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VOLUME IV

concerning
the Registration, Evaluation, Authorisation and Restrictions of Chemicals
(REACH)

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PART B: METHODS FOR THE DETERMINATION OF TOXICITY AND OTHER HEALTH EFFECTS

GENERAL INTRODUCTION: PART B

GENERAL DEFINITIONS FOR TERMS USED IN PART B OF THE TEST METHODS

(i) **Acute toxicity** comprises the adverse effects occurring within a given time (usually 14 days), after administration of a single dose of a substance.

(ii) **Evident toxicity** is a general term describing clear signs of toxicity following administration of test substance. These should be sufficient for hazard assessment and should be such that an increase in the dose administered can be expected to result in the development of severe toxic signs and probable mortality.

(iii) **Dose** is the amount of test substance administered. Dose is expressed as weight (grams or milligrams) or as weight of test substance per unit weight of test animal (e.g. milligrams per kilogram body weight), or as constant dietary concentrations (parts per million or milligrams per kilogram of food).

(iv) **Discriminating dose** is the highest out of the four fixed dose levels which can be administered without causing compound-related mortality (including human kills).

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

(vi) **LD_{50}** (median lethal dose) is a statistically derived single dose of a substance that can be expected to cause death in 50 % of dosed animals. The LD_{50} value is expressed in terms of weight of test substance per unit weight of test animal (milligrams per kilogram).

(vii) **LC_{50}** (median lethal concentration) is a statistically derived concentration of a substance that can be expected to cause death during exposure or within a fixed time after exposure in 50 % of animals exposed for a specified time. The LC_{50} value is expressed as weight of test substance per standard volume of air (milligrams per litre).

(viii) **NOAEL** is the abbreviation for no observed adverse effect level and is the highest dose or exposure level where no adverse treatment-related findings are observed.

(ix) **Repeated dose/Sub-chronic toxicity** comprises the adverse effects occurring in experimental animals as a result of repeated daily dosing with, or exposure to, a chemical for a short part of their expected life-span.

(x) **Maximum Tolerated Dose** (MTD) is the highest dose level eliciting signs of toxicity in animals without having major effects on survival relative to the test in which it is used.

(xi) **Skin irritation** is the production of inflammatory changes in the skin following the application of a test substance.

(xii) **Eye irritation** is the production of changes in the eye following the application of a test substance to the anterior surface of the eye.

(xiii) **Skin sensitisation** (allergic contact dermatitis) is an immunologically mediated cutaneous reaction to a substance.

(xiv) **Dermal corrosion** is the production of irreversible tissue damage in the skin following the application of a test substance for the duration period of 3 minutes up to 4 hours.

(xv) **Toxicokinetics** is the study of the absorption, distribution, metabolism and excretion of test substances.

(xvi) **Absorption** is the process(es) by which an administered substance enters the body.

(xvii) **Excretion** is the process(es) by which the administered substance and/or its metabolites are removed from the body.

(xviii) **Distribution** is the process(es) by which the absorbed substance and/or its metabolites partition within the body.

(xvv) **Metabolism** is the process(es) by which the administered substances are structurally changed in the body by either enzymatic or non enzymatic reactions.

B.I ACUTE - REPEATED DOSE / SUBCHRONIC AND CHRONIC TOXICITY

The acute toxic effects and organ or system toxicity of a substance may be evaluated using a variety of toxicity tests (Methods B.1 -B.5) from which, following a single dose, a preliminary indication of toxicity may be obtained.

Dependant on the toxicity of the substance, a limit test approach or a full LD50 may be considered, although no limit test is specified in inhalation studies, because it has not been possible to define a single inhalation exposure limit value.

Consideration should be given to methods which use as few animals as possible and minimise animal suffering, for example the fixed dose method (Method B.1 bis) and acute toxic class (Method B.1 tris). In level 1 testing, a study in a second species may complement the conclusions drawn from the first study. In this case, a standard test method may be used or the method may be adapted for a smaller number of animals.

The repeated dose toxicity test (Methods B.7, B.8 and B.9) includes assessment of toxic effects arising from repeated exposure. The need for careful clinical observations of the animals is stressed, so as to obtain as much information as possible. These tests should help to identify the target organs of toxicity and the toxic and toxic doses. Further in-depth investigation of these aspects may be required in long term studies (Methods B.26 - B.30 and B.33).

B.II MUTAGENICITY - GENOTOXICITY

Mutagenicity refers to the induction of permanent transmissible changes in the amount or structure of the genetic material of cells or organisms. These changes, 'mutations', may involve a single gene or gene segments, a block of genes, or whole chromosomes. Effects on whole chromosomes may be structural and / or numerical.

The mutagenic activity of a substance, for the base set information, is assessed by *in vitro* assays for gene (point) mutations in bacteria (Method B.13/14) and for structural chromosome aberrations in mammalian cells, (Method B.10).

Acceptable are also *in vivo* procedures, e.g. the micronucleus test (Method B.12) or the metaphase analysis of bone marrow cells, (Method B.11). However, in the absence of any contraindication the *in vitro* methods are strongly preferred.

Additional studies to investigate mutagenicity further or to pre-screen for carcinogenicity are required for higher production volumes and/or to conduct or follow-up a risk assessment, and these can be used for a number of purposes: to confirm results obtained in the base set; to investigate end-points not studied in the base set; to initiate or extend *in vivo* studies.

For these purposes, methods B.15 to B.25 include both *in vivo* and *in vitro* eukariotic systems and an extended range of biological end-points. These tests provide information on point mutations and other end-points in organisms more complex than the bacteria used for the base set.

As a general principle, when a programme of further mutagenicity studies is considered, it should be designed so as to provide relevant additional information on the mutagenic and/or carcinogenic potential of that substance.

The actual studies which may be appropriate in a specific instance will depend on numerous factors, including the chemical and physical characteristics of the substance, the results of the initial bacterial and cytogenetic assays, the metabolic profile of the substance, the results of other toxicity studies, and the known uses of the substance. A rigid schedule for selection of tests is therefore inappropriate in view of the variety of factors which may require consideration.

Some general principles for the testing strategy are laid down by Dir. 93/67/EEC, but clear testing strategies may be found in the technical guidance document for Risk Assessment, which nevertheless is flexible and can be adapted as appropriate to specific circumstances.

Methods for further investigation are however grouped below, on the basis of their principal genetic end-point:

Studies to investigate gene (point) mutations

(a) Forward or reverse mutation studies using eukaryotic micro-organisms (*Saccharomyces cerevisiae*) (Method B.15)

(b) *In vitro* studies to investigate forward mutation in mammalian cells, (Method B.17)

(c) The sex-linked recessive lethal assay in *Drosophila melanogaster*, (Method B.20)

(d) *In vivo* somatic cell mutation assay, the mouse spot test, (Method B.24)

Studies to investigate chromosome aberrations

(a) In vivo cytogenetic studies in mammals; In vivo metaphase analysis of bone marrow cells should be considered if it has not been included in the initial assessment (Method B.11). In addition, in vivo germ cell cytogenetics may be investigated, (Method B.23)

(b) In vitro cytogenetic studies in mammalian cells, if this has not been included in the initial assessment, (Method B.10)

(c) Dominant lethal studies in rodents, (Method B.22)

(d) Mouse heritable translocation test, (Method B.25)

Genotoxic effects - effects on DNA

Genotoxicity, identified as harmful effects on genetic material not necessarily associated with mutagenicity, may be indicated by induced damage to DNA without direct evidence of mutation. The following methods using eukaryotic micro-organisms or mammalian cells may be appropriate for such investigation:

(a) Mitotic recombination in *Saccharomyces cerevisiae*, (Methods B.16)

(b) DNA damage and repair - unscheduled DNA synthesis - mammalian cells - *in vitro*, (Method B.18)

(c) Sister chromatid exchange in mammalian cells - *in vitro*, (Method B.19)

Alternative methods for investigating carcinogenic potential

Mammalian cell-transformation assays are available which measure the ability of a substance to induce morphological and behavioural changes in cell cultures, which are thought to be associated with malignant transformation - *in vivo*, (Method B.21). A number of different cell types and criteria for transformation may be used.

Risk assessment for heritable effects in mammals

There are methods available to measure heritable effects in whole mammals produced by gene (point) mutations, e.g. the mouse specific locus test, to measure germ-cell mutation in the first generation, (not included in this Annex), or for chromosome aberrations, e.g. the mouse heritable translocation test, (Method B.25). Such methods may be used when estimating the possible genetic risk of a substance to man. However, in view of the complexities involved in these tests and the very large number of animals necessary, particularly for the specific locus test, a strong justification is needed before undertaking these studies.

B.III CARCINOGENICITY

Chemicals may be described as genotoxic or non-genotoxic carcinogens, dependant on the presumed mechanism of action.

Pre-screening information for genotoxic carcinogenic potential of a substance may be obtained from the mutagenicity/genotoxicity studies. Additional information may be obtained from the repeated dose, subchronic or chronic toxicity tests. The repeated dose toxicity test,

Method B.7 and longer repeated dose studies include assessment on histopathological changes observed in repeated dose toxicity tests, e.g. hyperplasia in certain tissues which could be of concern. These studies and toxicokinetic information may help to identify chemicals with carcinogenic potential, which may require further in-depth investigation of this aspect, in a carcinogenicity test (Method B.32) or often in a combined chronic toxicity/carcinogenicity study (Method B.33)

B.IV REPRODUCTIVE TOXICITY

Reproductive toxicity may be detected in different ways e.g. impairment of male and female reproductive functions or capacity, identified as 'effects on fertility', or induction of non-inheritable harmful effects on the progeny, identified as 'developmental toxicity' where teratogenicity and effects during lactation are also included.

For teratogenicity studies, as part of the developmental toxicity testing, the test method (Method B.31), is primarily directed to administration by the oral route. Alternatively, other routes may be used depending on the physical properties of the test substance or likely route of human exposure. In such cases, the test method should be suitably adapted taking into consideration the appropriate elements of the 28-day test methods.

Where a three-generation reproduction (fertility) test is required, the described method for the two-generation reproduction test (Method B.35), can be extended to cover a third generation.

B.V NEUROTOXICITY

Neurotoxicity may be detected in different ways e.g. functional changes and/or structural and biochemical changes in the central or peripheral nervous system. A preliminary indication of neurotoxicity may be obtained from acute toxicity tests. The repeated dose toxicity test, Method B.7, includes assessment of neurotoxicological effects, and the need for careful clinical observations of the animals is stressed, so as to obtain as much information as possible. The method should help to identify chemicals with neurotoxic potential, which may require further in-depth investigation of this aspect. Additionally, it is important to consider the potential of substances to cause specific neurotoxic effects that may not be detected in other toxicity studies. For example, certain organophosphorous substances have been observed to cause delayed neurotoxicity and can be evaluated in methods B.37 and B.38, following single or repeated-dose exposure.

B.VI IMMUNOTOXICITY

Immunotoxicity may be detected in different ways e.g. immunosupression and/or enhancement of the responsiveness of the immune system resulting in either hypersensitivity or induced autoimmunity. The repeated dose toxicity test, Method B.7, includes assessment of immunotoxic effects. The method should help to identify chemicals with immunotoxic potential, which may require further in-depth investigation of this aspect.

B.VII TOXICOKINETICS

Toxicokinetic studies help in the interpretation and evaluation of toxicity data. These studies are intended to elucidate particular aspects of the toxicity of the chemical under test and the

results may assist in the design of further toxicity studies. It is not envisaged that in every case all parameters will need to be determined. Only in rare cases will the whole sequence of toxicokinetic studies (absorption, excretion, distribution and metabolism) be necessary. For certain compounds, changes in this sequence may be advisable or a single-dose study may be sufficient (Method B.36).

Information on chemical structure (SAR) and physico-chemical properties may also provide an indication of the absorption characteristics by the intended route of administration and the metabolic and tissue distribution possibilities. There may also be information on toxicokinetic parameters from preceding toxicity and toxicokinetic studies.

C. CHARACTERISATION OF THE TEST SUBSTANCE

The composition of the test substance, including major impurities, and its relevant physico-chemical properties including stability, should be known prior to the initiation of any toxicity study.

The physico-chemical properties of the test substance provide important information for the selection of the route of administration, the design of each particular study and the handling and storage of the test substance.

The development of an analytical method for qualitative and quantitative determination of the test substance (including major impurities when possible) in the dosing medium and the biological material should precede the initiation of the study.

All information relating to the identification, the physico-chemical properties, the purity, and behaviour of the test substance should be included in the test report.

D. ANIMAL CARE

Stringent control of environmental conditions and proper animal care techniques are essential in toxicity testing.

(i) Housing conditions

The environmental conditions in the experimental animal rooms or enclosures should be appropriate to the test species. For rats, mice and guinea pigs, suitable conditions are a room temperature of 22°C ± 3°C with a relative humidity of 30 to 70 % ; for rabbits the temperature should be 20 ± 3°C with a relative humidity of 30 to 70 %.

Some experimental techniques are particularly sensitive to temperature effects and, in these cases, details of appropriate conditions are included in the description of the test method. In all investigations of toxic effects, the temperature and humidity should be monitored, recorded, and included in the final report of the study.

Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. Details of the lighting pattern should be recorded and included in the final report of the study.

Unless otherwise specified in the method, animals may be housed individually, or be caged in small groups of the same sex; for group caging, no more than five animals should be housed per cage.

In reports of animal experiments, it is important to indicate the type of caging used and the number of animals housed in each cage both during exposure to the chemical and any subsequent observation period.

(ii) Feeding conditions

Diets should meet all the nutritional requirements of the species under test. Where test substances are administered to animals in their diet the nutritional value may be reduced by interaction between the substance and a dietary constituent. The possibility of such a reaction should be considered when interpreting the results of tests. Conventional laboratory diets may be used with an unlimited supply of drinking water. The choice of the diet may be influenced by the need to ensure a suitable admixture of a test substance when administered by this method.

Dietary contaminants which are known to influence the toxicity should not be present in interfering concentrations.

E. ANIMAL WELFARE

When elaborating the test methods due consideration was given to animal welfare. Some examples are briefly given below, but this list is not exhaustive. The exact wording and/or conditions should be read in the text of the methods :

– For the determination of acute oral toxicity, two alternative methods, the 'Fixed Dose Procedure' and the ‘Acute Toxic Class method’ should be considered. The ‘Fixed Dose Procedure’ does not utilise death as specific endpoint and it uses fewer animals. The ‘Acute Toxic Class method’ uses on average 70% less animals than Method B.1 for Acute Oral toxicity. Both these alternative methods result in less pain and distress than the classical methodology.

– The number of animals used is reduced to the scientifically acceptable minimum : only 5 animals of the same sex are tested per dose level for methods B.1 and B.3; only 10 animals (and only 5 for the negative control group) are used for the determination of the skin sensitisation by the guinea pig maximisation test (method B.6); the number of animals needed for the positive control when testing mutagenicity in vivo is also lowered (methods B.11 and B.12)

– Pain and distress of animals during the tests are minimised : animals showing severe and enduring signs of distress and pain may need to be humanely killed; dosing test substances in a way known to cause marked pain and distress due to corrosive or irritating properties need not to be carried out (methods B.1, B.2 and B.3).

– Testing with irrelevantly high doses is avoided by the introduction of limit tests, not only in the acute toxicity tests (methods B.1, B.2 and B.3) but also in the in vivo tests for mutagenicity (methods B.11 and B.12).

– A strategy of testing for irritancy now allows the non-performance of a test, or its reduction to a single animal study, when sufficient scientific evidence can be provided.

Such scientific evidence can be based on the physico-chemical properties of the substance, the results of other tests already performed, or the results of well validated *in vitro* tests. For

example, if an acute toxicity study by the dermal route has been conducted at the limit test dose with the substance (method B.3), and no skin irritation was observed, further testing for skin irritation (method B.4) may be unnecessary; materials which have demonstrated definite corrosion or severe skin irritancy in a dermal irritation study (method B.4) should not be further tested for eye irritancy (method B.5).

F. ALTERNATIVE TESTING

A scientific objective for the European Union is the development and validation of alternative techniques which can provide the same level of information as current animal tests, but which use fewer animals, cause less suffering or avoid the use of animals completely.

Such methods, as they become available, must be considered wherever possible for hazard characterisation and consequent classification and labelling for intrinsic hazards.

G. EVALUATION AND INTERPRETATION

When tests are evaluated and interpreted, limitations in the extend to which the results of animal and *in vitro* studies can be extrapolated directly to man must be considered and therefore, evidence of adverse effects in humans, where available, may be used for confirmation of testing results.

These results, can be used for the classification and labelling of the new and the existing chemicals for human health effects, on the basis of their intrinsic properties, identified and quantified by these methods. Corresponding Annex VI criteria for classification and labelling relate also to the end-points of the testing protocols included in these testing methods.

These results can also be used for risk assessment studies, for new and existing chemicals, and appropriate testing strategies for these purposes are indicated in the corresponding guidance documents.

H. LITERATURE REFERENCES

Most of these methods are developed within the framework of the OECD programme for Testing Guidelines, and should be performed in conformity with the principles of Good Laboratory Practice, in order to ensure as wide as possible ‘mutual acceptance of data’.

Additional information may be found in the references found in the OECD guidelines and the relevant literature published elsewhere.

B.1BIS ACUTE TOXICITY (ORAL) -FIXED DOSE METHOD

1. METHOD

1.1. INTRODUCTION

See General Introduction Part B (A).

1.2. DEFINITIONS

See General Introduction Part B (B).

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLE OF THE TEST METHOD

The acute oral toxicity test provides information on the adverse effects which can follow, within a snort period of time, the ingestion of a single dose of the test substance.

The fixed dose method is conducted in two stages.

In a preliminary sighting study, the effects of various doses administered orally by gavage to single animals of one sex are investigated in a sequential manner. The sighting study yields information on the dose-toxicity relationship, including an estimate of the minimum lethal dose. Normally, no more than five animals are used in this first stage.

In the main study, the substance is administered orally by gavage to groups of five male and five female animals at one of the pre-set dose levels (5, 50, 500 or 2000 mg/kg). The dose used is derived from the sighting study and is that which is likely to produce 'evident toxicity' (see 1.2. Definitions) but no deaths.

Following administration, observations for effects are made.

When the initial dose level chosen produces evident toxicity but no compound-related mortality , no further testing is needed.

Where evident toxicity is not seen at the chosen dose level, the substance should be re-tested at the next higher dose level. Where animals die, or where a severe toxic reaction requires humane killing of animals, the substance should be re-tested at the next lower dose level.

This procedure permits the identification of the 'discriminating dose' (see 1.2. Definitions), that is the highest of the pre-set dose levels which can be administered without causing mortality (including humane kills).

Animals showing severe and enduring signs of distress and pain may need to be humanely killed. Dosing test substances in a way known to cause marked pain and distress due to corrosive or irritating properties need not be carried out.

1.5. QUALITY CRITERIA

None.

1.6. DESCRIPTION OF THE TEST METHOD

1.6.1. Preparations

1.6.1.1. Experimental Animals

Unless there are contra-indications the rat is the preferred species.

Commonly used laboratory strains should be employed. For each sex, at the start of the test, the range of weight variation in the animals used should not exceed ± 20 % of the appropriate mean value.

The animals are kept under the experimental housing and feeding conditions for at least five days prior to the test. Before the test, healthy young adult animals are randomized and assigned to the sighting study and main study treatment groups. In practice, only one group of each sex may be needed in the main study.

1.6.1.2. Dose preparation and administration

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 is considered first, followed by consideration of a solution in vegetable oil, then by possible solution in other vehicles, or in suspension. For non-aqueous vehicles the relevant toxic characteristics of the vehicle should be known or should be determined before or during the test. In rodents, normally the volume should not exceed 10 ml/kg body weight except in the case of aqueous solutions where 20 ml/kg may be used. Variability in test volume should be minimized by adjusting the concentration to ensure a constant volume at all dose levels.

Animals should be fasted prior to substance administration. For the rat, food should be withheld overnight; water is not restricted. The following day, the animals should be weighed and then the test substance administered by gavage in a single dose. If a single dose is not possible, the dose may be given in smaller fractions over a period not exceeding 24 hours. After the substance has been administered, food may be withheld for a further three to four hours. Where a dose is administered in fractions over a period it may be necessary to provide the animals with food and water depending on the length of the period.

1.6.2. Procedure

1.6.2.1. Sighting study

The effects of various doses are investigated in single animals. Normally female animals will be used in the absence of information indicating that males will be the more sensitive sex. Dosing is sequential, allowing at least 24 hours before dosing the next animal. All animals are carefully observed for signs of toxicity for at least seven days; if signs of moderate toxicity

persist at seven days, the animal should be observed for up to an additional seven days. The following initial dose levels are considered: 5, 50, 500 and 2000 mg/kg. If the initial dose chosen does not produce severe toxicity, and the next higher level produces mortality, then it will be necessary to investigate one or more intermediate dose levels as appropriate. In this way it should be possible to build up information on the dose level(s) that produce(s) some signs of toxicity and the minimum dose level that produces mortality.

An effort should be made to select the initial dose using evidence from related chemicals. In the absence of such information, it is suggested that the 500 mg/kg dose is used in the first instance. If no signs of toxicity are seen at the initial dose, then the next higher dose level is investigated. If no mortality occurs at 2000 mg/kg, the sighting test is complete and the main study should be conducted at this dose level. If severe effects, necessitating humane killing are seen at the initial dose (e.g. 500 mg/kg), the next lower dose (e.g. 50 mg/kg) is given to another animal. If this animal survives, further animals may then be dosed with the appropriate intermediate dose levels between the fixed doses. Normally, one would not expect to use more than five animals in this procedure.

1.6.2.2. Main study

At least 10 animals (five female and five male) should be used for each dose level which is investigated. The females should be nulliparous and non-pregnant.

It is a principle of the fixed dose method that only moderately toxic doses are used in the main study.

Administration of lethal doses of the test substance should be avoided.

The dose level to be used in the test should be selected from one of the four fixed dose levels, namely 5, 50, 500 or 2000 mg/kg body weight. The initial dose level chosen should be that which is likely to produce evident toxicity but no compound-related mortality (including humane kills; accidental deaths are not included but should be recorded). No further testing is necessary when this dose level produces evident toxicity but no compound-related mortality.

Where evident toxicity does not result from administration of the chosen dose level, the substance should be re-tested at the next higher dose level. The animals, however, should continue to be kept under observation until the observation period is complete. Where a severe toxic reaction requires animals to be humanely killed or there is compound-related mortality, the substance should be retested at the next lower dose level. Again, animals that do not need to be humanely killed should be kept under observation for the full observation period.

Following administration, observations are made and recorded systematically. Individual records should be maintained for each animal.

The observation period should be at least 14 days. However, the duration of observation should not be rigidly fixed. It should be determined by the toxic reactions, their rate of onset and the length of the recovery period; it may thus be extended when considered necessary. The time at which signs of toxicity appear and disappear and the time of death are important, especially if there is a tendency for toxic signs to be delayed.

A careful clinical examination should be made at least twice on the day of dosing and at least once each day thereafter. Animals obviously in pain or showing severe signs of distress should be humanely killed. Additional observations will be necessary during the first few

days after dosing if the animals continue to display signs of toxicity .The test may be terminated if it becomes apparent that the initial dose level chosen was too high.

Observations should include changes in the skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous systems, and somatomotor activity and behaviour pattern. Particular attention should be directed to observation of tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma.

Individual weights of animals should be determined shortly before the test substance is administered, daily for the next three days, and weekly thereafter. Animals that die during the test, and those surviving to termination of the test, are weighed and subjected to necropsy. All gross pathological changes should be recorded. Where indicated, tissues should be taken for histopathological examination.

The investigation of a second or, in exceptional circumstances, a third dose level may be required, dependent upon the results of the preceding dose level.

In the case in which a substance produces mortality at 5 mg/kg body weight (or where the sighting study indicates that mortality will result at that dose level) the acute toxicity of the substance may need to be further investigated.

