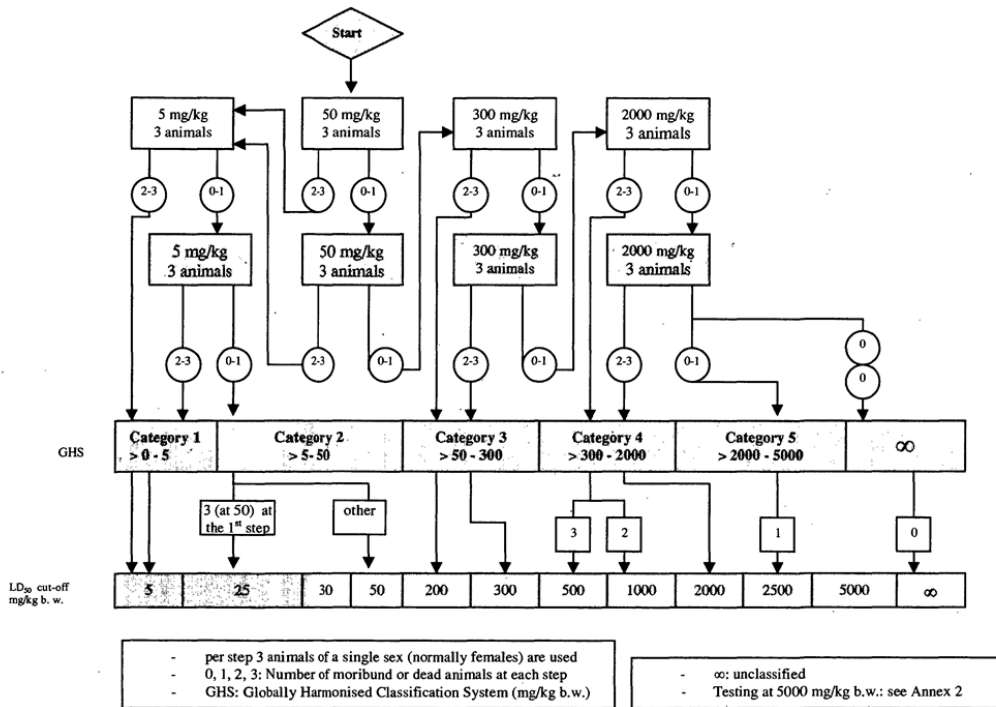


ANNEX 2: FLOW CHART FOR THE MAIN STUDY

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ANNEX 2: FLOW CHART FOR THE MAIN STUDY



**ANNEX 3
CRITERIA FOR CLASSIFICATION OF TEST SUBSTANCES WITH EXPECTED LD₅₀ VALUES EXCEEDING 2000 MG/KG WITHOUT THE NEED FOR TESTING.**

Criteria for hazard Category 5 are intended to enable the identification of test substances which are of relatively low acute toxicity hazard but which, under certain circumstances may present a danger to vulnerable populations. These substances are anticipated to have an oral or dermal

LD₅₀ in the range of 2000-5000 mg/kg or equivalent doses for other routes. Test substances could be classified in the hazard category defined by: 2000mg/kg <LD₅₀ < 5000mg/kg (Category 5 in the GHS) in the following cases:

- a) if directed to this category by any of the testing schemes of Annex 2, based on mortality incidences
- b) if reliable evidence is already available that indicates the LD⁵⁰ to be in the range of Category 5 values; or other animal studies or toxic effects in humans indicate a concern for human health of an acute nature.
- c) through extrapolation, estimation or measurement of data if assignment to a more hazardous class is not warranted, and
 - reliable information is available indicating significant toxic effects in humans, or
 - any mortality is observed when tested up to Category 4 values by the oral route, or
 - where expert judgement confirms significant clinical signs of toxicity, when tested up to Category 4 values, except for diarrhoea, piloerection or an ungroomed appearance, or
 - where expert judgement confirms reliable information indicating the potential for significant acute effects from the other animal studies.

TESTING AT DOSES ABOVE 2000 MG/KG

Exceptionally, and only when justified by specific regulatory needs, the use of an additional upper fixed dose level of 5000 mg/kg may be considered. Recognising the need to protect animal welfare, testing at 5000 mg/kg is discouraged and should only be considered when there is a strong likelihood that the results of such a test would have a direct relevance for protecting animal or human health (9).