2. DATA

Data from both the sighting study and the main study should be summarized in tabular form showing for each dose level tested the number of animals at the start of the test; the number of animals displaying signs of toxicity; the number of animals found dead during the test or killed for humane reasons; a description of the toxic effects and, for the main study, whether compound-related evident toxicity was observed; the time course of any toxic effects; and the necropsy findings. Changes in weight should be calculated and recorded when survival exceeds one day.

Animals which are humanely killed due to compound-related distress and pain are recorded as compound-related deaths.

3. REPORTING

3.1. TEST REPORT

The test report shall, if possible, include the following information, for both the sighting study and the main study, as appropriate :

– species, strain, source, environmental conditions, diet, etc.

– test conditions

– dose levels (with vehicle, if used, and concentration)

– full result of all dose levels investigated

– tabulation of response data by sex and dose level (i.e. number of animals used; changes in body weight; when applicable, number of animals that died or were killed

during the test; number of animals showing signs of toxicity; nature, severity and duration of effects)

– time course of onset of signs of toxicity and whether these were reversible

– when animals died or were killed, time of death after dosing, reasons and criteria used for humane killing of animals

– necropsy findings

– any histopathological findings

– discussion of the results

– interpretation of results, including the signs of evident toxicity and the discriminating dose level identified in the test.

3.2. EVALUATION AND INTERPRETATION

DOSE	RESULTS	INTERPRETATION
5 mg/kg b.w.	Less than 100 % survival	Compounds which are VERY TOXIC.
	100 % survival; but evident toxicity	Compounds which are TOXIC.
	100 % survival; no evident toxicity	See results at 50 mg/kg.
50 mg/kg b.w.	Less than 100 % survival	Compounds which may be TOXIC or VERY TOXIC. See results at 5 mg/kg.
	100 % survival; but evident toxicity	Compounds which are HARMFUL.
	100 % survival; no evident toxicity	See results at 500 mg/kg.
500 mg/kg b.w.	Less than 100 % survival	Compounds which may be TOXIC or HARMFUL. See results at 50 mg/kg.
	100 % survival; but evident toxicity	Compounds considered as having no significant acute toxicity.
	100 % survival; no evident toxicity	See results at 2 000 mg/kg.
2 000 mg/kg b.w.	Less than 100 % survival	See results at 500 mg/kg.
	100 % survival; with or without evident toxicity	Compounds which do not have significant acute toxicity.

See also General Introduction Part B (D).

4. REFERENCES

See General Introduction Part B (E).

B.1TRIS ACUTE TOXICITY (ORAL) – ACUTE TOXIC CLASS METHOD

1. METHOD

1.1. INTRODUCTION

The acute toxic class method provides information both for hazard assessment and for hazard classification purposes.

The method uses three fixed doses, adequately separated to enable a compound to be ranked, based on the results of the study. Besides, the procedure described in this test method allows for the selection of three additional fixed doses, which could either be used as alternative options at given decision points or as option for further testing. The use of (any of the) additional doses may be considered in case a further refinement may be desirable or necessary.

The method uses defined starting doses and is not intended to allow the calculation of a precise LD50, but does allow for the determination of a range of exposure where lethality is expected, since death of a proportion of the animals is still the major end-point of this test. The results of the test should allow for classification according to Annex VI criteria. Due to the sequential nature of the approach, the duration of the test could be longer than the procedure described in the B.1. The main advantage of this method is that it requires a smaller number of animals than both the acute toxicity (oral) (B.1) and the alternative Fixed Dose Method (B.1bis).

See also General Introduction Part B.

1.2. DEFINITIONS

See General Introduction Part B.

1.3. PRINCIPLE OF THE TEST METHOD

The substance is administered orally to a group of experimental animals at one of the defined doses. The substance is tested using a stepwise procedure, each step using three animals of one sex. It is not necessary to perform a preliminary sighting study. Absence or presence of substance related mortality of the animals dosed at one step will determine the next step, i.e.:

– no further testing is needed

– the next step will be performed with the same dose, but with animals of the other sex

– the next step will be performed at the next higher or the next lower dose level

1.4. DESCRIPTION OF THE TEST METHOD

1.4.1. Preparations

Healthy young adult animals are randomly selected, marked to permit individual identification, and kept in their cages for at least 5 days prior to the start of the test, to allow for acclimatisation to the laboratory conditions. The animals may be group-caged by sex and dose, but the number of animals per cage must not interfere with clear observations of each animal.

The test substance is administered in a single dose to the animals by gavage using a stomach tube or a suitable intubation cannula.

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 non-aqueous vehicles the toxic characteristics of the vehicle should be known, and if not known should be determined before the test.

Animals should be fasted prior to dosing (e.g. overnight for the rat or 3-4 hours for the mouse); water should not be withheld.

1.4.2. Test conditions

1.4.2.1. Test animals

Unless there are contraindications, rat is the preferred rodent species. The females should be nulliparous and non-pregnant.

At the commencement of the study, the weight variation of animals should be minimal and not exceed $\pm$ 20 per cent of the mean weight for each sex.

1.4.2.2. Number and sex

Three animals of one sex are used for each step. Either sex can be used in the initial step.

1.4.2.3. Dose levels

The dose level to be used as the starting dose is selected from one of three fixed levels i.e. 25, 200 and 2 000 mg/kg body weight. The starting dose level should be that which is most likely to produce mortality in at least some of the dosed animals. One of the flow charts of the procedures described in Annex 1 may be used depending on the starting dose.

For selecting the sex and the starting dose, all the available information should be used, including information on structure activity relationships. When the information suggest that mortality is unlikely at the highest dose level (2 000 mg/kg body weight), then a limit test should be conducted. When there is no information on a substance to be tested, for animal welfare reasons it is recommended to use the starting dose of 200 mg/kg body weight.

Occasionally, it may be desirable to achieve a refinement of information further than would be possible after conducting the test with the three fixed dose levels of 25, 200 and 2 000 mg/kg body weight. In these cases further testing at additional fixed dose levels of 5, 50 or 500 mg/kg body weight may be considered.

Doses that are known to cause marked pain and distress, due to corrosive or severely irritant actions, need not be administered.

The time interval between treatment groups is determined by the onset, duration, and severity of toxic signs. Treatment of animals of the other sex, or at the next dose, should be delayed until one is confident of survival of the previously dosed animals.

1.4.2.4. Limit test

A limit test at one dose level of 2 000 mg/kg body weight may be carried out with three animals of each sex. If substance related mortality is produced, further testing at 200 mg/kg (or 500 mg/kg) body weight may need to be carried out.

1.4.2.5. Observation period

The animals should normally be observed for 14 days, except where animals 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. All observations are systematically recorded with individual records being maintained for each animal.

1.4.3. Procedure

Following the period of fasting the animals should be weighed prior to test substance administration. After the substance has been administered, food may be withheld for a further 3-4 hours. Where a dose is administered in fractions over a period, it may be necessary to provide the animals with food and water, depending on the length of the period.

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 1 ml/100 g body weight; however, in the case of aqueous solutions 2 ml/100 g body weight can be considered. Variability in test volume should be minimised by adjusting the concentration to ensure a constant volume at all dose levels. If a single dose is not possible, the dose may be given in smaller fractions over a period not exceeding 24 hours.

Details of the test procedure are described in Annex 1.

1.4.3.1. General observations

Careful clinical observations should be made at least twice on the day of dosing, or more frequently when indicated by the response of the animals to the treatment, and at least once daily thereafter. Animals found in a moribund condition and animals showing severe pain and enduring signs of severe distress should be humanely killed. Animals killed for humane reasons are considered in the same way as animals that died on test.

When animals are killed for humane reasons or found dead, the time of death should be recorded as precisely as possible. 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.

All observations are systematically recorded with individual records being maintained for each animal.

1.4.3.2. Body weight

All animals should be weighed 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 before humanly killed.

1.4.3.3. Gross necropsy

All test animals, including those which die during the test or are removed from the study, 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, 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.

General guidance on the interpretation of the results for classification is given in Annex 2.

3. REPORTING

Test Report

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

Test animals:

– species/strain;

– microbiological status of the animals, when known;

– 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;

– details of the administration of the test substance including dosing volumes and time of dosing;

– details of food and water quality (including type/source, water source);

– the rationale for the selection of the starting dose.

Results:

– tabulation of response data by sex and dose level for each animal (i.e. animals showing signs of toxicity including mortality, nature, severity and duration of effects);

– 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.

Discussion of results.

Conclusions.

4. REFERENCES

This method is analogous to OECD TG 423.

ANNEX 1
TEST PROCEDURE

1. As indicated in point 1.4.2.3, the starting dose should be the one which is likely to produce mortality in at least some of the dosed animals. Information that could be used to select the starting dose include:

– data on physical chemical properties;

– structure-activity relationship;

– all data from other toxicity tests; and

– anticipated use of the test substance.

2. For each starting dose, the respective testing schemes, as included in this annex, outline the procedure to be followed. Depending on the number of humanely killed or dead animals the test procedure follows the indicated arrows.

3. When at a starting dose of 25 or 200 mg/kg body weight only one animal of the second sex dies, this would normally lead to no further testing. However, when no toxic signs are observed in the other five animals, during autopsy consideration should be given to the possibility that mortality may not have been substance related. In such a case, the test should be continued with dosing at the next higher level.

4. When at a dose of 2 000 mg/kg body weight, one animal per sex dies, the LD50 value is expected to exceed 2000 mg/kg body weight. However, because this is a borderline result, the response of the remaining two animals per sex should be carefully considered and the occurrence of distinct, marked toxic signs in these animals may well lead to classification corresponding to an LD50 value of 2 000 mg/kg body weight or less, or would justify further testing at this same level.

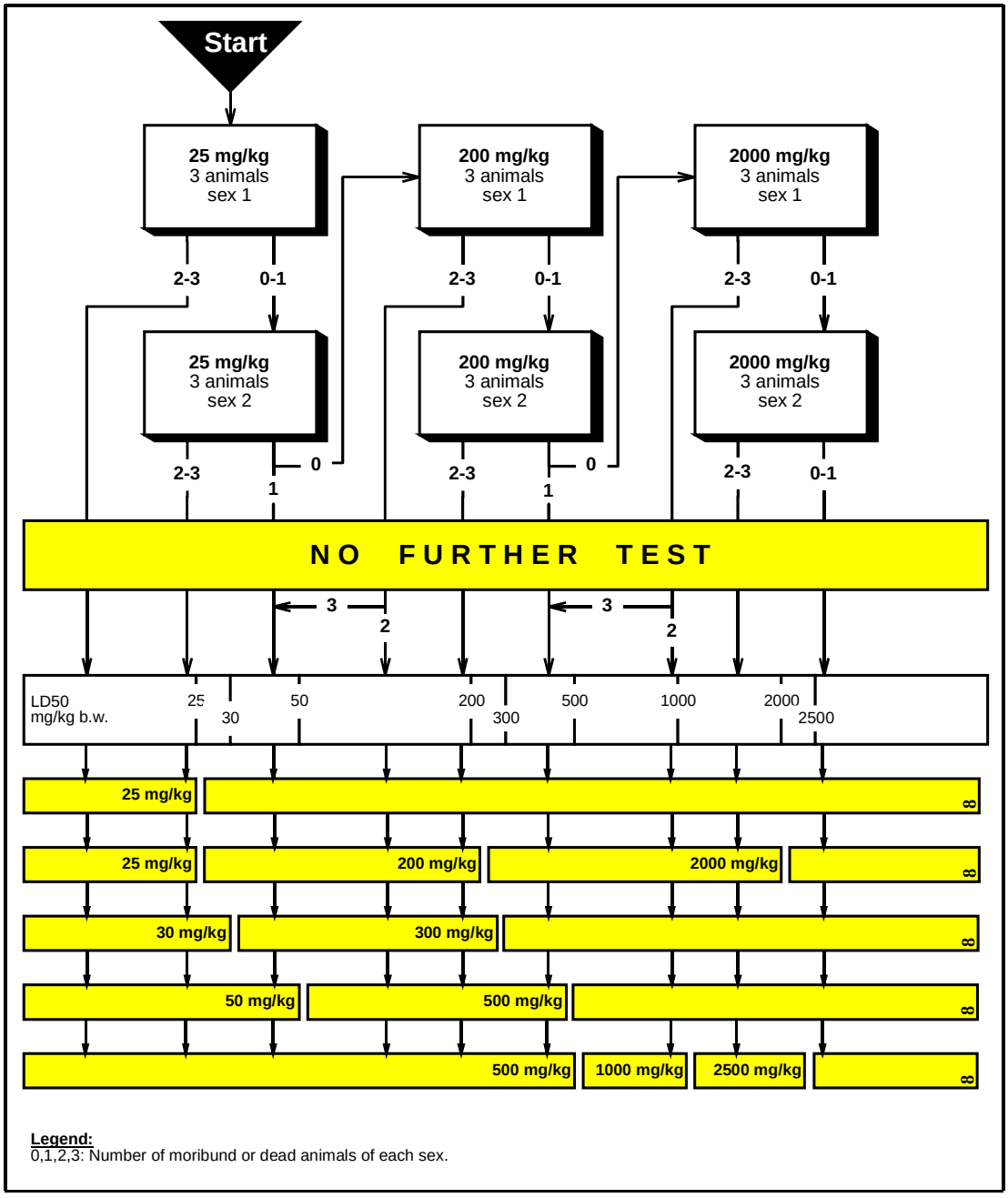
5. The procedure allows for testing at three additional fixed doses (option 2). This option could either be used to select an alternative dose at a given decision point, or for further testing after having completed the actual test (option 1). The option 1, test procedure is indicated with bold arrows, whereas for the option 2 test procedure, thin arrows are used.

ANNEX 2
INTERPRETATION OF RESULTS BASED ON OPTION 1 TESTING

The grey boxes below the 'no further testing' box in the schemes of this annex, represent cut off values for classification. Following the test procedure as outlined in option 1, the appropriate arrow should be followed further downwards, until it reaches the grey box of concern.

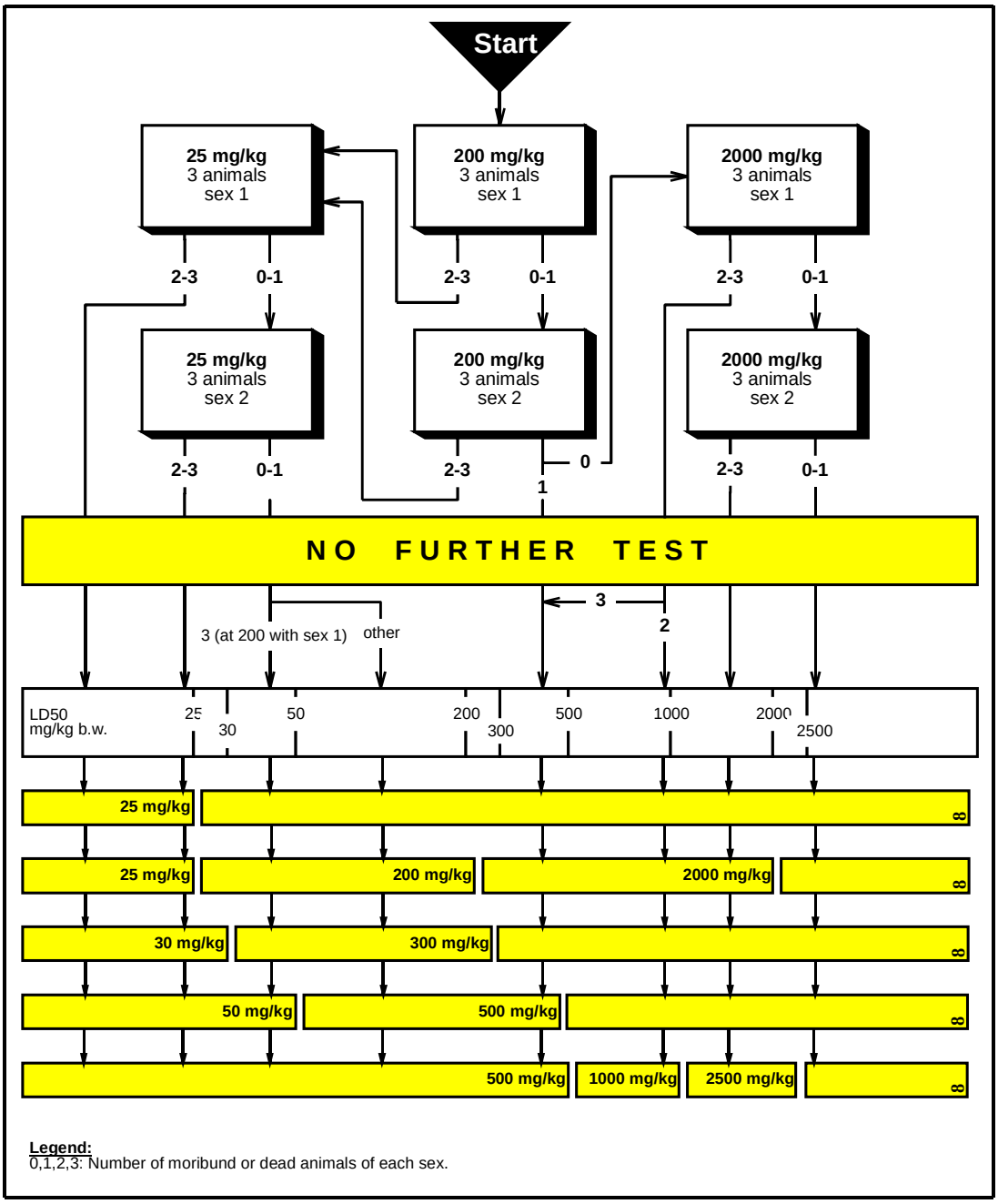
(a) Interpretation of results based on option 1 testing

Starting dose: 25 mg/kg body weight



(b) Interpretation of results based on option 1 testing

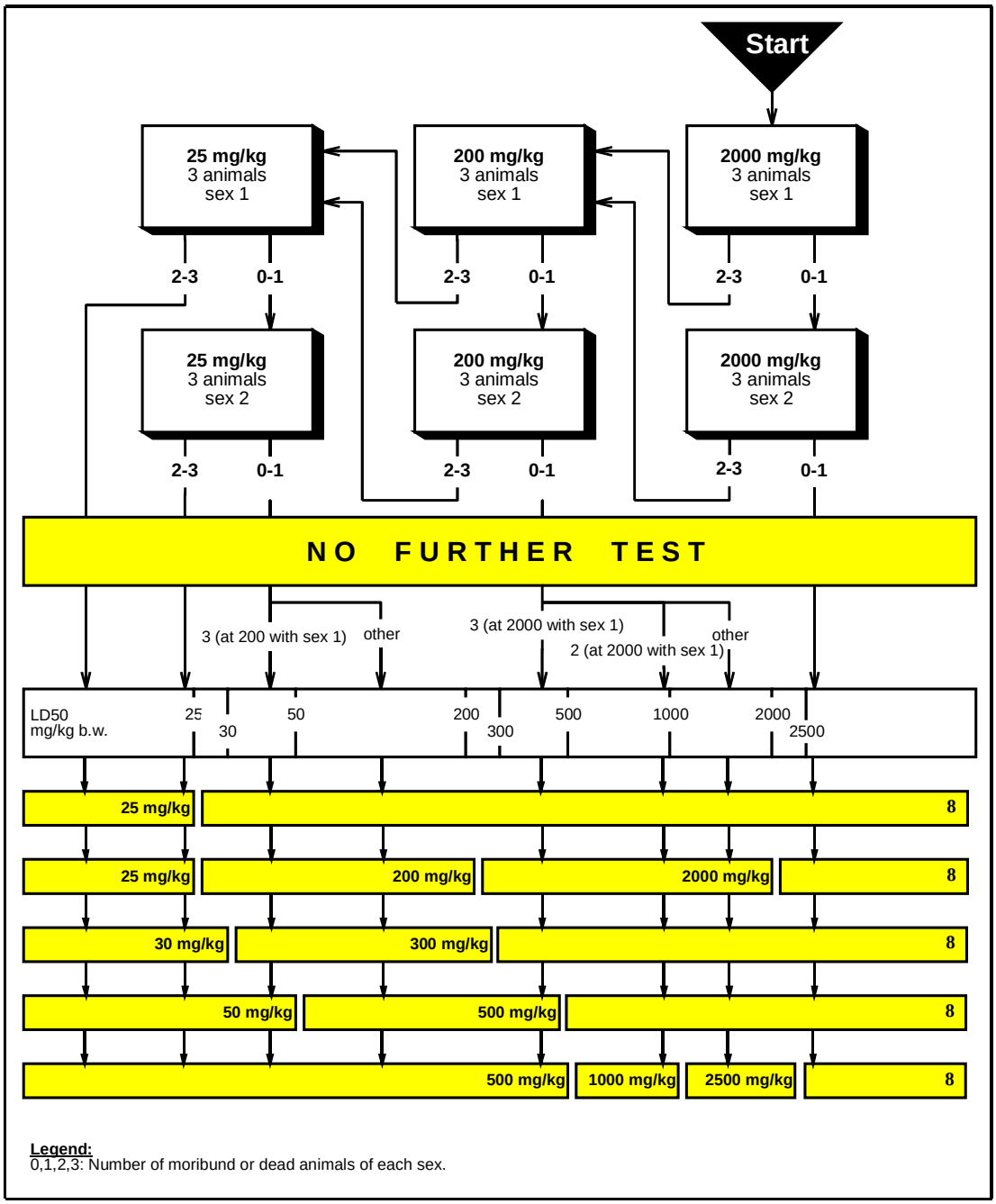
Starting dose: 200 mg/kg body weight



Legend:
0,1,2,3: Number of moribund or dead animals of each sex.

(c) Interpretation of results based on option 1 testing

Starting dose: 2000 mg/kg body weight



Legend:
0,1,2,3: Number of moribund or dead animals of each sex.

B.2. ACUTE TOXICITY (INHALATION)

1. METHOD

1.1. INTRODUCTION

It is useful to have preliminary information on the particle size distribution, the vapour pressure, the melting point, the boiling point, the flash point and explosivity (if applicable) of the substance.

See also General Introduction Part B (A).

1.2. DEFINITIONS

See General Introduction Part B (B).

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLE OF THE TEST METHOD

Several groups of experimental animals are exposed for a defined period to the test substance in graduated concentrations, one concentration being used per group. Subsequently observations of effects and deaths are made. Animals which die during the test are necropsied and at the conclusion of the test surviving animals are necropsied.

Animals showing severe and enduring signs of distress and pain may need to be humanely killed. Dosing test substances in a way known to cause marked pain and distress due to corrosive or severe irritating properties need not be carried out.

1.5. QUALITY CRITERIA

None.

1.6. DESCRIPTION OF THE TEST METHOD

1.6.1. Preparations

The animals are kept under the experimental housing and feeding conditions for at least five days prior to the experiment. Before the test healthy young animals are randomized and assigned to the required number of groups. They need not be subjected to simulated exposure unless this is indicated by the rype of exposure apparatus being used.

Solid test substances may need to be micronised in order to achieve particles of an appropriate size.

Where necessary a suitable vehicle may be added to the test substance to help generate an appropriate concentration of the test substance in the atmosphere and a vehicle control group should then be used. If a vehicle or other additives are used to facilitate dosing, they should be known not to produce toxic effects. Historical data can be used if appropriate.

1.6.2. Test Conditions

1.6.2.1. Experimental Animals

Unless there are contra-indications the rat is the preferred species. Commonly used laboratory strains should be employed. For each sex, at the start of the test the range of weight variation in the animals used should not exceed ± 20 % of the appropriate mean value.

1.6.2.2. Number and Sex

At least 10 rodents (five female and five male) are used at each concentration level. The females should be nulliparous and non-pregnant.

Note: In acute toxicity tests with animals of a higher order than rodents, the use of smaller numbers should be considered. Doses should be carefully selected, and every effort should be made not to exceed moderately toxic doses. In such tests administration of lethal doses of the test substance should be avoided.

1.6.2.3. Exposure Concentrations

These should be sufficient in number, at least three, and spaced appropriately to produce test groups with a range of toxic effects and mortality rates. The data should be sufficient to produce a concentration mortality curve and, where possible, permit an acceptable determination of an LC_{50}.1.6.2.4. Limit test

1.6.2.4. Limit test

If an exposure of five male and five female test animals to 20 mg per litre of a gas or 5 rug per litre of an aerosol or a particulate for four hours (or where this is not possible due to the physical or chemical, including explosive, properties of the test substance, the maximum attainable concentration) produces no compound related mortality within 14 days further testing may not be considered necessary. (18th ATP, dir. 93/21/EEC, Ll10/93)

1.6.2.5. Exposure time

The period of exposure should be four hours.

1.6.2.6. Equipment

The animals should be tested with inhalation equipment designed to sustain a dynamic airflow of at least 12 air changes per hour, to ensure an adequate oxygen content and an evenly distributed exposure atmosphere. Where a chamber is used its design should minimize crowding of the test animals and maximize their exposure by inhalation to the test substance. As a general rule to ensure stability of a chamber atmosphere the total 'volume' of the test animals should not exceed 5% of the volume of the test chamber. Oro-nasal, head only, or whole body individual chamber exposure may be used; the first two will help to minimize the uptake of the test substance by other routes.

1.6.2.7. Observation Period

The observation period should be at least 14 days. However, the duration of observations should not be rigidly fixed. It should be determined by the toxic reactions, their rate of onset and the length of the recovery period; it may thus be extended when considered necessary. The time at which signs of toxicity appear and disappear and the time of death are important, especially if there is a tendency for deaths to be delayed.

1.6.3. Procedure

Shortly before exposure, the animals are weighed, and then exposed to the test concentration in the designated apparatus for a period of four hours, after equilibration of the chamber concentration. Time for equilibration should be short. The temperature at which the test is performed should be maintained at 22 ± 3 °C. Ideally the relative humidity should be maintained between 30% and 70 %, but in certain instances (e.g. tests of some aerosols) this may not be practicable. Maintenance of a slight negative pressure inside the chamber (≥ 5 mm of water) will prevent leakage of the test substance into the surrounding area. Food and water should be withheld during exposure. Suitable systems for the generation and monitoring of the test atmosphere should be used. The system should ensure that stable exposure conditions are achieved as rapidly as possible. The chamber should be designed and operated in such a way that a homogeneous distribution of the test atmosphere within the chamber is maintained.

Measurements or monitoring should be made:

(a) of the rate of air flow (continuously).

(b) of the actual concentration of the test substance measured in the breathing zone at least three times during exposure (some atmospheres, e.g. aerosols at high concentrations, may need more frequent monitoring). During the exposure period the concentration should not vary by more than ± 15 % of the mean value. However in the case of some aerosols, this level of control may not be achievable and a wider range would then be acceptable. For aerosols, particle size analysis should be performed as often as necessary (at least once per test group).

(c) of temperature and humidity , continuously if possible.

During and following exposure, observations are made and recorded systematically; individual records should be maintained for each animal. Observations should be made frequently during the first day. A careful clinical examination should be made at least once each working day, other observations should be made daily with appropriate actions taken to minimize loss of animals from the study, e.g. necropsy or refrigeration of those animals found dead and isolation or sacrifice of weak or moribund animals.

Observations should include changes in the skin and fur, eyes, mucous membranes, respiratory, circulatory, autonomic and central nervous systems, and somatomotor activity and behaviour pattern. Particular attention should be directed to observation of respiratory behaviour, tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma. The time of death should be recorded as precisely as possible. Individual weights of animals should be determined weekly after exposure, and at death.

Animals that die during the test and those surviving at the termination of the test are subjected to necropsy with particular reference to any changes in the upper and lower respiratory tract.

All gross pathological changes should be recorded. Where indicated, tissues should be taken for histopathological examination.

2. DATA

Data should be summarized in tabular form showing for each test group the number of animals at the start of the test, time of death of individual animals, number of animals displaying other signs of toxicity, description of toxic effects and necropsy findings. Changes in weight must be calculated and recorded when survival exceeds one day. Animals which are humanely killed due to compound-related distress and pain are recorded as compound-related deaths. The LC_{50} should be determined by a recognized method. Data evaluation should

include the relationship, if any, between the animal's exposure to the test substance and the incidence and severity of all abnormalities, including behavioural and clinical abnormalities, gross lesions, body weight changes, mortality and any other toxic effects.

3. REPORTING

3.1. TEST REPORT

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

– species, strain, source, environmental conditions, diet etc.;

– test conditions: description of exposure apparatus

including design, type, dimensions, source of air, system for generating aerosols, method of conditioning air and the method of housing animals in a test chamber when this is used. The equipment for measuring temperature, humidity, and aerosol concentrations and particle size distribution should be described.

Exposure data

These should be tabulated and presented with mean values and a measure of variability (e.g. standard deviation) and shall, if possible, include:

(a) airflow rates through the inhalation equipment;

(b) temperature and humidity of the air;

(c) nominal concentrations (total amount of test substance fed into the inhalation equipment divided by volume of air);

(d) nature of vehicle, if used;

(e) actual concentrations in test breathing zone;

(f) The mass median aerodynamic diameter (MMAD) and the geometric standard deviation (GSD);

(g) equilibration period;

(h) exposure period;

– tabulation of response data by sex and exposure level (i.e. number of animals that died or were killed during the test; number of animals showing signs of toxicity; number of animals exposed);

– time of death during or following exposure, reasons and criteria used for humane killing of animals;

– all observations;

– LC50 value for each sex determined at the end of the observation period (with method of calculation specified);

– 95 % confidence interval for the LC50 (where this can be provided);

– dose/mortality curve and slope (where permitted by the method of determination);

– necropsy findings;

– any histopathological findings;

– discussions of the results (particular attention should be given to the effect that humane killing of animals during the test may have on the calculated LC50 value);

– interpretation of the results.

3.2. EVALUATION AND INTERPRETATION

See General Introduction Part B (D).

4. REFERENCES

See General Introduction Part B (E).

B.3. ACUTE TOXICITY (DERMAL)

1. METHOD

1.1. INTRODUCTION

See General Introduction Part B (A).

1.2. DEFINITION

See General Introduction Part B (B).

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLE OF THE TEST METHOD

The test substance is applied to the skin in graduated doses to several groups of experimental animals, one dose being used per group. Subsequently, observations of effects and deaths are made. Animals which die during the test are necropsied and at the conclusion of the test surviving animals are necropsied.

Animals showing severe and enduring signs of distress and pain may need to be humanely killed. Dosing test substances in a way known to cause marked pain and distress due to corrosive or irritating properties need not be carried out.

1.5. QUALITY CRITERIA

None.

1.6. DESCRIPTION OF THE TEST METHOD

1.6.1. Preparations

The animals are kept in their experimental cages under the experimental housing and feeding conditions for at least five days prior to the experiment. Before the test, healthy young adult animals are randomized and assigned to the treatment groups. Approximately 24 hours before the test, fur should be removed by clipping or shaving from the dorsal area of the trunk of the animals. When clipping or shaving the fur, care must be taken to avoid abrading the skin which could alter its permeability. Not less than 10% of the body surface should be clear for the application of the test substance. When testing solids, which may be pulverized if appropriate, the test substance should be moistened sufficiently with water or, where necessary, a suitable vehicle to ensure good contact with the skin. When a vehicle is used, the influence of the vehicle on penetration of skin by the test substance should be taken into account. Liquid test substances are generally used undiluted.

1.6.2. Test Conditions

1.6.2.1. Experimental Animals

The adult rat or rabbit may be used. Other species may be used but their use would require justification. Commonly used laboratory strains should be employed. For each sex, at the start of the test the range of weight variation in the animals used should not exceed ± 20 % of the appropriate mean value.

1.6.2.2. Number and Sex

At least 5 animals are used at each dose level. They should all be of the same sex. If females are used, they should be nulliparous and non-pregnant. Where information is available demonstrating that a sex is markedly more sensitive, animals of this sex should be dosed.

Note: In acute toxicity tests with animals of a higher order than rodents, the use of smaller numbers should be considered. Doses should be carefully selected, and every effort should be made not to exceed moderately toxic doses. In such tests, administration of lethal doses of the test substance should be avoided.

1.6.2.3. Dose Levels

These should be sufficient in number, at least three, and spaced appropriately to produce test groups with a range of toxic effects and mortality rates. Any irritant or corrosive effects should be taken into account when deciding on dose levels. The data should be sufficient to produce a dose/response curve and, where possible, permit an acceptable determination of the LD_{50}.

1.6.2.4. Limit Test

A limit test at one dose level of at least 2000 mg/kg bodyweight may be carried out in a group of 5 male and 5 female animals, using the procedures described above. If compound-related mortality is produced, a full study may need to be considered.

1.6.2.5. Observation Period

The observation period should be at least 14 days. However, the duration of observation should not be rigidly fixed. It should be determined by the toxic reactions, their rate of onset and the length of the recovery period; it may thus be extended when considered necessary. The time at which signs of toxicity appear and disappear, their duration and the time of death are important, especially if there is a tendency for deaths to be delayed.

1.6.3. Procedure

Animals should be caged individually. The test substance should be applied uniformly over an area which is approximately 10 % of the total body surface area. With highly toxic substances the surface area covered may be less but as much of the area should be covered with a layer as thin and uniform as possible.

Test substances should be held in contact with the skin with a porous gauze dressing and non-irritating tape throughout a 24-hour exposure period. The test site should be further covered in a suitable manner to retain the gauze dressing and test substance and ensure that the animals

cannot ingest the test substance. Restrainers may be used to prevent the ingestion of the test substance but complete immobilisation is not a recommended method.

At the end of the exposure period, residual test substance should be removed, where practicable, using water or some other appropriate method of cleansing the skin.

Observations should be recorded systematically as they are made. Individual records should be maintained for each animal. Observations should be made frequently during the first day. A careful clinical examination should be made at least once each working day, other observations should be made daily with appropriate actions taken to minimize 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.

Observations should include changes in fur, treated skin, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous systems, and somatomotor activity and behaviour pattern. Particular attention should be directed to observations of tremors, convulsions, salivation, diarrhoea, lethargy , sleep and coma. The time of death must be recorded as precisely as possible. Animals that die during the test and those surviving at the termination of the test are subjected to necropsy. All gross pathological changes should be recorded. Where indicated, tissues should be taken for histopathological examination.

Assessment of toxicity in the other sex

After completion of the study in one sex, at least one group of 5 animals of the other sex is dosed to establish that animals of this sex are not markedly more sensitive to the test substance. The use of fewer animals may be justified in individual circumstances. Where adequate information is available to demonstrate that animals of the sex tested are markedly more sensitive, testing in animals of the other sex may be dispensed with.

2. DATA

Data should be summarized in tabular form, showing for each test group the number of animals at the start of the test, time of death of individual animals, number of animals displaying other signs of toxicity , description of toxic effects and necropsy findings. Individual weights of animals should be determined and recorded shortly before the test substance is applied, weekly thereafter, and at death; changes in weight should be calculated and recorded when survival exceeds one day. Animals which are humanely killed due to compound-related distress and pain are recorded as compound-related deaths. The LD_{50} should be determined by a recognized method.

Data evaluation should include an evaluation of relationships, if any, between the animal's exposure to the test substance and the incidence and severity of all abnormalities, including behavioural and clinical abnormalities, gross lesions, body weight changes, mortality, and any other toxicological effects.

3. REPORTING

3.1. TEST REPORT

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

– species, strain, source, environmental conditions, diet, etc.;

– test conditions (including method of skin cleansing and type of dressing: occlusive or not occlusive);

– dose levels (with vehicle, if used, and concentrations),

– sex of animals dosed;

– tabulation of response data by sex and dose level (i.e. number of animals that died or were killed during the test; number of animals showing signs of toxicity; number of animals exposed);

– time of death after dosing, reasons and criteria used for humane killing of animals;

– all observations;

– LD50 value for the sex subjected to a full study, determined at 14 days with the method of determination specified;

– 95 % confidence interval for the LD50 (where this can be provided);

– dose/mortality curve and slope where permitted by the method of determination;

– necropsy findings;

– any histopathological findings;

– results of any test on the other sex;

– discussion of results (particular attention should be given to the effect that humane killing of animals during the test may have on the calculated LD50 value);

– interpretation of the results.

3.2. EVALUATION AND INTERPRETATION

See General Introduction Part B (D).

4. REFERENCES

See General Introduction Part B (E).

B.4. ACUTE TOXICITY (SKIN IRRITATION)

1. METHOD

1.1. INTRODUCTION

See General Introduction Part B (A).

1.2. DEFINITION

See General Introduction Part B (B).

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLE OF THE TEST METHOD

Initial considerations

Careful consideration needs to be given to all the available information on a substance to minimize the testing of substances under conditions that are likely to produce severe reactions. The following information may be useful when considering whether a complete test, a single-animal study, or no further testing is appropriate.

i) Physicochemical properties and chemical reactivity. Strongly acidic or alkaline substances (demonstrated pH of 2 or less or 11,5 or greater, for example) may not require testing for primary dermal irritation if corrosive properties can be expected. Alkaline or acidic reserve should also be taken into account.

ii) If convincing evidence of severe effects in well validated in vitro tests is available, a complete test may not be required.

iii) Results from acute toxicity studies. If an acute toxicity test by the dermal route has been conducted with the substance at the limit test dose level (2000 mg/kg body weight), and no skin irritation was observed, further testing for skin irritation may be unnecessary. In addition, testing of materials which have been shown to be highly toxic by the dermal route is unnecessary.

The substance to be tested is applied in a single dose to the skin of several experimental animals, each animal serving as its own control. The degree of irritation is read and graded after a specific interval, and is further described to provide a complete evaluation of the effects. The duration of the observations should be sufficient to evaluate fully the reversibility of the effects observed.

Animals showing severe and enduring signs of distress and pain may need to be humanely killed.

1.5. QUALITY CRITERIA

None.

1.6. DESCRIPTION OF THE TEST METHOD

1.6.1. Preparations

Approximately 24 hours before testing, fur should be removed, by clipping or shaving, from the dorsal area of the trunk of the animal.

When clipping or shaving the fur, care should be taken to avoid abrading the skin. Only animals with healthy intact skin should be used.

Some strains of rabbit have dense islets of hair which are more prominent at certain times of the year. Test substances should not be applied to these zones of dense hair growth.

When testing solids (which may be pulverized if considered necessary) the test substance should be moistened sufficiently with water or, where necessary, a suitable vehicle, to ensure good contact with the skin. When vehicles are used, the influence of the vehicle on irritation of skin by the test substance should be taken into account. Liquid test substances are generally used undiluted.

1.6.2. Test Conditions

1.6.2.1. Experimental Animals

Although several mammalian species may be used, the albino rabbit is the preferred species.

1.6.2.2. Number of Animals

If it is suspected from in vitro screening results or other considerations that the substance might produce necrosis (i.e. be corrosive) a single-animal test should be considered. If the results of this test do not indicate corrosivity, the test should be completed using at least two additional animals.

For the complete test, at least three healthy adult animals are used. Separate animals are not required for an untreated control group. Additional animals may be required to clarify equivocal responses.

1.6.2.3. Dose Level

Unless there are contra-indications 0,5 ml of liquid or 0,5 g of solid or semi-solid is applied to the test site. Adjacent areas of untreated skin of each animal serve as controls for the test.

1.6.2.4. Observation Period

The duration of the observation period should not be fixed rigidly. It should be sufficient to evaluate fully the reversibility or irreversibility of the effects observed, but need not normally exceed 14 days after application.

1.6.3. Procedure

Animals should be caged individually. The test substance should be applied to a small area (approximately 6 cm^2) of skin and covered with a gauze patch, which is held in place with non-irritating tape. In the case of liquids or some pastes it may be necessary to apply the test substance to the gauze patch and then apply that to the skin. The patch should be loosely held in contact with the skin by means of a suitable occlusive or semi-occlusive dressing for the duration of the exposure period. Access by the animal to the patch and resultant ingestion/inhalation of the test substance should be prevented.

At the end of the exposure period, residual test substance should be removed, where practicable, using water or an appropriate solvent, without altering the existing response or the integrity of the epidermis.

Exposure duration normally is four hours.

If it is suspected that the substance might produce necrosis (i.e. be corrosive), the duration of exposure should be reduced (e.g. to one hour or three minutes). Such testing may also employ a single animal in the first instance and, if not precluded by the acute dermal toxicity of the test compound, three patches may be applied simultaneously to this animal. The first patch is removed after three minutes. If no serious skin reaction is observed, the second patch is removed after one hour. If the observations at this stage indicate that a four-hour exposure is necessary and can be humanely conducted, the third patch is removed after four hours and the responses are graded. In this case (i.e. when a four-hour exposure has been possible), the test should then be completed using at least two additional animals, unless it is not considered humane to do so (e.g. if necrosis is observed following the four hour exposure).

If a serious skin reaction (e.g. necrosis) is observed at either three minutes or one hour, the test is immediately terminated.

Longer exposures may be indicated under certain conditions, e.g. expected pattern of human use and exposure.

1.6.3.1. Observation and Grading

Animals should be observed for signs of erythema and oedema and the response graded at 60 minutes, and then at 24, 48 and 72 hours after patch removal. Dermal irritation is graded and recorded according to the system in table 1. Further observations may be needed if reversibility has not been fully established within 72 hours. In addition to the observation of irritation, any serious lesions such as corrosion (irreversible destruction of skin tissue) and other toxic effects should be fully described.

Techniques such as histopathological examination or measurement of skin-fold thickness may be used to clarify doubtful reactions or responses masked by staining of the skin by test substance.

2. DATA

Data should be summarized in tabular form, showing for each individual animal the irritation gradings for erythema and oedema throughout the observation period. Any serious lesions, a description of the degree and nature of irritation, reversibility or corrosion and any other toxic effect observed should be recorded.

3. REPORTING

3.1. TEST REPORT

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

– species, strain, source, environmental conditions, diet, etc.;

– test conditions (including the relevant physicochemical properties of the chemical, the technique of skin preparation and cleansing, and the type of dressing: occlusive or semi-occlusive);

– tabulation of irritation response data for each individual animal for each observation time period (e.g. 1, 24, 48 and 72 hours, etc., after patch removal);

– description of any serious lesions observed, including corrosivity;

– description of the degree and nature of irritation observed and any histopathological findings;

– description of any toxic effects other than dermal irritation;

– discussion of the results;

– interpretation of the results.

3.2. EVALUATION AND INTERPRETATION

See General Introduction Part B (D).

4. REFERENCES

See General Introduction Part B (E).

Appendix
TABLE: GRADING OF SKIN REACTION

Erythema and Eschar Formation

	Value
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) or eschar formation (injuries in depth) preventing erythema reading	4

Oedema Formation

	Value
No oedema	0
Very slight oedema (barely perceptible)	1
Slight oedema (edges of area well defined by definite raising)	2
Moderate oedema (edges raised approximately l mm)	3
Severe oedema (raised more than 1 mm and extending beyond the area of exposure)	4

B.5. ACUTE TOXICITY (EYE IRRITATION)

1. METHOD

1.1. INTRODUCTION

See General Introduction Part B (A).

1.2. DEFINITION

See General Introduction Part B (B).

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLE OF THE TEST METHOD

Initial Considerations

Careful consideration needs to be given to all the available information on a substance to minimize the testing of substances under conditions that are likely to produce severe reactions. The following information may be useful in this regard.

i) Physicochemical properties and chemical reactivity. Strongly acidic or alkaline substances which, for example, can be expected to result in a pH in the eye of 2 or less, or 11,5 or greater, may not require testing if severe lesions can be expected. Alkaline or acidic reserve should also be taken into account.

ii) Results from well-validated alternative studies; materials which have been shown to have potential corrosive or severe irritant properties should not be further tested for eye irritation, it being presumed that such substances will produce severe effects on the eyes in a test using this method.

iii) Results from skin irritation studies. Materials which have demonstrated definite corrosive or severe skin irritancy in a dermal irritation study should not be further tested for eye irritancy, it being presumed that such substances might produce severe effects on the eyes.

The substance to be tested is applied in a single dose to one of the eyes in each of several experimental animals; the untreated eye is used to provide control information. The degree of irritation is evaluated and graded at specific intervals and is further described to provide a complete evaluation of the effects. The duration of the observations should be sufficient to evaluate fully reversibility or irreversibility of the effects observed.

Animals showing severe and enduring signs of distress and pain may need to be humanely killed.

1.5. QUALITY CRITERIA

None.

1.6. DESCRIPTION OF THE TEST METHOD

1.6.1. Preparations

Both eyes of each experimental animal provisionally selected for testing should be examined within 24 hours before testing starts. Animals showing eye irritation, ocular defects or pre-existing corneal injury should be not used.

1.6.2. Test Conditions

1.6.2.1. Experimental Animals

Although a variety of experimental animals have been used it is recommended that testing be performed using healthy adult albino rabbits.

1.6.2.2. Number of Animals

A single-animal test should be considered if marked effects are anticipated. If the results of this test in one rabbit suggest the substance to be severely irritant (reversible effect) or corrosive (irreversible effect) to the eye using the procedure described, further testing for ocular irritancy in subsequent animals may not need to be carried out. Occasionally, further testing in additional animals may be appropriate to investigate specific aspects.

In cases other than a single-animal test at least 3 animals should be used. Additional animals may be required to clarify equivocal responses.

1.6.2.3. Dose Level

For testing liquids, a dose of 0,1 ml is used. In testing solids, pastes, and particulate substances, the amount used should have a volume of 0,1 ml, or weigh approximately 0,1 g (the weight must always be recorded). If the test material is solid or granular it should be ground to a fine dust. The volume of particulates should be measured after gently compacting them, e.g. by tapping the measuring container.

For substances contained in pump sprays or pressurized aerosol containers the liquid should be expelled and 0,1 ml collected and instilled into the eye as described for liquids.

1.6.2.4. Observation Period

The duration of the observation period should not be rigidly fixed. It should be sufficient to evaluate the reversibility or irreversibility of the effects observed, but normally need not exceed 21 days after instillation.

1.6.3. Procedure

Animals should be caged individually. The test substance should be placed in the conjunctival sac of one eye of each animal after gently pulling the lower lid away from the eyeball. The lids are then gently held together for about one second to prevent loss of the material. The other eye, which remains untreated, serves as a control.

If it is thought that the substance could cause unreasonable pain, a local anaesthetic may be used prior to instillation of the test substance. The type, concentration, and the time of application of the local anaesthetic should be carefully selected to ensure that no significant differences in reaction to the test substance will result from its use. The control eye should be similarly anaesthetized.

The eyes of the test animals should not be washed out for 24 hours following instillation of the test substance. At 24 hours a washout may be used if considered appropriate.

For some substances shown to be irritating by this test, additional tests using rabbits with eyes washed soon after instillation of the substance may be indicated. In these cases it is recommended that three rabbits be used. Half a minute after instillation the eyes of the rabbits are washed for half a minute using a volume and velocity of flow which will not cause injury.

1.6.3.1. Observation and Grading

The eyes should be examined at 1, 24, 48 and 72 hours. If there is no evidence of ocular lesions at 72 hours the study may be ended.

Extended observation may be necessary if there is persistent corneal involvement or other ocular irritation in order to determine the progress of the lesions and their reversibility or irreversibility .In addition to the observations of the cornea, iris and conjunctiva, any other lesions which are noted should be recorded and reported. The grades of ocular reaction (table) should be recorded at each examination. (The grading of ocular responses is subject to various interpretations. To assist testing laboratories and those involved in making and interpreting the observations an illustrated guide to eye irritation may be used).

Examination of reactions can be facilitated by use of a binocular loupe, hand slit-lamp, biomicroscope, or other suitable device. After recording the observations at 24 hours, the eyes of any or all rabbits may be further examined with the aid of fluorescein.

2. DATA

Data should be summarized in tabular form, showing for each individual animal the irritation grades at the designated observation time. A description of the degree and nature of irritation, the presence of serious lesions and any effects other than ocular which were observed, shall be reported.