Sighting Study

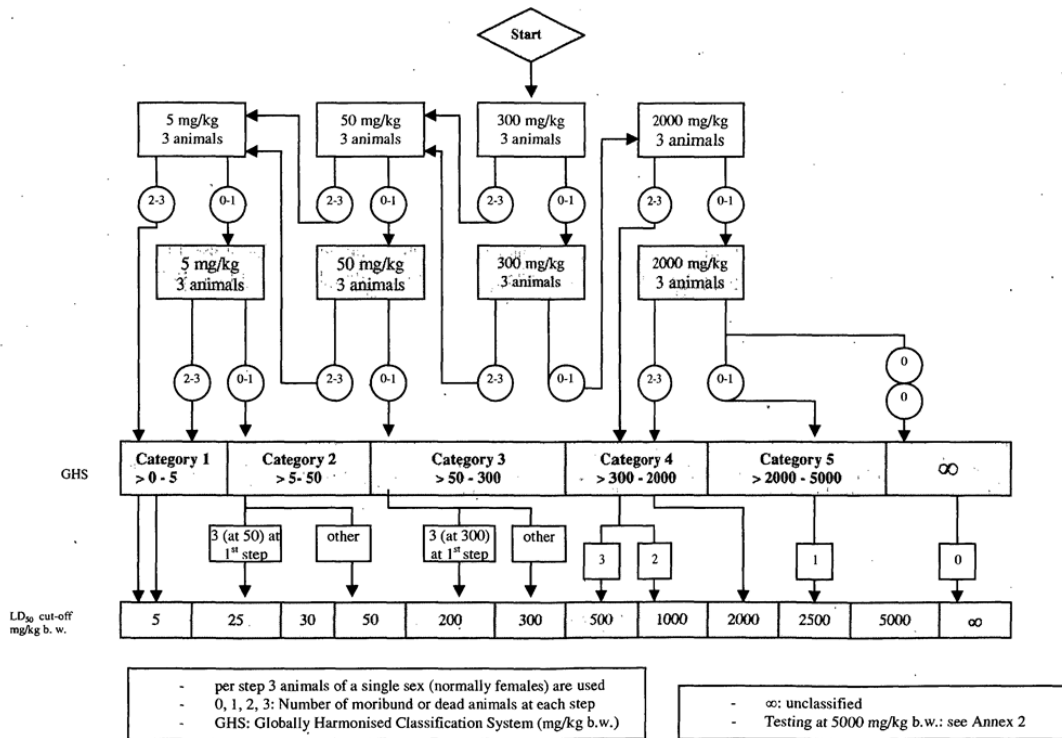
The decision rules governing the sequential procedure presented in Annex 1 are extended to include a 5000 mg/kg dose level. Thus, when a sighting study starting dose of 5000 mg/kg is used outcome A (death) will require a second animal to be tested at 2000 mg/kg; outcomes B and C (evident toxicity or no toxicity) will allow the selection of 5000 mg/kg as the main study starting dose. Similarly, if a starting dose other than 5000 mg/kg is used then testing will progress to 5000 mg/kg in the event of outcomes B or C at 2000 mg/kg; a subsequent 5000 mg/kg outcome A will dictate a main study starting dose of 2000 mg/kg and outcomes B and C will dictate a main study starting dose of 5000 mg/kg.

Main Study

The decision rules governing the sequential procedure presented in Annex 2 are extended to include a 5000 mg/kg dose level. Thus, when a main study starting dose of 5000 mg/kg is used, outcome A (≥ 2 deaths) will require the testing of a second group at 2000 mg/kg; outcome B (evident toxicity and/or ≤ 1 death) or C (no toxicity) will result in the substance being unclassified according to GHS. Similarly, if a starting dose other than 5000 mg/kg is used then testing will progress to 5000 mg/kg in the event of outcome C at 2000 mg/kg; a subsequent 5000 mg/kg outcome A will result in the substance being assigned to GHS Category 5 and outcomes B or C will lead to the substance being unclassified.

ANNEX TEST METHOD B.1 bis - Guidance on classification according to the EU 4: scheme to cover the transition period until full implementation of the Globally Harmonised Classification System (GHS) (taken from reference (8))

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ANNEX TEST METHOD B. 1 bis - Guidance on classification according to the EU scheme to cover the transition period until full implementation of the Globally Harmonised Classification System (GHS) (taken from reference (8))