3. REPORTING

3.1. TEST REPORT

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

– animal data (species, strain, source, environmental conditions, diet, etc.);

– test conditions (including relevant physicochemical properties of the test substance);

– tabulation of irritant/corrosive response data for each individual animal at each observation time point (e.g. 1, 24, 48 and 72 hours);

– description of any serious lesions observed;

– narrative description of the degree and nature of irritation or corrosion observed, including the area of the cornea involved, and the reversibility;

– description of the method used to grade the irritation at 1, 24, 48 and 72 hours (e.g. hand slit-lamp, biomicroscope, fluorescein);

– description of any non-ocular topical effects noted;

– discussion of the results;

– interpretation of the results.

3.2. EVALUATION AND INTERPRETATION

See General Introduction Part B (D).

4. REFERENCES

See General Introduction Part B (E).

Appendix
GRADING OF OCULAR LESIONS

Cornea

Opacity: degree of density (area most dense taken for reading)

No ulceration or opacity	0
Scattered or diffuse areas of opacity (other than slight dulling of normal lustre), details of iris clearly visible.	1
Easily discernible translucent area, details of iris slightly obscured.	2
Nacreous area, no details of iris visible, size of pupil barely discernible.	3
Opaque cornea, iris not discernible through the opacity	4

Iris

Normal	0
Markedly deepened rugae, congestion, swelling, moderate circumcorneal hyperaemia, or injection, any of these or combination of any thereof, iris still reacting to light (sluggish reaction is positive).	1
No reaction to light, haemorrhage, gross destruction (any or all of these)	2

Conjunctivae

Redness (refers to the most severe reading of palpebral and bulbar conjunctivae as compared to the control eye)

Blood vessels normal	0
Some blood vessels definitely hyperaemic (injected)	1
Diffuse, crimson colour, individual vessels not easily discernible.	2
Diffuse beefy red	3

Chemosis: lids and/or nictitating membranes

No swelling.	0
Any swelling above normal (including nictitating membranes).	1
Obvious swelling with partial eversion of lids.	2
Swelling with lids about half closed.	3

Swelling with lids more than half closed.	4

B.6. SKIN SENSITISATION

1. METHOD

1.1. INTRODUCTION

Remarks:

The sensitivity and ability of tests to detect potential human skin sensitisers are considered important in a classification system for toxicity relevant to public health.

There is no single test method which will adequately identify all substances with a potential for sensitising human skin and which is relevant for all substances.

Factors such as the physical characteristics of a substance, including its ability to penetrate the skin, must be considered in the selection of a test.

Two types of tests using guinea-pigs have been developed: the adjuvant-type tests, in which an allergic state is potentiated by dissolving or suspending the test substance in Freunds Complete Adjuvant (FCA), and the non-adjuvant tests.

Adjuvant-type tests are likely to be more accurate in predicting a probable skin sensitising effect of a substance in humans than those methods not employing Freunds Complete Adjuvant and are thus the preferred methods.

The Guinea-Pig Maximisation Test (GPMT) is a widely used adjuvant-type test. Although several other methods can be used to detect the potential of a substance to provoke skin sensitisation reaction, the GPMT is considered to be the preferred adjuvant technique.

With many chemical classes, non-adjuvant tests (the preferred one being the Buehler test) are considered to be less sensitive.

In certain cases there may be good reasons for choosing the Buehler test involving topical application rather than the intradermal injection used in the Guinea-Pig Maximisation Test. Scientific justification should be given when the Buehler test is used.

The Guinea-Pig Maximisation Test (GPMT) and the Buehler test are described in this method. Other methods may be used provided that they are well-validated and scientific justification is given.

If a positive result is seen in a recognised screening test, a test substance may be designated as a potential sensitiser, and it may not be necessary to conduct a further guinea pig test. However, if a negative result is seen in such a test, the guinea pig test must be conducted using the procedure described in this tes method.

See also General Introduction Part B.

1.2. DEFINITIONS

Skin sensitisation: (allergic contact dermatitis) is an immunologically mediated cutaneous reaction to a substance. In the human, the responses may be characterised by pruritis, erythema, oedema, papules,vesicles, bullae or a combination of these. In other species the reactions may differ and only erythema and oedema may be seen.

Induction exposure: an experimental exposure of a subject to a test substance with the intention of inducing a hypersensitive state.

Induction period: a period of at least one week following an induction exposure during which a hypersensitive state may be developed.

Challenge exposure: an experimental exposure of a previously treated subject to a test substance following an induction period, to determine if the subject reacts in a hypersensitive manner.

1.3. REFERENCE SUBSTANCES

The sensitivity and reliability of the experimental technique used should be assessed every six months by use of substances which are known to have mild-to-moderate skin sensitisation properties.

In a properly conducted test, a response of at least 30% in an adjuvant test and at least 15% in a non-adjuvant test should be expected for mild/moderate sensitisers.

The following substances are preferred.

CAS numbers	EINECS numbers	EINECS names	Common names
101-86-0	202-983-3	α-hexylcinnamaldehyde	α-hexylcinnamaldehyde
149-30-4	205-736-8	benzothiazole-2-thiol (mercaptobenzothiazole)	kaptax
94-09-7	202-303-5	benzocaine	norcaine

There may be circumstances where, given adequate justification other control substances meeting the above criteria may be used.

1.4. PRINCIPLE OF THE TEST METHOD

The test animals are initially exposed to the test substance by intradermal injections and/or epidermal application (induction exposure). Following a rest period of 10 to 14 days (induction period), during which an immune response may develop, the animals are exposed to a challenge dose. The extent and degree of skin reaction to the challenge exposure in the test animals is compared with that demonstrated by control animals which undergo sham treatment during induction and receive the challenge exposure.

1.5. DESCRIPTION OF THE TEST METHODS

If removal of the test substance is considered necessary, this should be achieved using water or an appropriate solvent without altering the existing response or the integrity of the epidermis.

1.5.1. Guinea-Pig Maximisation Test (GPMT)

1.5.1.1. *Preparations*

Healthy young adult albino guinea-pigs are acclimatised to the laboratory conditions for at least 5 days prior to the test. Before the test, animals are randomised and assigned to the treatment groups. Removal of hair is by clipping, shaving or possibly by chemical depilation, depending on the test method used. Care should be taken to avoid abrading the skin. The animals are weighed before the test commences and at the end of the test.

1.5.1.2. Test conditions

1.5.1.2.1. Test animals

Commonly used laboratory strains of albino guinea-pigs are used.

1.5.1.2.2. Number and sex

Male and/or female animals can be used. If females are used, they should be nulliparous and non-pregnant.

A minimum of 10 animals is used in the treatment group and at least 5 animals in the control group. When fewer than 20 test and 10 control guinea pigs have been used, and it is not possible to conclude that the test substance is a sensitiser, testing in additional animals to give a total of at least 20 test and 10 control animals is strongly recommended.

1.5.1.2.3. Dose levels

The concentration of the test substance used for each induction exposure should be well-tolerated systemically and should be the highest to cause mild-to-moderate skin irritation. The concentration used for the challenge exposure should be the highest non-irritant dose. The appropriate concentrations should be determined from a pilot study using two or three animals, if other information are not available. Consideration should be given to the use of FCA-treated animals for this purpose.

1.5.1.3. Procedure

1.5.1.3.1. Induction

Day 0-treated group

Three pairs of intradermal injections of 0.1 ml volume are given in the shoulder region which is cleared of hair so that one of each pair lies on each side of the midline.

Injection 1: a 1:1 mixture (v/v) FCA/water or physiological saline

Injection 2: the test substance in an appropriate vehicle at the selected concentration

Injection 3: the test substance at the selected concentration formulated in a 1:1 mixture (v/v) FCA/water or physiological saline

In injection 3, water soluble substances are dissolved in the aqueous phase prior to mixing with FCA. Liposoluble or insoluble substances are suspended in FCA prior to combining with the aqueous phase. The final concentration of test substance shall be equal to that used in injection 2.

Injections 1 and 2 are given close to each other and nearest the head, while 3 is given towards the caudal part of the test area.

Day 0-control group

Three pairs of intradermal injections of 0.1 ml volume are given in the same sites as in the treated animals.

Injection 1: a 1:1 mixture (v/v) FCA/water or physiological saline

Injection 2: the undiluted vehicle

Injection 3: a 50% w/v formulation of the vehicle in a 1:1 mixture (v/v) FCA/water or physiological saline.

Day 5-7-treated and control groups

Approximately twenty-four hours before the topical induction application, if the substance is not a skin irritant, the test area, after close-clipping and/or shaving is treated with 0.5 ml of 10% sodium lauryl sulphate in vaseline, in order to create a local irritation.

Day 6-8-treated group

The test area is again cleared of hair. A filter paper (2 x 4 cm) is fully-loaded with test substance in a suitable vehicle and applied to the test area and held in contact by an occlusive dressing for 48 hours. The choice of the vehicle should be justified. Solids are finely pulverised and incorporated in a suitable vehicle. Liquids can be applied undiluted, if appropriate.

Day 6-8-control group

The test area is again cleared of hair. The vehicle only is applied in a similar manner to the test area and held in contact by an occlusive dressing for 48 hours.

1.5.1.3.2. Challenge

Day 20-22-treated and control groups

The flanks of treated and control animals are cleared of hair. A patch or chamber loaded with the test substance is applied to one flank of the animals and, when relevant, a patch or chamber loaded with the vehicle only may also be applied to the other flank. The patches are held in contact by an occlusive dressing for 24 hours.

Observation and Grading: treated and control groups

– approximately 21 hours after removing the patch the challenge area is cleaned and closely-clipped and/or shaved and depilated if necessary;

– approximately 3 hours later (approximately 48 hours from the start of the challenge application) the skin reaction is observed and recorded according to the grades shown in appendix;

– approximately 24 hours after this observation a second observation (72 hours) is made and once again recorded.

Blind reading of test and control animals is encouraged.

If it is necessary to clarify the results obtained in the first challenge, a second challenge (i.e. a rechallenge), where appropriate with a new control group, should be considered approximately one week after the first one. A rechallenge may also be performed on the original control group.

All skin reactions and any unusual findings, including systemic reactions, resulting from induction and challenge procedures should be observed and recorded according to the grading scale of Magnusson/Kligman (See appendix). Other procedures, e.g. histopathological examination, the measurement of skin fold thickness, may be carried out to clarify doubtful reactions.

1.5.2. Buehler test

1.5.2.1. *Preparations*

Healthy young adult albino guinea-pigs are acclimatised to the laboratory conditions for at least 5 days prior to the test. Before the test, animals are randomised and assigned to the treatment groups. Removal of hair is by clipping, shaving or possibly by chemical depilation, depending on the test method used. Care should be taken to avoid abrading the skin. The animals are weighed before the test commences and at the end of the test.

1.5.2.2. Test conditions

1.5.2.2.1. Test animals

Commonly used laboratory strains of albino guinea-pigs are used.

1.5.2.2.2. Number and sex

Male and/or female animals can be used. If females are used, they should be nulliparous and non-pregnant.

A minimum of 20 animals is used in the treatment group and at least 10 animals in the control group.

1.5.2.2.3. Dose levels

The concentration of test substance used for each induction exposure should be the highest possible to produce a mild but not excessive irritation. The concentration used for the challenge exposure should be the highest non-irritating dose. If necessary, the appropriate concentration can be determined from a pilot study using two or three animals.

For water soluble test materials, it is appropriate to use water or a dilute non-irritating solution of surfactant as the vehicle. For other test materials 80% ethanol/water is preferred for induction and acetone for challenge.

1.5.2.3. Procedure

1.5.2.3.1. Induction

Day 0-treated group

One flank is cleared of hair (closely-clipped). The test patch system should be fully loaded with test substance in a suitable vehicle (the choice of the vehicle should be justified; liquid test substances can be applied undiluted, if appropriate).

The test patch system is applied to the test area and held in contact with the skin by an occlusive patch or chamber and a suitable dressing for 6 hours.

The test patch system must be occlusive. A cotton pad is appropriate and can be circular or square, but should approximate 4-6 cm^2. Restraint using an appropriate restrainer is preferred to assure occlusion. If wrapping is used, additional exposures may be required.

Day 0-control group

One flank is cleared of hair (closely-clipped). The vehicle only is applied in a similar manner to that used for the treated group. The test patch system is held in contact with the skin by an occlusive patch or chamber and a suitable dressing for 6 hours. If it can be demonstrated that a sham control group is not necessary, a naive control group may be used.

Days 6-8 and 13-15-treated and control group

The same application as on day 0 is carried out on the same test area (cleared of hair if necessary) of the same flank on day 6-8, and again on day 13-15.

1.5.2.3.2. Challenge

Day 27-29-treated and control group

The untreated flank of treated and control animals is cleared of hair (closely-clipped). An occlusive patch or chamber containing the appropriate amount of test substance is applied, at the maximum non-irritant concentration, to the posterior untreated flank of treated and control animals.

When relevant, an occlusive patch or chamber with vehicle only is also applied to the anterior untreated flank of both treated and control animals. The patches or chambers are held in contact by a suitable dressing for 6 hours.

1.5.2.3.3. Observation and grading

– approximately 21 hours after removing the patch the challenge area is cleared of hair;

– approximately three hours later (approximately 30 hours after application of the challenge patch) the skin reactions are observed and recorded according to the grades shown in the appendix;

– approximately 24 hours after the 30 hour observation (approximately 54 hours after application of the challenge patch) skin reactions are again observed and recorded.

Blind reading of the test and control animals is encouraged.

If it is necessary to clarify the results obtained in the first challenge, a second challenge (i.e. a rechallenge), where appropriate with a new control group, should be considered approximately one week after the first one. A rechallenge may also be performed on the original control group.

All skin reactions and any unusual findings, including systemic reactions, resulting from induction and challenge procedures should be observed and recorded according to the Magnusson/Kligman grading scale (See appendix). Other procedures, e.g. histopathological examination, the measurement of skin fold thickness, may be carried out to clarify doubtful reactions.

2. DATA (GPMT AND BUEHLER TEST)

Data should be summarised in tabular form, showing for each animal the skin reactions at each observation.

3. REPORTING (GPMT AND BUEHLER TEST)

If a screening assay is performed before the guinea pig test the description or reference of the test (e.g. Local Lymph Node Assasy (LLNA), Mouse Ear Swelling Test (MEST)), including details of the procedure, must be given together with results obtained with the test and reference substances.

Test report (GMPT and Buehler test)

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

Test animals:

– strain of guinea-pig used;

– number, age and sex of animals;

– source. housing conditions. diet. etc.;

– individual weights of animals at the start of the test.

Test conditions:

– technique of patch site preparation;

– details of patch materials used and patching technique;

– result of pilot study with conclusion on induction and challenge concentrations to be used in the test;

– details of test substance preparation, application and removal;

– justification for choice of vehicle;

– vehicle and test substance concentrations used for induction and challenge exposures and the total amount of substance applied for induction and challenge.

Results:

– a summary of the results of the latest sensitivity and reliability check (see 1.3) including information on substance, concentration and vehicle used;

– on each animal including grading system;

– narrative description of the nature and degree effects observed;

– any histopathological findings.

Discussion of results.

Conclusions.

4. REFERENCES

This method is analogous to OECD TG 406.

Appendix

TABLE:

Magnusson/Kligman grading scale for the evaluation of challenge patch test reactions

0 = no visible change

1 = discrete or patchy erythema

2 = moderate and confluent erythema

3 = intense erythema and swelling

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 ± 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[1]. 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.

1 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.

B.8. REPEATED DOSE (28 DAYS) TOXICITY (INHALATION)

1. METHOD

1.1. INTRODUCTION

It is useful to have preliminary information on the particle size distribution, the vapour pressure, the melting point, the boiling point, the flash point and explosivity (if applicable) of the substance.

See also General Introduction Part B (A).

1.2. DEFINITION

See General Introduction Part B (B).

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLE OF THE TEST METHOD

Several groups of experimental animals are exposed daily for a defined period to the test substance in graduated concentrations, one concentration being used per group, for a period of 28 days. Where a vehicle is used to help generate an appropriate concentration of the test substance in the atmosphere, a vehicle control group should be used. During the period of administration the animals are observed daily to detect signs of toxicity. Animals which die during the test are necropsied and at the conclusion of the test surviving animals are necropsied.

1.5. QUALITY CRITERIA

None.

1.6. DESCRIPTION OF THE TEST METHOD

1.6.1. Preparations

The animals are kept under the experimental housing and feeding conditions for at least five days prior to the experiment. Before the test, healthy young animals are randomized and assigned to the required number of groups. Where necessary, a suitable vehicle may be added to the test substance to help generate an appropriate concentration of the substance in the atmosphere. If a vehicle or other additive is used to facilitate dosing, it should be known not to produce toxic effects. Historical data can be used if appropriate.

1.6.2. Test Conditions

1.6.2.1. Experimental Animals

Unless there are contra-indications, the rat is the preferred species. Commonly used laboratory strains of young healthy animals should be employed.

At the commencement of the study the range of weight variation in the animals used should not exceed ± 20 % of the appropriate mean value.

1.6.2.2. Number and Sex

At least 10 animals (five female and five male) should be used for each test group. The females should be nulliparous and non-pregnant. If interim sacrifices are planned, the numbers should be increased by the number of animals scheduled to be sacrificed before the completion of the study. In addition, a satellite group of 10 animals (five animals per sex) may be treated with the high concentration 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.

1.6.2.3. Exposure Concentration

At least three concentrations are required, with a control or a vehicle control (corresponding to the concentration of vehicle at the highest level) if a vehicle is used. Except for treatment with the test substance, animals in the control group should be handled in an identical manner to the test-group animals. The highest concentration should result in toxic effects but no, or few, fatalities. The lowest concentration should not produce any evidence of toxicity .Where there is a usable estimation of human exposure, the lowest concentration should exceed this. Ideally, the intermediate concentration should produce minimal observable toxic effects. If more than one intermediate concentration is used the concentrations 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 to permit a meaningful evaluation of the results.

1.6.2.4. Exposure Time

The duration of daily exposure should be six hours but other periods may be needed to meet specific requirements.

1.6.2.5. Equipment

The animals should be tested in inhalation equipment designed to sustain a dynamic airflow of at least 12 air changes per hour to ensure an adequate oxygen content and an evenly distributed exposure atmosphere. Where a chamber is used its design should minimize crowding of the test animals and maximize their exposure by inhalation of the test substance. As a general rule to ensure stability of a chamber atmosphere the total 'volume' of the test animals should not exceed 5% of the volume of the test chamber. Oro-nasal, head only, or individual whole body chamber exposure may be used; the first two will minimize uptake by other routes.

1.6.2.6. Observation Period

The experimental animals should be observed daily for signs of toxicity during the entire treatment and recovery period. The time of death and the time at which signs of toxicity appear and disappear should be recorded.

1.6.3. Procedure

The animals are exposed to the test substance daily, five to seven days per week, for a period of 28 days. Animals in any satellite groups scheduled for follow-up observations should be kept for a further 14 days without treatment to detect recovery from, or persistence of toxic effects. The temperature at which the test is performed should be maintained at 22 ± 3 °C.

Ideally, the relative humidity should be maintained between 30 and 70 %, but in certain instances (e.g. tests of some aerosols) this may not be practicable. Maintenance of a slight negative pressure inside the chamber (≤ 5 mm of water) will prevent leakage of the test substance into the surrounding area. Food and water should be withheld during exposure.

A dynamic inhalation system with a suitable analytical concentration control system should be used. To establish suitable exposure concentrations a trial test is recommended. The airflow should be adjusted to ensure that conditions throughout the exposure chamber are homogeneous. The system should ensure that stable exposure conditions are achieved as rapidly as possible.

Measurements or monitoring should be made:

(a) of the rate of airflow (continuously);

(b) of the actual concentration of the test substance measured in the breathing zone. During the daily exposure period the concentration should not vary by more than ± 15% of the mean value. However, in the case of some aerosols, this level of control may not be achievable and a wider range would then be acceptable. During the total duration of the study, the day-to-day concentrations should be held as constant as practicable. For aerosols, at least one particle size analysis should be performed per test group weekly;

(c) of temperature and humidity, continuously if possible.

During and following exposure observations are made and recorded systematically; individual records should be maintained for each animal. All the animals should be observed daily and signs of toxicity recorded including the time of onset, their degree and duration. Observations should include changes in the skin and fur, eyes, mucous membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behaviour pattern. Measurements should be made weekly of the animals' weight. It is also recommended that food consumption is measured 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 study period, all survivors in the non-satellite treatment groups are necropsied. Moribund animals and animals in severe distress or pain should be removed when noticed, humanely killed and necropsied.

The following examinations shall be made at the end of the test on all animals including the controls:

(i) haematology, including at least haematocrit, haemoglobin concentration, erythrocyte count, total and differential leucocyte count and a measure of clotting potential;

(ii) clinical blood biochemistry including at least one parameter of liver and kidney function: serum alanine aminotransferase (formerly known as glutamic pyruvic transaminase), serum aspartate aminotransferase (formerly known as glutamic oxaloacetic transaminase), urea nitrogen, albumin, blood creatinine, total bilirubin and total serum protein measurements;

Other determinations which may be necessary for an adequate toxicological evaluation include calcium, phosphorus, chloride, sodium, potassium, fasting glucose analysis of lipids, hormones, acid/base balance, methaemoglobin and cholinesterase activity.

Additional clinical biochemistry may be employed, where necessary, to extend the investigation of observed toxic effects.

1.6.3.1. Gross Necropsy

All animals in the study should be subjected to a full gross necropsy. At least the liver, kidneys, adrenals, lungs, and testes should be weighed wet as soon as possible after dissection to avoid drying. Organs and tissues (the respiratory tract, liver, kidneys, spleen, testes, adrenals, heart, and any organs showing gross lesions or changes in size) should be preserved in a suitable medium for possible future histopathological examination. The lungs should be removed intact, weighed and treated with a suitable fixative to ensure that lung structure is maintained.

1.6.3.2. Histopathological Examination

In the high-concentration group and in the control(s), histological examination should be performed on preserved organs and tissues. Organs and tissues showing defects attributable to the test substance at the highest dosage level should be examined in all lower-dosage groups. Animals in any satellite groups should be examined histologically with particular emphasis on those organs and tissues identified as showing effects in the other treated groups.

2. DATA

Data should be summarized in tabular form, showing for each test group the number of animals at the start of the test and the number of animals displaying each type of lesion.

All observed results should be evaluated by an appropriate statistical method. Any recognized statistical method may be used.

3. REPORTING

3.1. TEST REPORT

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

– species, strain, source, environmental conditions, diet, etc.;

– test conditions:

Description of exposure apparatus including design, type, dimensions, source of air, system for generating aerosols, method of conditioning air, treatment of exhaust air and the method of housing animals in a test chamber when this is used. The equipment for measuring temperature, humidity and, where appropriate, stability of aerosol concentrations or particle size distribution, should be described.

Exposure data:

These should be tabulated and presented with mean values and a measure of variability (e.g. standard deviation) and shall, if possible, include:

a) airflow rates through the inhalation equipment;

b) temperature and humidity of air;

c) nominal concentrations (total amount of test substance fed into the inhalation equipment divided by the volume of air);

d) nature of vehicle, if used;

e) actual concentrations in test breathing zone;

f) the mass median aerodynamic diameter (MMAD) and the geometric standard deviation (GSD);

– toxic response data by sex and concentration;

– time of death during the study or whether animals survived to termination;

– description of toxic or other effects; no-effect level;

– the time of observation of each abnormal sign and its subsequent course;

– food and body-weight data;

– haematological tests employed and results;

– clinical biochemistry tests employed and results;

– necropsy findings;

– a detailed description of all histopathological findings;

– a statistical treatment of results where possible;

– discussion of the results;

– interpretation of results.

3.2. EVALUATION AND INTERPRETATION

See General Introduction Part B (D).

4. REFERENCES

See General Introduction Part B (E).

8.9. REPEATED DOSE (28 DAYS) TOXICITY (DERMAL)

1. METHOD

1.1. INTRODUCTION

See General Introduction Part B (A).

1.2. DEFINITIONS

See General Introduction Part B (B).

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLE OF THE TEST METHOD

The test substance is applied daily to the skin in graduated doses to several groups of experimental animals, one dose per group, for a period of 28 days. During the period of application, the animals are observed daily to detect signs of toxicity. Animals which die during the test are necropsied and at the conclusion of the test surviving animals are necropsied.

1.5. QUALITY CRITERIA

None.

1.6. DESCRIPTION OF THE TEST METHOD

1.6.1. Preparations

The animals are kept under the experimental housing and feeding conditions for at least five days prior to the test. Before the test, healthy young animals are randomized and assigned to the treatment and control groups. Shortly before testing, fur is clipped from the dorsal area of the trunk of the test animals. Shaving may be employed but it should be carried out approximately 24 hours before the test. Repeat clipping or shaving is usually needed at approximately weekly intervals. When clipping or shaving the fur, care must be taken to avoid abrading the skin. Not less than 10% of the body surface area should be clear for the application of the test substance. The weight of the animal should be taken into account when deciding on the area to be cleared and on the dimensions of the covering. When testing solids, which may be pulverized if appropriate, the test substance should be moistened sufficiently with water or, where necessary, a suitable vehicle to ensure good contact with the skin. Liquid test substances are generally used undiluted. Daily application on a five to seven-day per week basis is used.

1.6.2. Test Conditions

1.6.2.1. Experimental Animals

The adult rat, rabbit or guinea-pig may be used. Other species may be used but their use would require justification.

At the commencement of the study, the range of weight variation in the animals used should not exceed ± 20 % of the appropriate mean value.

1.6.2.2. Number and Sex

At least 10 animals (five female and five male) with healthy skin should be used at each dose level. The females should be nulliparous and non-pregnant. If interim sacrifices are planned, the numbers should be increased by the number of animals scheduled to be sacrificed 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.

1.6.2.3. Dose levels

At least three dose levels are required with a control or a vehicle control if a vehicle is used. The exposure period should be at least six hours per day. The application of the test substance should be made 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. Except for treatment with the test substance, animals in the control group should be handled in an identical manner to the test group subjects. Where a vehicle is used to facilitate dosing, the vehicle control group should be dosed in the same way as the treated groups, and receive the same amount as that received by the highest dose level group. The highest dose level should result in toxic effects but produce no, or few, fatalities. The lowest dose level should not produce any evidence or toxicity .Where there is a usable estimation of human exposure, the lowest level should exceed this. Ideally, the intermediate dose level 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.

If application of the test substance produces severe skin irritation, the concentrations should be reduced and this may result in a reduction in, or absence of, other toxic effects at the high dose level. Moreover if the skin has been badly damaged it may be necessary to terminate the study and undertake a new study at lower concentrations.

1.6.2.4. Limit Test

If a preliminary study at a dose level of 1000 mg/kg, or a higher dose level related to possible human exposure where this is known, produces no toxic effects, further testing may not be considered necessary.

1.6.2.5. Observation Period

The experimental animals should be observed daily for signs of toxicity .The time of death and the time at which signs of toxicity appear and disappear should be recorded.

1.6.3. Procedure

Animals should be caged individually. The animals are treated with the test substance, ideally on seven days per week, for a period of 28 days. Animals in any satellite groups scheduled for follow-up observations should be kept for a further 14 days without treatment to detect recovery from or persistence of toxic effects. Exposure time should be at least six hours per day.

The test substance should be applied uniformly over an area which is approximately 10 % of the total body surface area. With highly toxic substances, the surface area covered may be less but as much of the area as possible should be covered with as thin and uniform a layer as possible.

During exposure the test substance is held in contact with the skin with porous gauze dressing and non-irritating tape. The test site should be further covered in a suitable manner to retain the gauze dressing and test substance and ensure that the animals cannot ingest the test substance. Restrainers may be used to prevent the ingestion of the test substance but complete immobilization is not a recommended method. As an alternative a 'collar protective device' may be used.

At the end of the exposure period, residual test substance should be removed, where practicable, using water or some other appropriate method of cleansing the skin.

All the animals should be observed daily and signs of toxicity recorded including the time of onset, their degree and duration. Observations should include changes in skin and fur, eyes and mucous membranes as well as respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behaviour pattern. Measurements should be made weekly of the animals' weight. It is also recommended that food consumption is measured 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 study period, all survivors in the non-satellite treatment groups are necropsied. Moribund animals and animals in severe distress or pain should be removed when noticed, humanely killed and necropsied.

The following examinations shall be made at the end of the test on all animals including the controls:

1) haematology, including at least haematocrit, haemoglobin concentration, erythrocyte count, total and differential leucocyte count, and a measure of clotting potential;

2) clinical blood biochemistry including at least one parameter of liver and kidney function: serum alanine aminotransferase (formerly known as glutamic pyruvic transaminase), serum aspartate aminotransferase (formerly known as glutamic oxaloacetic transaminase), urea nitrogen, albumin, blood creatinine, total bilirubin and total serum protein;

Other determinations which may be necessary for an adequate toxicological evaluation include calcium, phosphorus, chloride, sodium, potassium, fasting glucose, analysis of lipids, hormones, acid/base balance, methaemoglobin and cholinesterase activity .

Additional clinical biochemistry may be employed, where necessary, to extend the investigation of observed effects.

1.6.4. Gross Necropsy

All animals in the study should be subjected to a full gross necropsy. At least the liver, kidneys, adrenals, and testes should be weighed wet as soon as possible after dissection, to avoid drying. Organs and tissues, i.e. normal and treated skin, liver, kidney, spleen, testes, adrenals, heart, and target organs (that is those organs showing gross lesions or changes in size), should be preserved in a suitable medium for possible future histopathological examination.

1.6.5. Histopathological Examination

In the high dose group and in the control group, histological examination should be performed on the preserved organs and tissues. Organs and tissues showing defects attributable to the test substance at the highest dosage level should be examined in all lower-dosage groups. Animals in the satellite group should be examined histologically with particular emphasis on those organs and tissues identified as showing effects in the other treated groups.

2. DATA

Data should be summarized in tabular form, showing for each test group the number of animals at the start of the test and the number of animals displaying each type of lesion.

All observed results should be evaluated by an appropriate statistical method. Any recognized statistical method may be used.

3. REPORTING

3.1. TEST REPORT

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

– animal data (species, strain, source, environmental conditions, diet, etc.);

– test conditions (including the type of dressing: occlusive or not-occlusive);

– dose levels (including vehicle, if used) and concentrations;

– no-effect level, where possible;

– 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;

– haematological tests employed and results;

– clinical biochemistry tests employed and results;

– necropsy findings;

– a detailed description of all histopathological findings;

– statistical treatment of results where possible;

– discussion of the results;

– interpretation of the results.

3.2. EVALUATION AND INTERPRETATION

See General Introduction Part B (D).

4. REFERENCES

See General Introduction Part B (E).

B. 10. MUTAGENICITY – *IN VITRO* MAMMALIAN CHROMOSOME ABERRATION TEST

1. METHOD

This method is a replicate of the OECD TG 473, *In Vitro* Mammalian Chromosome Aberration Test (1997).

1.1. INTRODUCTION

The purpose of the *in vitro* chromosomal aberration test is to identify agents that cause structural chromosome aberrations in cultured mammalian cells (1)(2)(3). Structural aberrations may be of two types, chromosome or chromatid. With the majority of chemical mutagens, induced aberrations are of the chromatid type, but chromosome-type aberrations also occur. An increase in polyploidy may indicate that a chemical has the potential to induce numerical aberrations. However, this method is not designed to measure numerical aberrations and is not routinely used for that purpose. Chromosome mutations and related events are the cause of many human genetic diseases and there is substantial evidence that chromosome mutations and related events causing alterations in oncogenes and tumour suppressor genes of somatic cells are involved in cancer induction in humans and experimental animals.

The *in vitro* chromosome aberration test may employ cultures of established cell lines, cell strains or primary cell cultures. The cells used are selected on the basis of growth ability in culture, stability of the karyotype, chromosome number, chromosome diversity and spontaneous frequency of chromosome aberrations.

Tests conducted *in vitro* generally require the use of an exogenous source of metabolic activation. This metabolic activation system cannot mimic entirely the mammalian *in vivo* conditions. Care should be taken to avoid conditions which would lead to positive results which do not reflect intrinsic mutagenicity and may arise from changes in pH, osmolality or high levels of cytotoxicity (4)(5).

This test is used to screen for possible mammalian mutagens and carcinogens. Many compounds that are positive in this test are mammalian carcinogens; however, there is not a perfect correlation between this test and carcinogenicity. Correlation is dependent on chemical class and there is increasing evidence that there are carcinogens that are not detected by this test because they appear to act through mechanisms other than direct DNA damage.

See also General Introduction Part B.

1.2 DEFINITIONS

Chromatid-type aberration: structural chromosome damage expressed as breakage of single chromatids or breakage and reunion between chromatids.

Chromosome-type aberration: structural chromosome damage expressed as breakage, or breakage and reunion, of both chromatids at an identical site

Endoreduplication: a process in which after an S period of DNA replication, the nucleus does not go into mitosis but starts another S period. The result is chromosomes with 4, 8, 16, …chromatids.

Gap: an achromatic lesion smaller than the width of one chromatid, and with minimum misalignment of the chromatids).

Mitotic index: the ratio of cells in metaphase divided by the total number of cells observed in a population of cells; an indication of the degree of proliferation of that population.

Numerical aberration: a change in the number of chromosomes from the normal number characteristic of the cells utilised.

Polyploidy: a multiple of the haploid chromosome number (*n*) other than the diploid number (i.e. 3*n*, 4*n* and so on).

Structural aberration: a change in chromosome structure detectable by microscopic examination of the metaphase stage of cell division, observed as deletions and fragments, intrachanges or interchanges.

1.3 PRINCIPLE OF THE TEST METHOD

Cell cultures are exposed to the test substance both with and without metabolic activation. At predetermined intervals after exposure of cell cultures to the test substance, they are treated with a metaphase-arresting substance (e.g. Colcemid® or colchicine), harvested, stained and metaphase cells are analysed microscopically for the presence of chromosome aberrations.

1.4 DESCRIPTION OF THE TEST METHOD

1.4.1 Preparations

1.4.1.1 Cells

A variety of cell lines, strains or primary cell cultures, including human cells, may be used (e.g. Chinese hamster fibroblasts, human or other mammalian peripheral blood lymphocytes).

1.4.1.2 Media and culture conditions

Appropriate culture media, and incubation conditions (culture vessels, CO_2 concentration, temperature and humidity) should be used in maintaining cultures. Established cell lines and strains should be checked routinely for stability in the modal chromosome number and the absence of mycoplasma contamination and should not be used if contaminated. The normal cell cycle time for the cells and culture conditions used should be known.

1.4.1.3 Preparation of cultures

Established cell lines and strains: cells are propagated from stock cultures, seeded in culture medium at a density such that the cultures will not reach confluency before the time of harvest, and incubated at 37°C.

Lymphocytes: whole blood treated with an anti-coagulant (e.g. heparin) or separated lymphocytes obtained from healthy subjects are added to the culture medium containing a mitogen (e.g. phytohaemagglutinin) and incubated at 37°C.

1.4.1.4 Metabolic activation

Cells should be exposed to the test substance both in the presence and absence of an appropriate metabolic activation system. The most commonly used system is a cofactor-supplemented post-mitochondrial fraction (S9) prepared from the livers of rodents treated with enzyme-inducing agents such as: Aroclor 1254 (6)(7)(8)(9), or a mixture of phenobarbitone and ß–naphthoflavone (10)(11)(12).

The post-mitochondrial fraction is usually used at concentrations in the range from 1-10% v/v in the final test medium. The condition of a metabolic activation system may depend upon the class of chemical being tested. In some cases it may be appropriate to utilise more than one concentration of post-mitochondrial fraction.

A number of developments, including the construction of genetically engineered cell lines expressing specific activating enzymes, may provide the potential for endogenous activation. The choice of the cell lines used should be scientifically justified (e.g. by the relevance of the cytochrome P450 isoenzyme for the metabolism of the test substance).

1.4.1.5 Test substance/Preparation

Solid test substances should be dissolved or suspended in appropriate solvents or vehicles and diluted if appropriate prior to treatment of the cells. Liquid test substances may be added directly to the test systems and/or diluted prior to treatment. Fresh preparations of the test substance should be employed unless stability data demonstrate the acceptability of storage.

1.4.2 Test conditions

1.4.2.1 Solvent/vehicle

The solvent/vehicle should not be suspected of chemical reaction with the test substance and should be compatible with the survival of the cells and the S9 activity. If other than well-known solvent/vehicles are used, their inclusion should be supported by data indicating their compatibility. It is recommended that wherever possible, the use of an aqueous solvent/vehicle be considered first. When testing water-unstable substances, the organic solvents used should be free of water. Water can be removed by adding a molecular sieve.

1.4.2.2 Exposure concentrations

Among the criteria to be considered when determining the highest concentration are cytotoxicity, solubility in the test system and changes in pH or osmolality.

Cytotoxicity should be determined with and without metabolic activation in the main experiment using an appropriate indication of cell integrity and growth, such as degree of confluency, viable cell counts, or mitotic index. It may be useful to determine cytotoxicity and solubility in a preliminary experiment.

At least three analysable concentrations should be used. Where cytotoxicity occurs, these concentrations should cover a range from the maximum to little or no toxicity; this will usually mean that the concentrations should be separated by no more than a factor between 2

and √10. At the time of harvesting, the highest concentration should show a significant reduction in degree of confluency, cell count or mitotic index, (all greater than 50%). The mitotic index is only an indirect measure of cytotoxic/cytostatic effects and depends on the time after treatment. However, the mitotic index is acceptable for suspension cultures in which other toxicity measurements may be cumbersome and impractical. Information on cell cycle kinetics, such as average generation time (AGT), could be used as supplementary information. AGT, however, is an overall average that does not always reveal the existence of delayed subpopulations, and even slight increases in average generation time can be associated with very substantial delay in the time of optimal yield of aberrations.

For relatively non-cytotoxic substances, the maximum test concentration should be 5 µl/ml, 5 mg/ml or 0.01 M, whichever is the lowest.

For relatively insoluble substances that are not toxic at concentrations lower than the insoluble concentration, the highest dose used should be a concentration above the limit of solubility in the final culture medium at the end of the treatment period. In some cases (e.g. when toxicity occurs only at higher than the lowest insoluble concentration) it is advisable to test at more than one concentration with visible precipitation. It may be useful to assess solubility at the beginning and the end of the treatment, as solubility can change during the course of exposure in the test system due to presence of cells, S9, serum etc. Insolubility can be detected by using the unaided eye. The precipitate should not interfere with the scoring.

1.4.2.3 Negative and positive controls

Concurrent positive and negative (solvent or vehicle) controls, both with and without metabolic activation, should be included in each experiment. When metabolic activation is used, the positive control chemical should be the one that requires activation to give a mutagenic response.

Positive controls should employ a known clastogen at exposure levels expected to give a reproducible and detectable increase over background which demonstrates the sensitivity of the test system.

Positive control concentrations should be chosen so that the effects are clear but do not immediately reveal the identity of the coded slides to the reader. Examples of positive control substances include:

Metabolic Activation condition	**Substance**	**CAS No.**	**EINECS No.**
Absence of Exogenous Metabolic Activation	Methyl methanesulphonate	66-27-3	200-625-0
	Ethyl methanesulphonate	62-50-0	200-536-7
	Ethyl nitrosourea	759-73-9	212-072-2
	Mitomycin C	50-07-7	200-008-6
	4–Nitroquinoline–*N*–	56-57-5	200-281-1

	oxide		
Presence of Exogenous Metabolic Activation	Benzo[*a*]pyrene	50-32-8	200-028-5
	Cyclophosphamide	50-18-0	200-015-4
	Cyclophosphamide monohydrate	6055-19-2	

Other appropriate positive control substances may be used. The use of chemical class-related positive control chemicals should be considered, when available.

Negative controls, consisting of solvent or vehicle alone in the treatment medium, and treated in the same way as the treatment cultures, should be included for every harvest time. In addition, untreated controls should also be used unless there are historical control data demonstrating that no deleterious or mutagenic effects are induced by the chosen solvent.

1.4.3 Procedure

1.4.3.1 Treatment with the test substance

Proliferating cells are treated with the test substance in the presence and absence of a metabolic activation system. Treatment of lymphocytes should commence at about 48 hours after mitogenic stimulation.

1.4.3.2 Duplicate cultures should normally be used at each concentration, and are strongly recommended for negative/solvent control cultures. Where minimal variation between duplicate cultures can be demonstrated (13)(14), from historical data, it may be acceptable for single cultures to be used at each concentration.

Gaseous or volatile substances should be tested by appropriate methods, such as in sealed culture vessels (15)(16).

1.4.3.3 Culture harvest time

In the first experiment, cells should be exposed to the test substance, both with and without metabolic activation, for 3-6 hours, and sampled at a time equivalent to about 1.5 normal cell cycle length after the beginning of treatment (12). If this protocol gives negative results both with and without activation, an additional experiment without activation should be done, with continuous treatment until sampling at a time equivalent to about 1.5 normal cell cycle lengths. Certain chemicals may be more readily detected by treatment/sampling times longer than 1.5 cycle lengths. Negative results with metabolic activation need to be confirmed on a case-by-case basis. In those cases where confirmation of negative results is not considered necessary, justification should be provided.

1.4.3.4 Chromosome preparation

Cell cultures are treated with Colcemid® or colchicine usually for one to three hours prior to harvesting. Each cell culture is harvested and processed separately for the preparation of chromosomes. Chromosome preparation involves hypotonic treatment of the cells, fixation and staining.

1.4.3.5 Analysis

All slides, including those of positive and negative controls, should be independently coded before microscopic analysis. Since fixation procedures often result in the breakage of a proportion of metaphase cells with loss of chromosomes, the cells scored should therefore contain a number of centromeres equal to the modal number ± 2 for all cell types. At least 200 well spread metaphases should be scored per concentration and control, equally divided amongst the duplicates, if applicable. This number can be reduced when high number of aberrations are observed.

Though the purpose of the test is to detect structural chromosome aberrations, it is important to record polyploidy and endoreduplication when these events are seen.

2. DATA

2.1. TREATMENT OF RESULTS

The experimental unit is the cell, and therefore the percentage of cells with structural chromosome aberration(s) should be evaluated. Different types of structural chromosome aberrations should be listed with their numbers and frequencies for experimental and control cultures. Gaps are recorded separately and reported but generally not included in the total aberration frequency.

Concurrent measures of cytotoxicity for all treated and negative control cultures in the main aberration experiments should also be recorded.

Individual culture data should be provided. Additionally, all data should be summarised in tabular form.

There is no requirement for verification of a clear positive response. Equivocal results should be clarified by further testing preferably using modification of experimental conditions. The need to confirm negative results has been discussed in 1.4.3.3. Modification of study parameters to extend the range of conditions assessed should be considered in follow-up experiments. Study parameters that might be modified include the concentration spacing and the metabolic activation conditions.

2.2. EVALUATION AND INTERPRETATION OF RESULTS

There are several criteria for determining a positive result, such as a concentration-related increase or a reproducible increase in the number of cells with chromosome aberrations. Biological relevance of the results should be considered first. Statistical methods may be used as an aid in evaluating the test results (3)(13). Statistical significance should not be the only determining factor for a positive response.

An increase in the number of polyploid cells may indicate that the test substance has the potential to inhibit mitotic processes and to induce numerical chromosome aberrations. An increase in the number of cells with endoreduplicated chromosomes may indicate that the test substance has the potential to inhibit cell cycle progression (17)(18).

A test substance for which the results do not meet the above criteria is considered non-mutagenic in this system.

Although most experiments will give clearly positive or negative results, in rare cases the data set will preclude making a definite judgement about the activity of the test substance. Results may remain equivocal or questionable regardless of the number of times the experiment is repeated.

Positive results from the *in vitro* chromosome aberration test indicate that the test substance induces structural chromosome aberrations in cultured mammalian somatic cells. Negative results indicate that, under the test conditions, the test substance does not induce chromosome aberrations in cultured mammalian somatic cells.

3. REPORTING

TEST REPORT

The test report must include the following information:

Solvent/Vehicle:

– justification for choice of vehicle;

– solubility and stability of the test substance in solvent/vehicle, if known;

Cells:

– type and source of cells;

– karyotype features and suitability of the cell type used;

– absence of mycoplasma, if applicable;

– information on cell cycle length;

– sex of blood donors, whole blood or separated lymphocytes, mitogen used;

– number of passages, if applicable;

– methods for maintenance of cell culture, if applicable;

– modal number of chromosomes.

Test conditions :

– identity of metaphase arresting substance, its concentration and duration of cell exposure;

– rationale for selection of concentrations and number of cultures including, e.g. cytotoxicity data and solubility limitations, if available;

– composition of media, CO2 concentration if applicable;

– concentration of test substance;

– volume of vehicle and test substance added;

– incubation temperature;

– incubation time;

– duration of treatment;

– cell density at seeding, if appropriate;

– type and composition of metabolic activation system, including acceptability criteria;

– positive and negative controls;

– methods of slide preparation;

– criteria for scoring aberrations;

– number of metaphases analysed;

– methods for the measurements of toxicity;

– criteria for considering studies as positive, negative or equivocal.

Results :

– signs of toxicity, e.g. degree of confluency, cell cycle data, cell counts, mitotic index;

– signs of precipitation;

– data on pH and osmolality of the treatment medium, if determined;

– definition for aberrations, including gaps;

– number of cells with chromosome aberrations and type of chromosome aberrations given separately for each treated and control culture;

– changes in ploidy if seen;

– dose-response relationship, where possible;

– statistical analyses, if any;

– concurrent negative (solvent/vehicle) and positive control data;

– historical negative (solvent/vehicle) and positive control data, with ranges, means and standard deviations.

Discussion of results.

Conclusions.

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B.11. MUTAGENICITY – *IN VIVO* MAMMALIAN BONE MARROW CHROMOSOME ABERRATION TEST

1. METHOD

This method is a replicate of the OECD TG 475, Mammalian Bone Marrow Chromosome Aberration Test (1997).

1.1 INTRODUCTION

The mammalian *in vivo* chromosome aberration test is used for the detection of structural chromosome aberrations induced by the test substance to the bone marrow cells of animals, usually rodents (1)(2)(3)(4). Structural chromosome aberrations may be of two types, chromosome or chromatid. An increase in polyploidy may indicate that a chemical has the potential to induce numerical aberrations. With the majority of chemical mutagens, induced aberrations are of the chromatid-type, but chromosome-type aberrations also occur. Chromosome mutations and related events are the cause of many human genetic diseases and there is substantial evidence that chromosome mutations and related events causing alterations in oncogenes and tumour suppressor genes are involved in cancer in humans and experimental systems.

Rodents are routinely used in this test. Bone marrow is the target tissue in this test, since it is a highly vascularised tissue, and it contains a population of rapidly cycling cells that can be readily isolated and processed. Other species and target tissues are not the subject of this method.

This chromosome aberration test is especially relevant to assessing mutagenic hazard in that it allows consideration of factors of *in vivo* metabolism, pharmacokinetics and DNA-repair processes although these may vary among species and among tissues. An *in vivo* test is also useful for further investigation of a mutagenic effect detected by *in vitro* test.

If there is evidence that the test substance, or a reactive metabolite, will not reach the target tissue, it is not appropriate to use this test.

See also General Introduction Part B.

1.2 DEFINITIONS

Chromatid-type aberration: structural chromosome damage expressed as breakage of single chromatids or breakage and reunion between chromatids.

Chromosome-type aberration: structural chromosome damage expressed as breakage, or breakage and reunion, of both chromatids at an identical site.

Endoreduplication: a process in which after an S period of DNA replication, the nucleus does not go into mitosis but starts another S period. The result is chromosomes with 4, 8, 16, ...chromatids.

Gap: an achromatic lesion smaller than the width of one chromatid, and with minimum misalignment of the chromatid(s).

Numerical aberration: a change in the number of chromosomes from the normal number characteristic of the cells utilised.

Polyploidy: a multiple of the haploid chromosome number (*n*) other than the diploid number (i.e. 3*n*, 4*n* and so on).

Structural aberration: a change in chromosome structure detectable by microscopic examination of the metaphase stage of cell division, observed as deletions and fragments, intrachanges or interchanges.

1.3 PRINCIPLE OF THE TEST METHOD

Animals are exposed to the test substance by an appropriate route of exposure and are sacrificed at appropriate times after treatment. Prior to sacrifice, animals are treated with a metaphase arresting agent (e.g. colchicine or Colcemid®). Chromosome preparations are then made from the bone marrow cells and stained, and metaphase cells are analysed for chromosome aberrations.

1.4 DESCRIPTION OF THE TEST METHOD

1.4.1 Preparations

1.4.1.1 Selection of animal species

Rats, mice and Chinese hamsters are commonly used, although any appropriate mammalian species may be used. Commonly used laboratory strains of young healthy adult animals should be employed. At the commencement of the study, the weight variation of animals should be minimal and not exceed ± 20% of the mean weight of each sex.

1.4.1.2 Housing and feeding conditions

General conditions referred in the General Introduction to Part B are applied although the aim for humidity should be 50-60%.

1.4.1.3 Preparation of the animals

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 minimised. The animals are identified uniquely. The animals are acclimated to the laboratory conditions for at least five days.

1.4.1.4 Preparation of doses

Solid test substances should be dissolved or suspended in appropriate solvents or vehicles and diluted, if appropriate, prior to dosing of the animals. Liquid test substances may be dosed directly or diluted prior to dosing. Fresh preparations of the test substance should be employed unless stability data demonstrate the acceptability of storage.

1.4.2 Test conditions

1.4.2.1 Solvent/Vehicle

The solvent/vehicle should not produce toxic effects at the dose levels used, and should not be suspected of chemical reaction with the test substance. If other than well-known solvents/vehicles are used, their inclusion should be supported with data indicating their compatibility. It is recommended that wherever possible, the use of an aqueous solvent/vehicle should be considered first.

1.4.2.2 Controls

Concurrent positive and negative (solvent/vehicle) controls should be included for each sex in each test. Except for treatment with the test substance, animals in the control groups should be handled in an identical manner to the animals in the treated groups.

Positive controls should produce structural aberrations *in vivo* at exposure levels expected to give a detectable increase over background. Positive control doses should be chosen so that the effects are clear but do not immediately reveal the identity of the coded slides to the reader. It is acceptable that the positive control be administered by a route different from the test substance and sampled at only a single time. The use of chemical class related positive control chemicals may be considered, when available. Examples of positive control substances include:

Substance	**CAS No.**	**EINECS No.**
Ethyl methanesulphonate	62-50-0	200-536-7
Ethyl nitrosourea	759-73-9	212-072-2
Mitomycin C	50-07-7	200-008-6
Cyclophosphamide Cyclophosphamide monohydrate	50-18-0 6055-19-2	200-015-4
Triethylenemelamine	51-18-3	200-083-5

Negative controls, treated with solvent or vehicle alone, and otherwise treated in the same way as the treatment groups, should be included for every sampling time, unless acceptable inter-animal variability and frequencies of cells with chromosome aberrations are available from historical control data. If single sampling is applied for negative controls, the most appropriate time is the first sampling time. In addition, untreated controls should also be used unless there are historical or published control data demonstrating that no deleterious or mutagenic effects are induced by the chosen solvent/vehicle.

1.5 PROCEDURE

1.5.1 Number and sex of animals

Each treated and control group must include at least 5 analysable animals per sex. If at the time of the study there are data available from studies in the same species and using the same route of exposure that demonstrate that there are no substantial differences in toxicity between sexes, then testing in a single sex will be sufficient. Where human exposure to chemicals may be sex-specific, as for example with some pharmaceutical agents, the test should be performed with animals of the appropriate sex.

1.5.2 Treatment schedule

Test substances are preferably administered as a single treatment. Test substances may also be administered as a split dose, i.e. two treatments on the same day separated by no more than a few hours, to facilitate administering a large volume of material. Other dose regimens should be scientifically justified.

Samples should be taken at two separate times following treatment on one day. For rodents, the first sampling interval is 1.5 normal cell cycle length (the latter being normally 12-18 hr) following treatment. Since the time required for uptake and metabolism of the test substance as well as its effect on cell cycle kinetics can affect the optimum time for chromosome aberration detection, a later sample collection 24 hr after the first sample time is recommended. If dose regimens of more than one day are used, one sampling time at 1.5 normal cell cycle lengths after the final treatment should be used.

Prior to sacrifice, animals are injected intraperitoneally with an appropriate dose of a metaphase arresting agent (e.g. Colcemid® or colchicine). Animals are sampled at an appropriate interval thereafter. For mice this interval is approximately 3-5 hours; for Chinese hamsters this interval is approximately 4-5 hours. Cells are harvested from the bone marrow and analysed for chromosome aberrations.

1.5.3 Dose levels

If a range finding study is performed because there are no suitable data available, it should be performed in the same laboratory, using the same species, strain, sex, and treatment regimen to be used in the main study (5). If there is toxicity, three dose levels are used for the first sampling time. These dose levels should cover a range from the maximum to little or no toxicity. At the later sampling time only the highest dose needs to be used. The highest dose is defined as the dose producing signs of toxicity such that higher dose levels, based on the same dosing regimen, would be expected to produce lethality. Substances with specific biological activities at low non-toxic doses (such as hormones and mitogens) may be exceptions to the dose-setting criteria and should be evaluated on a case-by-case basis. The highest dose may also be defined as a dose that produces some indication of toxicity in the bone marrow (e.g. greater than 50% reduction in mitotic index).

1.5.4 Limit test

If a test at one dose level of at least 2000 mg/kg body weight using a single treatment, or as two treatments on the same day, produces no observable toxic effects, and if genotoxicity would not be expected based on data from structurally related substances, then a full study using three dose levels may not be considered necessary. For studies of a longer duration, the limit dose is 2000 mg/kg/body weight/day for treatment up to 14 days, and 1000 mg/kg/body

weight/day for treatment longer than 14 days. Expected human exposure may indicate the need for a higher dose level to be used in the limit test.

1.5.5 Administration of doses

The test substance is usually administered by gavage using a stomach tube or a suitable intubation cannula, or by intraperitoneal injection. Other routes of exposure may be acceptable where they can be justified. The maximum volume of liquid that can be administered by gavage or injection at one time depends on the size of the test animal. The volume should not exceed 2 ml/100g body weight. The use of volumes higher than these must be justified. 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.

1.5.6 Chromosome preparation

Immediately after sacrifice, bone marrow is obtained, exposed to hypotonic solution and fixed. The cells are then spread on slides and stained.

1.5.7 Analysis

The mitotic index should be determined as a measure of cytotoxicity in at least 1000 cells per animal for all treated animals (including positive controls) and untreated negative control animals.

At least 100 cells should be analysed for each animal. This number could be reduced when high numbers of aberrations are observed. All slides, including those of positive and negative controls, should be independently coded before microscopic analysis. Since slide preparation procedures often result in the breakage of a proportion of metaphases with loss of chromosomes, the cells scored should therefore contain a number of centromeres equal to the number $2n \pm 2$.

2. DATA

2.1 TREATMENT OF RESULTS

Individual animal data should be presented in tabular form. The experimental unit is the animal. For each animal the number of cells scored, the number of aberrations per cell and the percentage of cells with structural chromosome aberration(s) should be evaluated. Different types of structural chromosome aberrations should be listed with their numbers and frequencies for treated and control groups. Gaps are recorded separately and reported but generally not included in the total aberration frequency. If there is no evidence for a difference in response between the sexes, the data from both sexes may be combined for statistical analysis.

2.2 EVALUATION AND INTERPRETATION OF RESULTS

There are several criteria for determining a positive result, such as a dose-related increase in the relative number of cells with chromosome aberrations or a clear increase in the number of cells with aberrations in a single dose group at a single sampling time. Biological relevance of

the results should be considered first. Statistical methods may be used as an aid in evaluating the test results (6). Statistical significance should not be the only determining factor for a positive response. Equivocal results should be clarified by further testing preferably using a modification of experimental conditions.

An increase in polyploidy may indicate that the test substance has the potential to induce numerical chromosome aberrations. An increase in endoreduplication may indicate that the test substance has the potential to inhibit cell cycle progression (7)(8).

A test substance for which the results do not meet the above criteria is considered non-mutagenic in this test.

Although most experiments will give clearly positive or negative results, in rare cases the data set will preclude making a definite judgement about the activity of the test substance. Results may remain equivocal or questionable regardless of the number of experiments performed.

Positive results from the *in vivo* chromosome aberration test indicate that a substance induces chromosome aberrations in the bone marrow of the species tested. Negative results indicate that, under the test conditions, the test substance does not induce chromosome aberrations in the bone marrow of the species tested.

The likelihood that the test substance or its metabolites reach the general circulation or specifically the target tissue (e.g. systemic toxicity) should be discussed.

3. REPORTING

TEST REPORT

The test report must include the following information :

Solvent/Vehicle:

– justification for choice of vehicle;

– solubility and stability of the test substance in solvent/vehicle, if known;

Test animals:

– species/strain used;

– number, age and sex of animals;

– source, housing conditions, diet, etc.;

– individual weight of the animals at the start of the test, including body weight range, mean and standard deviation for each group;

Test conditions :

– positive and negative (vehicle/solvent) controls;

– data from range-finding study, if conducted;

– rationale for dose level selection;

– details of test substance preparation;

– details of the administration of the test substance;

– rationale for route of administration;

– methods for verifying that the test substance reached the general circulation or target tissue, if applicable;

– 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;

– detailed description of treatment and sampling schedules;

– methods for measurements of toxicity;

– identity of metaphase arresting substance, its concentration and duration of treatment;

– methods of slide preparation;

– criteria for scoring aberrations;

– number of cells analysed per animal;

– criteria for considering studies as positive, negative or equivocal.

Results :

– signs of toxicity;

– mitotic index;

– type and number of aberrations, given separately for each animal;

– total number of aberrations per group with means and standard deviations;

– number of cells with aberrations per group with means and standard deviations;

– changes in ploidy, if seen;

– dose-response relationship, where possible;

– statistical analyses, if any;

– concurrent negative control data;

– historical negative control data with ranges, means and standard deviations;

– concurrent positive control data.

Discussion of the results.

Conclusions.

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B. 12. MUTAGENICITY – *IN VIVO* MAMMALIAN ERYTHROCYTE MICRONUCLEUS TEST

1. METHOD

This method is a replicate of the OECD TG 474, Mammalian Erythrocyte Micronucleus Test (1997).

1.1 INTRODUCTION

The mammalian *in vivo* micronucleus test is used for the detection of damage induced by the test substance to the chromosomes or the mitotic apparatus of erythroblasts by analysis of erythrocytes as sampled in bone marrow and/or peripheral blood cells of animals, usually rodents.

The purpose of the micronucleus test is to identify substances that cause cytogenetic damage which results in the formation of micronuclei containing lagging chromosome fragments or whole chromosomes.

When a bone marrow erythroblast develops into a polychromatic erythrocyte, the main nucleus is extruded; any micronucleus that has been formed may remain behind in the otherwise anucleated cytoplasm. Visualisation of micronuclei is facilitated in these cells because they lack a main nucleus. An increase in the frequency of micronucleated polychromatic erythrocytes in treated animals is an indication of induced chromosome damage.

The bone marrow of rodents is routinely used in this test since polychromatic erythrocytes are produced in that tissue. The measurement of micronucleated immature (polychromatic) erythrocytes in peripheral blood is equally acceptable in any species in which the inability of the spleen to remove micronucleated erythrocytes has been demonstrated, or which has shown an adequate sensitivity to detect agents that cause structural or numerical chromosome aberrations. Micronuclei can be distinguished by a number of criteria. These include identification of the presence or absence of a kinetochore or centromeric DNA in the micronuclei. The frequency of micronucleated immature (polychromatic) erythrocytes is the principal endpoint. The number of mature (normochromatic) erythrocytes in the peripheral blood that contain micronuclei among a given number of mature erythrocytes can also be used as the endpoint of the assay when animals are treated continuously for 4 weeks or more.

This mammalian *in vivo* micronucleus test is especially relevant to assessing mutagenic hazard in that it allows consideration of factors of *in vivo* metabolism, pharmacokinetics and DNA-repair processes although these may vary among species, among tissues and among genetic endpoints. An *in vivo* assay is also useful for further investigation of a mutagenic effect detected by an *in vitro* system.

If there is evidence that the test substance, or a reactive metabolite, will not reach the target tissue, it is not appropriate to use this test.

See also General Introduction Part B.

1.2 DEFINITIONS

Centromere (Kinetochore): Region(s) of a chromosome with which spindle fibers are associated during cell division, allowing orderly movement of daughter chromosomes to the poles of the daughter cells.

Micronuclei: Small nuclei, separate from and additional to the main nuclei of cells, produced during telophase of mitosis (meiosis) by lagging chromosome fragments or whole chromosomes.

Normochromatic erythrocyte: Mature erythrocyte that lacks ribosomes and can be distinguished from immature, polychromatic erythrocytes by stains selective for ribosomes.

Polychromatic erythrocyte: Immature erythrocyte, in an intermediate stage of development, that still contains ribosomes and therefore can be distinguished from mature, normochromatic erythrocytes by stains selective for ribosomes.

1.3 PRINCIPLE OF THE TEST METHOD

Animals are exposed to the test substance by an appropriate route. If bone marrow is used, the animals are sacrificed at appropriate times after treatment, the bone marrow extracted, and preparations made and stained (1)(2)(3)(4)(5)(6)(7). When peripheral blood is used, the blood is collected at appropriate times after treatment and smear preparations are made and stained (4)(8)(9)(10). For studies with peripheral blood, as little time as possible should elapse between the last exposure and cell harvest. Preparations are analysed for the presence of micronuclei.

1.4 DESCRIPTION OF THE TEST METHOD

1.4.1 Preparations

1.4.1.1 Selection of animal species

Mice or rats are recommended if bone marrow is used, although any appropriate mammalian species may be used. When peripheral blood is used, mice are recommended. However, any appropriate mammalian species may be used provided it is a species in which the spleen does not remove micronucleated erythrocytes or a species which has shown an adequate sensitivity to detect agents that cause structural or numerical chromosome aberrations. Commonly used laboratory strains of young healthy animals should be employed. At the commencement of the study, the weight variation of animals should be minimal and not exceed ±20% of the mean weight of each sex.

1.4.1.2 Housing and feeding conditions

General conditions referred in the General Introduction to Part B are applied although the aim for humidity should be 50-60%.

1.4.1.3 Preparation of the animals

Healthy young adult animals are randomly assigned to the control and treatment groups. The animals are identified uniquely. The animals are acclimated to the laboratory conditions for at

least five days. Cages should be arranged in such a way that possible effects due to cage placement are minimised.

1.4.1.4 Preparation of doses

Solid test substances should be dissolved or suspended in appropriate solvents or vehicles and diluted, if appropriate, prior to dosing of the animals. Liquid test substances may be dosed directly or diluted prior to dosing. Fresh preparations of the test substance should be employed unless stability data demonstrate the acceptability of storage.

1.4.2 Test conditions

1.4.2.1 Solvent/Vehicle

The solvent/vehicle should not produce toxic effects at the dose levels used, and should not be suspected of chemical reaction with the test substance. If other than well-known solvents/vehicles are used, their inclusion should be supported with reference data indicating their compatibility. It is recommended that wherever possible, the use of an aqueous solvent/vehicle should be considered first.

1.4.2.2 Controls

Concurrent positive and negative (solvent/vehicle) controls should be included for each sex in each test. Except for treatment with the test substance, animals in the control groups should be handled in an identical manner to animals of the treatment groups.

Positive controls should produce micronuclei *in vivo* at exposure levels expected to give a detectable increase over background. Positive control doses should be chosen so that the effects are clear but do not immediately reveal the identity of the coded slides to the reader. It is acceptable that the positive control be administered by a route different from the test substance and sampled at only a single time. In addition, the use of chemical class-related positive control chemicals may be considered, when available. Examples of positive control substances include:

Substance	**CAS No.**	**EINECS No.**
Ethyl methanesulphonate	62-50-0	200-536-7
N-ethyl-N-nitrosourea	759-73-9	212-072-2
Mitomycin C	50-07-7	200-008-6
Cyclophosphamide Cyclophosphamide monohydrate	50-18-0 6055-19-2	200-015-4
Triethylenemelamine	51-18-3	200-083-5

Negative controls, treated with solvent or vehicle alone, and otherwise treated in the same way as the treatment groups should be included for every sampling time, unless acceptable inter-animal variability and frequencies of cells with micronuclei are demonstrated by

historical control data. If single sampling is applied for negative controls, the most appropriate time is the first sampling time. In addition, untreated controls should also be used unless there are historical or published control data demonstrating that no deleterious or mutagenic effects are induced by the chosen solvent/vehicle.

If peripheral blood is used, a pre-treatment sample may also be acceptable as a concurrent negative control, but only in the short peripheral blood studies (e.g., 1-3 treatment(s)) when the resulting data are in the expected range for the historical control.

1.5 PROCEDURE

1.5.1 Number and sex of animals

Each treated and control group must include at least 5 analysable animals per sex (11). If at the time of the study there are data available from studies in the same species and using the same route of exposure that demonstrate that there are no substantial differences between sexes in toxicity, then testing in a single sex will be sufficient. Where human exposure to chemicals may be sex-specific, as for example with some pharmaceutical agents, the test should be performed with animals of the appropriate sex.

1.5.2 Treatment schedule

No standard treatment schedule (i.e. 1, 2 or more treatments at 24 h intervals) can be recommended. The samples from extended dose regimens are acceptable as long as a positive effect has been demonstrated for this study or, for a negative study, as long as toxicity has been demonstrated or the limit dose has been used, and dosing continued until the time of sampling. Test substances may also be administered as a split dose, i.e., two treatments on the same day separated by no more than a few hours, to facilitate administering a large volume of material.

The test may be performed in two ways:

(a) Animals are treated with the test substance once. Samples of bone marrow are taken at least twice, starting not earlier than 24 hours after treatment, but not extending beyond 48 hours after treatment with appropriate intervals between samples. The use of sampling times earlier than 24 hours after treatment should be justified. Samples of peripheral blood are taken at least twice, starting not earlier than 36 hours after treatment, with appropriate intervals following the first sample, but not extending beyond 72 hours. When a positive response is recognised at one sampling time, additional sampling is not required.

(b) If 2 or more daily treatments are used (e.g. two or more treatments at 24 hour intervals), samples should be collected once between 18 and 24 hours following the final treatment for the bone marrow and once between 36 and 48 hours following the final treatment for the peripheral blood (12).

Other sampling times may be used in addition, when relevant.

1.5.3 Dose levels

If a range finding study is performed because there are no suitable data available, it should be performed in the same laboratory, using the same species, strain, sex, and treatment regimen

to be used in the main study (13). If there is toxicity, three dose levels are used for the first sampling time. These dose levels should cover a range from the maximum to little or no toxicity. At the later sampling time only the highest dose needs to be used. The highest dose is defined as the dose producing signs of toxicity such that higher dose levels, based on the same dosing regimen, would be expected to produce lethality. Substances with specific biological activities at low non-toxic doses (such as hormones and mitogens) may be exceptions to the dose-setting criteria and should be evaluated on a case-by-case basis. The highest dose may also be defined as a dose that produces some indication of toxicity in the bone marrow (e.g. a reduction in the proportion of immature erythrocytes among total erythrocytes in the bone marrow or peripheral blood).

1.5.4 Limit test

If a test at one dose level of at least 2000 mg/kg body weight using a single treatment, or as two treatments on the same day, produces no observable toxic effects, and if genotoxicity would not be expected based upon data from structurally related substances, then a full study using three dose levels may not be considered necessary. For studies of a longer duration, the limit dose is 2000 mg/kg/body weight/day for treatment up to 14 days, and 1000 mg/kg/body weight/day for treatment longer than 14 days. Expected human exposure may indicate the need for a higher dose level to be used in the limit test.

1.5.5 Administration of doses

The test substance is usually administered by gavage using a stomach tube or a suitable intubation cannula, or by intraperitoneal injection. Other routes of exposure may be acceptable where they can be justified. The maximum volume of liquid that can be administered by gavage or injection at one time depends on the size of the test animal. The volume should not exceed 2 ml/100g body weight. The use of volumes higher than these must be justified. 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.

1.5.6 Bone marrow / blood preparation

Bone marrow cells are usually obtained from the femurs or tibias immediately following sacrifice. Commonly, cells are removed from femurs or tibias, prepared and stained using established methods. Peripheral blood is obtained from the tail vein or other appropriate blood vessel. Blood cells are immediately stained supravitally (8)(9)(10) or smear preparations are made and then stained. The use of a DNA specific stain [e.g. acridine orange (14) or Hoechst 33258 plus pyronin-Y (15)] can eliminate some of the artifacts associated with using a non DNA specific stain. This advantage does not preclude the use of conventional stains (e.g., Giemsa). Additional systems [e.g. cellulose columns to remove nucleated cells (16)] can also be used provided that these systems have been shown to adequately work for micronucleus preparation in the laboratory.

1.5.7 Analysis

The proportion of immature among total (immature + mature) erythrocytes is determined for each animal by counting a total of at least 200 erythrocytes for bone marrow and 1000 erythrocytes for peripheral blood (17). All slides, including those of positive and negative controls, should be independently coded before microscopic analysis. At least 2000 immature erythrocytes per animal are scored for the incidence of micronucleated immature erythrocytes. Additional information may be obtained by scoring mature erythrocytes for micronuclei.

When analysing slides, the proportion of immature erythrocytes among total erythrocytes should not be less than 20% of the control value. When animals are treated continuously for 4 weeks or more, at least 2000 mature erythrocytes per animal can also be scored for the incidence of micronuclei. Systems for automated analysis (image analysis and cell suspensions flow cytometry) are acceptable alternatives to manual evaluation if appropriately justified and validated.

2. DATA

2.1 TREATMENT OF RESULTS

Individual animal data should be presented in tabular form. The experimental unit is the animal. The number of immature erythrocytes scored, the number of micronucleated immature erythrocytes, and the number of immature among total erythrocytes should be listed separately for each animal analysed. When animals are treated continuously for 4 weeks or more, the data on mature erythrocytes should also be given if it is collected. The proportion of immature among total erythrocytes and, if considered applicable, the percentage of micronucleated erythrocytes is given for each animal. If there is no evidence for a difference in response between the sexes, the data from both sexes may be combined for statistical analysis.

2.2 EVALUATION AND INTERPRETATION OF RESULTS

There are several criteria for determining a positive result, such as a dose-related increase in the number of micronucleated cells or a clear increase in the number of micronucleated cells in a single dose group at a single sampling time. Biological relevance of the results should be considered first. Statistical methods may be used as an aid in evaluating the test results (18)(19). Statistical significance should not be the only determining factor for a positive response. Equivocal results should be clarified by further testing preferably using a modification of experimental conditions.

A test substance for which the results do not meet the above criteria is considered non-mutagenic in this test.

Although most experiments will give clearly positive or negative results, in rare cases the data set will preclude making a definite judgement about the activity of the test substance. Results may remain equivocal or questionable regardless of the number of times the experiment is repeated.

Positive results in the micronucleus test indicate that the substance induces micronuclei which are the result of chromosomal damage or damage to the mitotic apparatus in the erythroblasts of the test species. Negative results indicate that, under the test conditions, the test substance does not produce micronuclei in the immature erythrocytes of the test species.

The likelihood that the test substance or its metabolites reach the general circulation or specifically the target tissue (e.g. systemic toxicity) should be discussed.

3. REPORTING

TEST REPORT

The test report should include the following information :

Solvent/Vehicle:

– justification for choice of vehicle;

– solubility and stability of the test substance in solvent/vehicle, if known;

Test animals:

– species/strain used;

– number, age and sex of animals;

– source, housing conditions, diet, etc.;

– individual weight of the animals at the start of the test, including body weight range, mean and standard deviation for each group;

Test conditions :

– positive and negative (vehicle/solvent) control data;

– data from range-finding study, if conducted;

– rationale for dose level selection;

– details of test substance preparation;

– details of the administration of the test substance;

– rationale for route of administration;

– methods for verifying that the test substance reached the general circulation or target tissue, if applicable;

– 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;

– detailed description of treatment and sampling schedules;

– methods of slide preparation;

– methods for measurements of toxicity;

– criteria for scoring micronucleated immature erythrocytes;

– number of cells analysed per animal;

– criteria for considering studies as positive, negative or equivocal.

Results :

– signs of toxicity;

– proportion of immature erythrocytes among total erythrocytes;

– number of micronucleated immature erythrocytes, given separately for each animal;

– mean ± standard deviation of micronucleated immature erythrocytes per group;

– dose-response relationship, where possible;

– statistical analyses and methods applied;

– concurrent and historical negative control data;

– concurrent positive control data.

Discussion of the results.

Conclusions.

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B. 13/14. MUTAGENICITY : REVERSE MUTATION TEST USING BACTERIA

1. METHOD

This method is a replicate of the OECD TG 471, Bacterial Reverse Mutation Test (1997).

1.1. INTRODUCTION

The bacterial reverse mutation test uses amino-acid requiring strains of *Salmonella typhimurium* and *Escherichia coli* to detect point mutations, which involve substitution, addition or deletion of one or a few DNA base pairs (1)(2)(3). The principle of this bacterial reverse mutation test is that it detects mutations which revert mutations present in the test strains and restore the functional capability of the bacteria to synthesise an essential amino acid. The revertant bacteria are detected by their ability to grow in the absence of the amino-acid required by the parent test strain.

Point mutations are the cause of many human genetic diseases and there is substantial evidence that point mutations in oncogenes and tumour suppressor genes of somatic cells are involved in tumour formation in humans and experimental animals. The bacterial reverse mutation test is rapid, inexpensive and relatively easy to perform. Many of the test strains have several features that make them more sensitive for the detection of mutations including responsive DNA sequences at the reversion sites, increased cell permeability to large molecules and elimination of DNA repair systems or enhancement of error-prone DNA repair processes. The specificity of the test strains can provide some useful information on the types of mutations that are induced by genotoxic agents. A very large data base of results for a wide variety of structures is available for bacterial reverse mutation tests and well-established methodologies have been developed for testing chemicals with different physico-chemical properties, including volatile compounds.

See also General Introduction Part B.

1.2. DEFINITIONS

A reverse mutation test in either *Salmonella typhimurium* or *Escherichia coli* detects mutation in an amino-acid requiring strain (histidine or tryptophan, respectively) to produce a strain independent of an outside supply of amino-acid.

Base pair substitution mutagens are agents that cause a base change in DNA. In a reversion test this change may occur at the site of the original mutation, or at a second site in the bacterial genome.

Frameshift mutagens are agents that cause the addition or deletion of one or more base pairs in the DNA, thus changing the reading frame in the RNA.

1.3. INITIAL CONSIDERATIONS

The bacterial reverse mutation test utilises prokaryotic cells, which differ from mammalian cells in such factors as uptake, metabolism, chromosome structure and DNA repair processes. Tests conducted *in vitro* generally require the use of an exogenous source of metabolic activation. *In vitro* metabolic activation systems cannot mimic entirely the mammalian *in vivo* conditions. The test therefore does not provide direct information on the mutagenic and carcinogenic potency of a substance in mammals.

The bacterial reverse mutation test is commonly employed as an initial screen for genotoxic activity and, in particular, for point mutation-inducing activity. An extensive data base has demonstrated that many chemicals that are positive in this test also exhibit mutagenic activity in other tests. There are examples of mutagenic agents which are not detected by this test; reasons for these shortcomings can be ascribed to the specific nature of the endpoint detected, differences in metabolic activation, or differences in bioavailability. On the other hand, factors which enhance the sensitivity of the bacterial reverse mutation test can lead to an overestimation of mutagenic activity.

The bacterial reverse mutation test may not be appropriate for the evaluation of certain classes of chemicals, for example highly bactericidal compounds (e.g. certain antibiotics) and those which are thought (or known) to interfere specifically with the mammalian cell replication system (e.g. some topoisomerase inhibitors and some nucleoside analogues). In such cases, mammalian mutation tests may be more appropriate.

Although many compounds that are positive in this test are mammalian carcinogens, the correlation is not absolute. It is dependent on chemical class and there are carcinogens that are not detected by this test because they act through other, non-genotoxic, mechanisms or mechanisms absent in bacterial cells**.**

1.4. PRINCIPLE OF THE TEST METHOD

Suspensions of bacterial cells are exposed to the test substance in the presence and in the absence of an exogenous metabolic activation system. In the plate incorporation method, these suspensions are mixed with an overlay agar and plated immediately onto minimal medium. In the preincubation method, the treatment mixture is incubated and then mixed with an overlay agar before plating onto minimal medium. For both techniques, after two or three days of incubation, revertant colonies are counted and compared to the number of spontaneous revertant colonies on solvent control plates.

Several procedures for performing the bacterial reverse mutation test have been described. Among those commonly used are the plate incorporation method (1)(2)(3)(4), the preincubation method (2)(3)(5)(6)(7)(8), the fluctuation method (9)(10), and the suspension method (11). Modifications for the testing of gases or vapours have been described (12).

The procedures described in the method pertain primarily to the plate incorporation and preincubation methods. Either of them is acceptable for conducting experiments both with and without metabolic activation. Some substances may be detected more efficiently using the preincubation method. These substances belong to chemical classes that include short chain aliphatic nitrosamines, divalent metals, aldehydes, azo-dyes and diazo compounds, pyrollizidine alkaloids, allyl compounds and nitro compounds (3). It is also recognised that certain classes of mutagens are not always detected using standard procedures such as the plate incorporation method or preincubation method. These should be regarded as “special

cases" and it is strongly recommended that alternative procedures should be used for their detection. The following "special cases" could be identified (together with examples of procedures that could be used for their detection): azo-dyes and diazo compounds (3)(5)(6)(13), gases and volatile chemicals (12)(14)(15)(16) and glycosides (17)(18). A deviation from the standard procedure needs to be scientifically justified.

1.5. DESCRIPTION OF THE TEST METHOD

1.5.1. Preparations

1.5.1.1. Bacteria

Fresh cultures of bacteria should be grown up to the late exponential or early stationary phase of growth (approximately 10^9 cells per ml). Cultures in late stationary phase should not be used. It is essential that the cultures used in the experiment contain a high titre of viable bacteria. The titre may be demonstrated either from historical control data on growth curves, or in each assay through the determination of viable cell numbers by a plating experiment.

The recommended incubation temperature is 37°C.

At least five strains of bacteria should be used. These should include four strains of *S. typhimurium* (TA 1535; TA 1537 or TA97a or TA97; TA98; and TA100) that have been shown to be reliable and reproducibly responsive between laboratories. These four *S. typhimurium* strains have GC base pairs at the primary reversion site and it is known that may not detect certain oxidising mutagens, cross-linking agents and hydrazines. Such substances may be detected by *E. coli* WP2 strains or *S. typhimurium* TA102 (19) which have an AT base pair at the primary reversion site. Therefore the recommended combination of strains is:

– S. typhimurium TA1535, and

– S. typhimurium TA1537 or TA97 or TA97a, and

– S. typhimurium TA98, and

– S. typhimurium TA100, and

– E. coli WP2 uvrA, or E. coli WP2 uvrA (pKM101), or S. typhimurium TA102.

In order to detect cross-linking mutagens it may be preferable to include TA102 or to add a DNA repair-proficient strain of *E. coli* [e.g. *E. coli* WP2 or *E. coli* WP2 (pKM101)]

Established procedures for stock culture preparation, marker verification and storage should be used. The amino-acid requirement for growth should be demonstrated for each frozen stock culture preparation (histidine for *S. typhimurium* strains, and tryptophan for *E. coli* strains). Other phenotypic characteristics should be similarly checked, namely: the presence or absence of R-factor plasmids where appropriate [i.e. ampicillin resistance in strains TA98, TA100 and TA97a or TA97, WP2 uvrA and WP2 uvrA (pKM101), and ampicillin + tetracycline resistance in strain TA102]; the presence of characteristic mutations (i.e. rfa mutation in *S. typhimurium* through sensitivity to crystal violet, and uvrA mutation in *E. coli* or uvrB mutation in *S. typhimurium*, through sensitivity to ultra-violet light) (2)(3). The strains should also yield spontaneous revertant colony plate counts within the frequency

ranges expected from the laboratory's historical control data and preferably within the range reported in the literature.

1.5.1.2. Medium

An appropriate minimal agar (e.g. containing Vogel-Bonner minimal medium E and glucose), and an overlay agar containing histidine and biotin or tryptophan to allow for a few cell divisions, is used (1)(2)(9).

1.5.1.3. Metabolic activation

Bacteria should be exposed to the test substance both in the presence and absence of an appropriate metabolic activation system. The most commonly used system is a cofactor-supplemented post-mitochondrial fraction (S9) prepared from the livers of rodents treated with enzyme-inducing agents such as Aroclor 1254 (1)(2) or a combination of Phenobarbitone and ß–naphthoflavone (18)(20)(21). The post-mitochondrial fraction is usually used at concentrations in the range from 5 to 30% v/v in the S9-mix. The choice and condition of a metabolic activation system may depend upon the class of chemical being tested. In some cases, it may be appropriate to utilize more than one concentration of post-mitochondrial fraction. For azo-dyes and diazo-compounds, using a reductive metabolic activation system may be more appropriate (6)(13).

1.5.1.4. Test substance/Preparation

Solid test substances should be dissolved or suspended in appropriate solvents or vehicles and diluted if appropriate prior to treatment of the bacteria. Liquid test substances may be added directly to the test systems and/or diluted prior to treatment. Fresh preparations should be employed unless stability data demonstrate the acceptability of storage.

The solvent/vehicle should not be suspected of chemical reaction with the test substance and should be compatible with the survival of the bacteria and the S9 activity (22). If other than well-known solvent/vehicles are used, their inclusion should be supported by data indicating their compatibility. It is recommended that wherever possible, the use of an aqueous solvent/vehicle be considered first. When testing water-unstable substances, the organic solvents used should be free of water.

1.5.2. Test conditions

1.5.2.1. Test strains (see 1.5.1.1)

1.5.2.2. Exposure concentration

Amongst the criteria to be taken into consideration when determining the highest amount of the test substance to_be used are the cytotoxicity and the solubility in the final treatment mixture.

It may be useful to determine toxicity and insolubility in a preliminary experiment. Cytotoxicity may be detected by a reduction in the number of revertant colonies, a clearing or diminution of the background lawn, or the degree of survival of treated cultures. The cytotoxicity of a substance may be altered in the presence of metabolic activation systems. Insolubility should be assessed as precipitation in the final mixture under the actual test conditions and evident to the unaided eye.

The recommended maximum test concentration for soluble non-cytotoxic substances is 5 mg/plate or 5 µl/plate. For non-cytotoxic substances that are not soluble at 5 mg/plate or 5 µl/plate, one or more concentrations tested should be insoluble in the final treatment mixture. Test substances that are cytotoxic already below 5 mg/plate or 5 µl/plate should be tested up to a cytotoxic concentration. The precipitate should not interfere with the scoring.

At least five different analysable concentrations of the test substance should be used with approximately half log (i.e. √10) intervals between test points for an initial experiment. Smaller intervals may be appropriate when a concentration-response is being investigated. Testing above the concentration of 5 mg/plate or 5 µl/plate may be considered when evaluating substances containing substantial amounts of potentially mutagenic impurities.

1.5.2.3. Negative and positive controls

Concurrent strain-specific positive and negative (solvent or vehicle) controls, both with and without metabolic activation, should be included in each assay. Positive control concentrations that demonstrate the effective performance of each assay should be selected.

For assays employing a metabolic activation system, the positive control reference substance(s) should be selected on the basis of the type of bacteria strains used.

The following substances are examples of suitable positive controls for assays with metabolic activation:

CA numbers	EINECS numbers	Names
781-43-1	212-308-4	9,10-dimethylanthracene
57-97-6	200-359-5	7,12-dimethylbenz[*a*]anthracene
50-32-8	200-028-5	benzo[a]pyrene
613-13-8	210-330-9	2-aminoanthracene
50-18-0 6055-19-2	200-015-4	cyclophosphamide cyclophosphamide monohydrate

The following substance is a suitable positive control for the reductive metabolic activation method:

573-58-0	209-358-4	Congo Red

2–Aminoanthracene should not be used as the sole indicator of the efficacy of the S9-mix. If 2-aminoanthracene is used, each batch of S9 should also be characterised with a mutagen that requires metabolic activation by microsomal enzymes, e.g., benzo[a]pyrene, dimethylbenzanthracene.

The following substances are examples of strain-specific positive controls for assays performed without exogenous metabolic activation system:

CAS numbers	EINECS numbers	Names	Strain
26628-22-8	247-852-1	sodium azide	TA 1535 and TA 100
607-57-8	210-138-5	2-nitrofluorene	TA 98
90-45-9	201-995-6	9-aminoacridine	TA 1537, TA 97 and TA 97a
17070-45-0	241-129-4	ICR 191	TA 1537, TA 97 and TA 97a
80-15-9	201-254-7	cumene hydroperoxide	TA 102
50-07-7	200-008-6	mitomycin C	WP2 uvrA and TA102
70-25-7	200-730-1	N-ethyl-N-nitro-N-nitrosoguanidine	WP2, WP2uvrA and WP2uvrA(pKM101)
56-57-5	200-281-1	4-nitroquinoline-1-oxide	WP2, WP2uvrA and WP2uvrA(pKM101)
3688-53-7		furylfuramide (AF2)	plasmid-containing strains

Other appropriate positive control reference substances may be used. The use of chemical class-related positive control chemicals should be considered, when available.

Negative controls, consisting of solvent or vehicle alone, without test substance, and otherwise treated in the same way as the treatment groups, should be included. In addition, untreated controls should also be used unless there are historical control data demonstrating that no deleterious or mutagenic effects are induced by the chosen solvent.

1.5.3. Procedure

For the plate incorporation method (1)(2)(3)(4), without metabolic activation, usually 0.05 ml or 0.1 ml of the test solutions, 0.1 ml of fresh bacterial culture (containing approximately 10^8 viable cells) and 0.5 ml of sterile buffer are mixed with 2.0 ml of overlay agar. For the assay with metabolic activation, usually 0.5 ml of metabolic activation mixture containing an

adequate amount of post-mitochondrial fraction (in the range from 5 to 30% v/v in the metabolic activation mixture) are mixed with the overlay agar (2.0 ml), together with the bacteria and test substance/test solution. The contents of each tube are mixed and poured over the surface of a minimal agar plate. The overlay agar is allowed to solidify before incubation.

For the preincubation method (2)(3)(5)(6), the test substance/test solution is preincubated with the test strain (containing approximately 10^8 viable cells) and sterile buffer or the metabolic activation system (0.5 ml) usually for 20 min. or more at 30-37°C prior to mixing with the overlay agar and pouring onto the surface of a minimal agar plate. Usually, 0.05 or 0.1 ml of test substance/test solution, 0.1 ml of bacteria, and 0.5 ml of S9-mix or sterile buffer are mixed with 2.0 ml of overlay agar. Tubes should be aerated during pre-incubation by using a shaker.

For an adequate estimate of variation, triplicate plating should be used at each dose level. The use of duplicate plating is acceptable when scientifically justified. The occasional loss of a plate does not necessarily invalidate the assay.

Gaseous or volatile substances should be tested by appropriate methods, such as in sealed vessels (12)(14)(15)(16).

1.5.4. Incubation

All plates in a given assay should be incubated at 37°C for 48-72 hours. After the incubation period, the number of revertant colonies per plate is counted.

2. DATA

2.1. TREATMENT OF RESULTS

Data should be presented as the number of revertant colonies per plate. The number of revertant colonies on both negative (solvent control, and untreated control if used) and positive control plates should also be given. Individual plate counts, the mean number of revertant colonies per plate and the standard deviation should be presented for the test substance and positive and negative (untreated and/or solvent) controls.

There is no requirement for verification of a clear positive response. Equivocal results should be clarified by further testing preferably using a modification of experimental conditions. Negative results need to be confirmed on a case-by-case basis. In those cases where confirmation of negative results is not considered necessary, justification should be provided. Modification of study parameters to extend the range of conditions assessed should be considered in follow-up experiments. Study parameters that might be modified include the concentration spacing, the method of treatment (plate-incorporation or liquid pre-incubation), and metabolic activation conditions.

2.2. EVALUATION AND INTERPRETATION OF RESULTS

There are several criteria for determining a positive result, such as a concentration-related increase over the range tested and/or a reproducible increase at one or more concentrations in the number of revertant colonies per plate in at least one strain with or without metabolic activation system (23). Biological relevance of the results should be considered first. Statistical methods may be used as an aid in evaluating the test results (24). However, statistical significance should not be the only determining factor for a positive response.

A test substance for which the results do not meet the above criteria is considered non-mutagenic in this test.

Although most experiments will give clearly positive or negative results, in rare cases the data set will preclude making a definite judgement about the activity of the test substance. Results may remain equivocal or questionable regardless of the number of times the experiment is repeated.

Positive results from the bacterial reverse mutation test indicate that the substance induces point mutations by base substitutions or frameshifts in the genome of either *Salmonella typhimurium* and/or *Escherichia coli.* Negative results indicate that under the test conditions, the test substance is not mutagenic in the tested species.

3. REPORTING

TEST REPORT

The test report must include the following information :

Solvent/Vehicle:

– justification for choice of solvent/vehicle;

– solubility and stability of the test substance in solvent/vehicle, if known.

– Strains :

– strains used;

– number of cells per culture;

– strain characteristics.

Test conditions :

– amount of test substance per plate (mg/plate or µl/plate) with rationale for selection of dose and number of plates per concentration;

– media used;

– type and composition of metabolic activation system, including acceptability criteria;

– treatment procedures.

Results :

– signs of toxicity;

– signs of precipitation;

– individual plate counts;

– the mean number of revertant colonies per plate and standard deviation;

– dose-response relationship, where possible;

– statistical analyses, if any;

– concurrent negative (solvent/vehicle) and positive control data, with ranges, means and standard deviations;

– historical negative (solvent/vehicle) and positive control data with ranges, means and standard deviations.

Discussion of results.

Conclusions.

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B. 15 MUTAGENICITY TESTING AND SCREENING FOR CARCINOGENICITY GENE MUTATION - *SACCHAROMYCES CEREVISIAE*

1. METHOD

1.1. INTRODUCTION

See General Introduction Part B.

1.2. DEFINITION

See General Introduction Part B.

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLE OF THE TEST METHOD

A variety of haploid and diploid strains of the yeast *Saccharomyces cerevisiae* can be used to measure the production of gene mutations induced by chemical agents with and without metabolic activation.

Forward mutation systems in haploid strains, such as the measurement of mutation from red, adenine-requiring mutants (*ade*-1 , *ade*-2) to double adenine-requiring white mutants and selective systems such as the induction of resistance to canavnaine and cycloheximide, have been utilized.

The most extensively validated reverse mutation system involves the use of the haploid strain XV 185-14C which carries the ochre nonsense mutations *ade* 2-1, *arg* 4-17, *lys* 1-1 and *trp* 5-48, which are reversible by base substitution mutagens that induce site specific mutations or ochre suppressor mutations. XV 185-14C also carries the *his* 1-7 marker, a missense mutation reverted mainly by second site mutations, and the marker *hom* 3-10 which is reverted by frameshift mutagens.

In diploid strains of *S. cerevisiae* the only extensively used strain is D_7 which is homozygous for *ilv* 1-92.

1.5. QUALITY CRITERIA

None.

1.6. DESCRIPTION OF THE TEST METHOD

Preparations

Solutions of test chemicals and control should be prepared just prior to testing, using an appropriate vehicle. In the case of organic compounds which are not water soluble, not more than a 2% solution v/v of organic solvents such as ethanol, acetone or dimethylsulphoxide (DMSO) should be used. The final concentration of the vehicle should not significantly affect cell viability and growth characertistics.

Metabolic activation

Cells should be exposed to test chemicals both in the presence and absence of an appropriate exogenous metabolic activation system.

The most commonly used system is a co-factor supplemented post-mitochondrial fraction from the livers of rodents pre-treated with enzyme inducing agents. The use of other species, tissues, post-mitochondrial fractions, or procedures may also be appropriate for metabolic activation.

Test conditions

Tester strains

The haploid strain XV 185-14C and the diploid strain D_7 are the most used in gene mutation studies. Other strains may also be appropriate.

Media

Appropriate culture media are used for the determination of survival and mutant numbers.

Use of negative and positive controls

Positive, untreated and solvent controls should be performed concurrently. Appropriate positive control chemicals should be used for each specific mutational endpoint.

Exposure concentration

At least five adequately spaced concentrations of the test substance should be used. For toxic substances, the highest concentration tested should not reduce survival below 5 to 10% .Relatively water-insoluble substances should be tested up to their limit of solubility , using appropriate procedures. For freely water-soluble non-toxic substances, the upper concentration should be determined on a case by case basis.

Incubation conditions

The plates are incubated four to seven days at 28 to 30 °C in the dark.

Spontaneous mutation frequencies

Sub-cultures should be used with spontaneous mutation frequencies within the accepted normal range.

Number of replicates

At least three replicate plates should be used per concentration for the assay of prototrophs produced by gene mutation and for cell viability. In the case of experiments using markers

such as *hom* 3-10 with a low mutation rate, the number of plates used must be increased to provide statistically relevant data.

Procedure

Treatment of *S. cerevisiae* strains is usually performed in a liquid test procedure involving either stationary or growing cells. Initial experiments should be carried out on growing cells: 1-5 x 10^7 cells/ml are exposed to the test chemical for up to 18 hours at 28 to 37 °C with shaking; an adequate amount of metabolic activation system is added during treatment when appropriate. At the end of the treatment, cells are centrifuged, washed and seeded upon an appropriate culture medium. After incubation, plates are scored for survival and the induction of gene mutation.

If the first experiment is negative, then a second experiment should be carried out using stationary phase cells. If the first experiment is positive it is confirmed in an appropriate independent experiment.

2. DATA

Data should be presented in tabular form indicating the number of colonies counted, number of mutants, survival and mutant frequency. All results should be confirmed in an independent experiment. The dara should be evaluated using appropriate statistical methods.

3. REPORTING

3.1. TEST REPORT

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

– strain used,

– test conditions: stationary phase or growing cells, compositions of media, incubation temperature and duration, metabolic activation system,

– treatment conditions: exposure levels, procedure and duration of treatment, treatment temperature, positive and negative controls,

– number of colonies counted, number of mutants, survival and mutant frequency, dose/response relationship if applicable, statistical evaluation of data,

– discussion of results,

– interpretation of results.

3.2. EVALUATION AND INTERPRETATION

See General Introduction Part B.

4. REFERENCES

B. 16 MITOTIC RECOMBINATION - SACCHAROMYCES CEREVISIAE

1. METHOD

1.1. INTRODUCTION

See General Introduction Part B.

1.2. DEFINITION

See General Introduction Part B.

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLE OF THE TEST METHOD

Mitotic recombination in *Saccharomyces cerevisiae* can be detected between genes (or more generally between a gene and its centromere) and within genes. The former event is called mitotic crossing-over and generates reciprocal products whereas the latter event is most frequently non-reciprocal and is called gene conversion. Crossing-over is generally assayed by the production of recessive homozygous colonies or sectors produced in a heterozygous strain, whereas gene conversion is assayed by the production of prototrophic revertants produced in an auxotrophic heteroallelic strain carrying two different defective alleles of the same gene. The most commonly used strains for the detection of mitotic gene conversion are D_4 (heteroallelic at *ade* 2 and *trp* 5) D_7 (heteroallelic at *trp* 5) BZ_{34} (heteroallelic at *arg* 4) and JDl (heteroallelic at *his* 4 and *trp* 5). Mitotic crossing-over producing red and pink homozygous sectors can be assayed in D_5 or in D_7 (which also measures mitotic gene conversion and reverse mutation at *ilv* 1-92) both strains being heteroallelic for complementing alleles of *ade* 2.

1.5. QUALITY CRITERIA

None.

1.6. DESCRIPTION OF THE TEST METHOD

Preparations

Solutions of test chemicals and control or reference compounds should be prepared just prior to testing, using an appropriate vehicle. With organic compounds that are water insoluble not more than a 2% solution v/v of organic solvents such as ethanol, acetone or

dimethylsulphoxide (DMSO) should be used. The final concentration of the vehicle should not significantly affect cell viability and growth characteristics.

Metabolic activation

Cells should be exposed to test chemicals both in the presence and absence of an appropriate exogenous metabolic activation system. The system most commonly used is a co-factor supplemented post-mitochondrial fraction from the livers of rodents pre-treated with enzyme inducing agents. The use of other species, tissues, post-mitochondrial fractions, or procedures may also be appropriate for metabolic activation.

Test conditions

Tester strains

The most frequently used strains are the diploids D_4, D_5, D_7 and JD1. The use of other strains may be appropriate.

Media

Appropriate culture media are used for the determination of survival and the frequency of mitotic recombination.

Use of negative and positive controls

Positive, untreated and solvent controls should be performed concurrently. Appropriate positive control chemicals should be used for each specific recombination endpoint.

Exposure concentrations

At least five adequately spaced concentrations of the test substance should be used. Among the factors to be taken into consideration are cytotoxicity and solubility. The lowest concentration must have no effect on celf viability. For toxic chemicals, the highest concentration tested should not reduce survival below 5 to 10% .Relatively water-insoluble chemicals should be tested up to the limit of solubility using appropriate procedures. For freely water-soluble non-toxic substances the upper concentration should be determined on a case by case basis.

Cells may be exposed to test chemicals in either the stationary phase or during growth for periods of up to 18 hours. However, for long treatment times cultures should be microscopically inspected for spore formation, the presence of which invalidates the test.

Incubation conditions

The plates are incubated in the dark for four to seven days at 28 to 30 °C. Plates used for the assay of red and pink homozygous sectors produced by mitotic crossing-over should be kept in a refrigerator (about 4 °C) for a further one to two days before scoring to allow for the development of the appropriate pigmented colonies.

Spontaneous mitotic recombination frequencies

Sub-cultures should be used with spontaneous mitotic recombination mutation frequencies within the accepted normal range.

Number of replicates

A minimum of three replicate plates should be used per concentration for the assay of prototrophs produced by mitotic gene conversion and for viability. In the case of the assay of recessive homozygosis produced by mitotic crossing-over, the plate number should be increased to provide an adequate number of colonies.

Procedures

Treatment of *S. cerevisiae* strains is usually performed in a liquid test procedure involving either stationary or growing cells. Initial experiments should be done: on growing cells. 1-5 x 10^7 celles/ml are exposed to the test chemical for up to 18 hours at 28 to 37 °C with shaking; an adequate amount of metabolic activation system is added during treatment when appropriate.

At the end of the treatment, cells are centrifuged, washed and seeded upon appropriate culutre medium. After incubation plates are scored for survival and the induction of mitotic recombination.

If the first experiment is negative, then a second experiment should be carried out using stationary phase cells. If the first experiment is positive it is confirmed in an independent experiment.

2. DATA

Data should be presented in tabular form indicating the number of colonies counted, the number of recombinants, survival and the frequency of recombinants.

Results should be confirmed in an independent experiment.

The data should be evaluated using appropriate statistical methods.

3. REPORTING

3.1. TEST REPORT

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

– strain used,

– test conditions: stationary phase or growing cells, composition of media, incubation temperature and duration, metabolic activation system,

– treatment conditions: exposure concentration, procedure and duration of treatment, treatment temperature, positive and negative controls,

– number of colonies counted, number of recombinants; survival and recombination frequency, dose/response relationship if applicable, statistical evaluation of data,

– discussion of the results,

– interpretation of the results.

3.2. EVALUATION AND INTERPRETATION

See General Introduction Part B.

4. REFERENCES

See General Introduction Part B.

B. 17. MUTAGENICITY – IN VITRO MAMMALIAN CELL GENE MUTATION TEST

1. METHOD

This method is a replicate of the OECD TG 476, *In Vitro* Mammalian Cell Gene Mutation Test (1997).

1.1 INTRODUCTION

The *in vitro* mammalian cell gene mutation test can be used to detect gene mutations induced by chemical substances. Suitable cell lines include L5178Y mouse lymphoma cells, the CHO, CHO-AS52 and V79 lines of Chinese hamster cells, and TK6 human lymphoblastoid cells (1). In these cell lines the most commonly-used genetic endpoints measure mutation at thymidine kinase (TK) and hypoxanthine-guanine phosphoribosyl transferase (HPRT), and a transgene of xanthine-guanine phosphoribosyl transferase (XPRT). The TK, HPRT and XPRT mutation tests detect different spectra of genetic events. The autosomal location of TK and XPRT may allow the detection of genetic events (e.g. large deletions) not detected at the HPRT locus on X-chromosomes (2)(3)(4)(5)(6).

In the *in vitro* mammalian cell gene mutation test, cultures of established cell lines or cell strains can be used. The cells used are selected on the basis of growth ability in culture and stability of the spontaneous mutation frequency.

Tests conducted *in vitro* generally require the use of an exogenous source of metabolic activation. This metabolic activation system cannot mimic entirely the mammalian *in vivo* conditions. Care should be taken to avoid conditions which would lead to results not reflecting intrinsic mutagenicity. Positive results which do not reflect intrinsic mutagenicity may arise from changes in pH, osmolality or high levels of cytotoxicity (7).

This test is used to screen for possible mammalian mutagens and carcinogens. Many compounds that are positive in this test are mammalian carcinogens; however, there is not a perfect correlation between this test and carcinogenicity. Correlation is dependent on chemical class and there is increasing evidence that there are carcinogens that are not detected by this test because they appear to act through other, non genotoxic mechanisms or mechanisms absent in bacterial cells (6).

See also General Introduction Part B.

1.2 DEFINITIONS

Forward mutation: a gene mutation from the parental type to the mutant form which gives rise to an alteration or a loss of the enzymatic activity of the function of the encoded protein.

Base pair substitution mutagens: substances which cause substitution of one or several base pairs in the DNA.

Frameshift mutagens: Substances which cause the addition or deletion of single or multiple base pairs in the DNA molecule.

Phenotypic expression time: a period during which unaltered gene products are depleted from newly mutated cells.

Mutant frequency: the number of mutant cells observed divided by the number of viable cells.

Relative total growth: increase in cell number over time compared to a control population of cells; calculated as the product of suspension growth relative to the negative control times cloning efficiency relative to negative control.

Relative suspension growth: increase in cell number over the expression period relative to the negative control.

Viability: the cloning efficiency of the treated cells at the time of plating in selective conditions after the expression period.

Survival: the cloning efficiency of the treated cells when plated at the end of the treatment period; survival is usually expressed in relation to the survival of the control cell population.

1.3 PRINCIPLE OF THE TEST METHOD

Cells deficient in thymidine kinase (TK) due to the mutation $TK^{+/-}$ -> $TK^{-/-}$ are resistant to the cytotoxic effects of the pyrimidine analogue trifluorothymidine (TFT). Thymidine kinase proficient cells are sensitive to TFT, which causes the inhibition of cellular metabolism and halts further cell division. Thus mutant cells are able to proliferate in the presence of TFT, whereas normal cells, which contain thymidine kinase, are not. Similarly, cells deficient in HPRT or XPRT are selected by resistance to 6-thioguanine (TG) or 8-azaguanine (AG). The properties of the test substance should be considered carefully if a base analogue or a compound related to the selective agent is tested in any of the mammalian cell gene mutation tests. For example, any suspected selective toxicity by the test substance for mutant and non-mutant cells should be investigated. Thus, performance of the selection system/agent must be confirmed when testing chemicals structurally related to the selective agent (8).

Cells in suspension or monolayer culture are exposed to the test substance, both with and without metabolic activation, for a suitable period of time and subcultured to determine cytotoxicity and to allow phenotypic expression prior to mutant selection (9)(10)(11)(12)(13). Cytotoxicity is usually determined by measuring the relative cloning efficiency (survival) or relative total growth of the cultures after the treatment period. The treated cultures are maintained in growth medium for a sufficient period of time, characteristic of each selected locus and cell type, to allow near-optimal phenotypic expression of induced mutations. Mutant frequency is determined by seeding known numbers of cells in medium containing the selective agent to detect mutant cells and in medium without selective agent to determine the cloning efficiency (viability). After a suitable incubation time, colonies are counted. The mutant frequency is derived from the number of mutant colonies in selective medium and the number of colonies in non-selective medium.

1.4 DESCRIPTION OF THE TEST METHOD

1.4.1 Preparations

1.4.1.1 Cells

A variety of cell types are available for use in this test including subclones of L5178Y, CHO, CHO-AS52, V79 or TK6 cells. Cell types used in this test should have a demonstrated sensitivity to chemical mutagens, a high cloning efficiency and a stable spontaneous mutant frequency. Cells should be checked for mycoplasma contamination and should not be used if contaminated.

The test should be designed to have a predetermined sensitivity and power. The number of cells, cultures and concentrations of test substance used should reflect these defined parameters (14). The minimal number of viable cells surviving treatment and used at each stage in the test should be based on the spontaneous mutation frequency. A general guide is to use a cell number which is at least ten times the inverse of the spontaneous mutation frequency. However, it is recommended to utilise at least 10^6 cells. Adequate historical data on the cell system used should be available to indicate consistent performance of the test.

1.4.1.2 Media and culture conditions

Appropriate culture media, and incubation conditions (culture vessels, temperature, CO_2 concentration, and humidity) should be used. Media should be chosen according to the selective systems and cell type used in the test. It is particularly important that culture conditions should be chosen that ensure optimal growth of cells during the expression period and colony forming ability of both mutant and non-mutant cells.

1.4.1.3 Preparation of cultures

Cell are propagated from stock cultures, seeded in culture medium and incubated at 37°C. Prior to use in this test, cultures may need to be cleansed of pre-existing mutant cells.

1.4.1.4 Metabolic activation

Cells should be exposed to the test substance both in the presence and absence of an appropriate metabolic activation system. The most commonly used system is a cofactor-supplemented post-mitochondrial fraction (S9) prepared from the livers of rodents treated with enzyme-inducing agents such as Aroclor 1254 (15)(16)(17)(18) or a combination of phenobarbitone and ß–naphthoflavone (19)(20).

The post-mitochondrial fraction is usually used at concentrations in the range from 1-10% v/v in the final test medium. The choice and condition of a metabolic activation system may depend upon the class of chemical being tested. In some cases it may be appropriate to utilise more than one concentration of post-mitochondrial fraction.

A number of developments, including the construction of genetically engineered cell lines expressing specific activating enzymes, may provide the potential for endogenous activation. The choice of the cell lines used should be scientifically justified (e.g. by the relevance of the cytochrome P450 isoenzyme for the metabolism of the test substance).

1.4.1.5 Test substance/Preparation

Solid test substances should be dissolved or suspended in appropriate solvents or vehicles and diluted if appropriate prior to treatment of the cells. Liquid test substances may be added directly to the test systems and/or diluted prior to treatment. Fresh preparations of the test substance should be employed unless stability data demonstrate the acceptability of storage.

1.4.2 Test conditions

1.4.2.1 Solvent/vehicle

The solvent/vehicle should not be suspected of chemical reaction with the test substance and should be compatible with the survival of the cells and the S9 activity. If other than well-known solvent/vehicles are used, their inclusion should be supported by data indicating their compatibility. It is recommended that wherever possible, the use of an aqueous solvent/vehicle be considered first. When testing water-unstable substances, the organic solvents used should be free of water. Water can be removed by adding a molecular sieve.

1.4.2.2 Exposure concentrations

Among the criteria to be considered when determining the highest concentration are cytotoxicity, solubility in the test system and changes in pH or osmolality.

Cytotoxicity should be determined with and without metabolic activation in the main experiment using an appropriate indication of cell integrity and growth, such as relative cloning efficiency (survival) or relative total growth. It may be useful to determine cytotoxicity and solubility in a preliminary experiment.

At least four analysable concentrations should be used. Where there is cytotoxicity, these concentrations should cover a range from the maximum to little or no toxicity; this will usually mean that the concentration levels should be separated by no more than a factor between 2 and √10. If the maximum concentration is based on cytotoxicity then it should result in approximately 10-20% (but not less than 10%) relative survival (relative cloning efficiency) or relative total growth. For relatively non-cytotoxic substances, the maximum test concentration should be 5 mg/ml 5 µl/ml, or 0.01 M, whichever is the lowest.

Relatively insoluble substances should be tested up to or beyond their limit of solubility under culture conditions. Evidence of insolubility should be determined in the final treatment medium to which cells are exposed. It may be useful to assess solubility at the beginning and the end of the treatment, as solubility can change during the course of exposure in the test system due to presence of cells, S9, serum etc. Insolubility can be detected by using the unaided eye. The precipitate should not interfere with the scoring.

1.4.2.3 Controls

Concurrent positive and negative (solvent or vehicle) controls, both with and without metabolic activation should be included in each experiment. When metabolic activation is used, the positive control chemical should be the one that requires activation to give a mutagenic response.

Examples of positive control substances include:

Metabolic Activation condition	Locus	Substance	CAS No.	EINECS No.
Absence of Exogenous Metabolic Activation	HPRT	Ethyl methanesulphonate	62-50-0	200-536-7
		Ethyl nitrosourea	759-73-9	212-072-2
	TK (small and large colonies)	Methyl methanesulphonate	66-27-3	200-625-0
	XPRT	Ethyl methanesulphonate	62-50-0	200-536-7
		Ethyl nitrosourea	759-73-9	212-072-2
Presence of Exogenous Metabolic Activation	HPRT	3-Methylcholanthrene	56-49-5	200-276-4
		N-Nitrosodimethylamine	62-75-9	200-549-8
		7,12-Dimethylbenzanthracene	57-97-6	200-359-5
	TK (small and large colonies)	Cyclophosphamide	50-18-0	200-015-4
		Cyclophosphamide monohydrate	6055-19-2	
		Benzo[*a*]pyrene	50-32-8	200-028-5
		3-Methylcholanthrene	56-49-5	200-276-5
	XPRT	N-Nitrosodimethylamine (for high levels of S-9)	62-75-9	200-549-8
		Benzo[*a*]pyrene	50-32-8	200-028-5

Other appropriate positive control reference substances may be used, e.g., if a laboratory has a historical data base on 5-Bromo 2'-deoxyuridine [CAS n. 59-14-3, EINECS n. 200-415-9], this reference substance could be used as well. The use of chemical class-related positive control chemicals should be considered, when available.

Negative controls, consisting of solvent or vehicle alone in the treatment medium, and treated in the same way as the treatment groups, should be included. In addition, untreated controls should also be used unless there are historical control data demonstrating that no deleterious or mutagenic effects are induced by the chosen solvent.

1.4.3 Procedure

1.4.3.1 Treatment with the test substance

Proliferating cells should be exposed to the test substance both with and without metabolic activation. Exposure should be for a suitable period of time (usually three to six hours is effective). Exposure time may be extended over one or more cell cycles.

Either duplicate or single treated cultures may be used at each concentration tested. When single cultures are used, the number of concentrations should be increased to ensure an adequate number of cultures for analysis (e.g. at least 8 analysable concentrations). Duplicate negative (solvent) control cultures should be used.

Gaseous or volatile substances should be tested by appropriate methods, such as in sealed culture vessels (21)(22).

1.4.3.2 Measurement of survival, viability and mutant frequency

At the end of the exposure period, cells are washed and cultured to determine survival and to allow for expression of the mutant phenotype. Measurement of cytotoxicity by determining the relative cloning efficiency (survival) or relative total growth of the cultures is usually initiated after the treatment period.

Each locus has a defined minimum time requirement to allow near optimal phenotypic expression of newly induced mutants (HPRT and XPRT require at least 6-8 days, and TK at least 2 days). Cells are grown in medium with and without selective agent(s) for determination of numbers of mutants and cloning efficiency, respectively. The measurement of viability (used to calculate mutant frequency) is initiated at the end of the expression time by plating in non-selective medium.

If the test substance is positive in the L5178Y $TK^{+/-}$ test, colony sizing should be performed on at least one of the test cultures (the highest positive concentration) and on the negative and positive controls. If the test substance is negative in the L5178Y $TK^{+/-}$ test, colony sizing should be performed on the negative and positive controls. In studies using $TK6TK^{+/-}$, colony sizing may also be performed.

2. DATA

2.1 TREATMENT OF RESULTS

Data should include cytotoxicity and viability determination, colony counts and mutant frequencies for the treated and control cultures. In the case of a positive response in the L5178Y $TK^{+/-}$ test, colonies are scored using the criteria of small and large colonies on at least one concentration of the test substance (highest positive concentration) and on the negative and positive control. The molecular and cytogenetic nature of both large and small colony mutants has been explored in detail (23)(24). In the $TK^{+/-}$ test, colonies are scored using the criteria of normal growth (large) and slow growth (small) colonies (25). Mutant cells that have suffered the most extensive genetic damage have prolonged doubling times and thus form small colonies. This damage typically ranges in scale from the losses of the entire gene to karyotypically visible chromosome aberrations. The induction of small colony mutants has been associated with chemicals that induce gross chromosome aberrations (26). Less seriously affected mutant cells grow at rates similar to the parental cells and form large colonies.

Survival (relative cloning efficiencies) or relative total growth should be given. Mutant frequency should be expressed as number of mutant cells per number of surviving cells.

Individual culture data should be provided. Additionally, all data should be summarised in tabular form.

There is no requirement for verification of a clear positive response. Equivocal results should be clarified by further testing preferably using modification of experimental conditions. Negative results need to be confirmed on a case-by-case basis. In those cases where confirmation of negative results is not considered necessary, justification should be provided. Modification of study parameters to extend the range of conditions assessed should be considered in follow-up experiments for either equivocal or negative results. Study parameters that might be modified include the concentration spacing and the metabolic activation conditions.

2.2 EVALUATION AND INTERPRETATION OF RESULTS

There are several criteria for determining a positive result, such as a concentration-related increase or a reproducible increase in mutant frequency. Biological relevance of the results should be considered first. Statistical methods may be used as an aid in evaluating the test results. Statistical significance should not be the only determining factor for a positive response.

A test substance for which the results do not meet the above criteria is considered non-mutagenic in this system.

Although most studies will give clearly positive or negative results, in rare cases the data set will preclude making a definite judgement about the activity of the test substance. Results may remain equivocal or questionable regardless of the number of times the experiment is repeated.

Positive results from the *in vitro* mammalian cell gene mutation test indicate that the test substance induces gene mutations in the cultured mammalian cells used. A positive concentration-response that is reproducible is most meaningful. Negative results indicate that,

under the test conditions, the test substance does not induce gene mutations in the cultured mammalian cells used.

3. REPORTING

TEST REPORT

The test report must include the following information :

Solvent/Vehicle:

– justification for choice of vehicle/solvent;

– solubility and stability of the test substance in solvent/vehicle, if known;

Cells:

– type and source of cells;

– number of cell cultures;

– number of cell passages, if applicable;

– methods for maintenance of cell culture, if applicable;

– absence of mycoplasma.

Test conditions :

– rationale for selection of concentrations and number of cultures including, e.g. cytotoxicity data and solubility limitations, if available;

– composition of media, CO2 concentration;

– concentration of test substance;

– volume of vehicle and test substance added;

– incubation temperature;

– incubation time;

– duration of treatment;

– cell density during treatment;

– type and composition of metabolic activation system, including acceptability criteria;

– positive and negative controls;

– length of expression period (including number of cells seeded, and subcultures and feeding schedules, if appropriate);

– selective agents;

– criteria for considering tests as positive, negative or equivocal;

– methods used to enumerate numbers of viable and mutant cells.

– definition of colonies of which size and type are considered (including criteria for "small" and "large" colonies, as appropriate).

Results :

– signs of toxicity;

– signs of precipitation;

– data on pH and osmolality during the exposure to the test substance, if determined;

– colony size if scored for at least negative and positive controls;

– laboratory's adequacy to detect small colony mutants with the L5178Y TK+/- system, where appropriate;

– dose-response relationship, where possible;

– statistical analyses, if any;

– concurrent negative (solvent/vehicle) and positive control data;

– historical negative (solvent/vehicle) and positive control data with ranges, means and standard deviations;

– mutant frequency.

Discussion of results.

Conclusions.

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B. 18. DNA DAMAGE AND REPAIR - UNSCHEDULED DNA SYNTHESIS - MAMMALIAN CELLS *IN VITRO*

1. METHOD

1.1. INTRODUCTION

See General Introduction Part B.

1.2. DEFINITION

See General Introduction Part B.

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLE OF THE TEST METHOD

The Unscheduled DNA Synthesis (UDS) test measures the DNA repair synthesis after excision and removal of a stretch of DNA containing the region of damage induced by chemical and physical agents. The test is based on the incorporation of tritium labelled thymidine (^{3}H-TdR) into the DNA of mammalian cells which are not in the S phase of the cell cycle. The uptake of ^{3}H-TdR may be determined by autoradiography or by liquid scintillation counting (LSC) of DNA from the treated cells. Mammalian cells in culture, unless primary rat hepatocytes are used, are treated with the test agent with and without an exogenous metabolic activation system. UDS may also be measured in *in vivo* systems.

1.5. QUALITY CRITERIA

None.

1.6. DESCRIPTION OF THE TEST METHOD

Preparations

Test chemicals and control or reference substances should be prepared in growth medium or dissolved or suspended in appropriate vehicles and then further diluted in growth medium for use in the assay. The final concentration of the vehicle should not affect cell viability.

Primary cultures of rat hepatocytes, human lymphocytes or established cell lines (e.g. human diploid fibroblasts) may be used in the assay.

Cells should be exposed to the test chemical both in the presence and absence of an appropriate metabolic activation system.

Test conditions

Number of cultures

At least two cell cultures for autoradiography and six cultures (or less if scientifically justified) for LSC UDS determinations are necessary for each experimental point.

Use of negative and positive controls

Concurrent positive and negative (untreated and/or vehicle) controls with and without metabolic activation should be included in each experiment.

Examples of positive controls for the rat hepatocyte assay include 7,12-dimethylbenzathracene (7,12- DMBA) or 2-acetylaminofluorene (2-AAF). In the case of established cell lines 4-nitroquinoline-N-oxide (4-NQO) is an example of a positive control for both the autoradiographic and LSC assays performed without metabolic activation; N-dimethylnitrosamine is an example of a positive control compound when metabolic activation systems are used.

Exposure concentrations

Multiple concentrations of the test substance over a range adequate to define the response should be used. The highest concentration should elicit some cytotoxic effects. Relatively water-insoluble compounds should be tested up to the limit of solubility. For freely water-soluble non-toxic chemicals, the upper test chemical concentration should be determined on a case-by-case basis.

Cells

Appropriate growth media, CO_2 concentration, temperature and humidity should be used in maintaining cultures. Established cell lines should be periodically checked for *Mycoplasma* contamination.

Metabolic activation

A metabolic activation system is not used with primary hepatocyte cultures. Established cell lines and lymphocytes are exposed to test substance both in the presence and absence of an appropriate metabolic activation system.

Procedure

Preparation of cultures

Established cell lines are generated from stock cultures (e.g. by trypsinization or by shaking off), seeded in culture vessels at appropriate density, and incubated at 37 °C.

Short-term cultures of rat hepatocytes are established by allowing freshly dissociated hepatocytes in an appropriate medium to attach themselves to the growing surface.

Human lymphocyte cultures are set up using appropriate techniques.

Treatment of the cultures with the test substance

Primary rat hepatocytes

Freshly isolated rat hepatocytes are treated with the test substance in a medium containing ^{3}H-TdR for an appropriate length of time. At the end of the treatment period, medium should be drained off the cells, which are then rinsed fixed and dried. Slides should be dipped in autoradiographic emulsion (alternative stripping film may be used), exposed, developed, stained and counted.

Established cell lines and lymphocytes

Autoradiographic techniques: Cell cultures are exposed to the test substance for appropriate durations followed by treatment with ^{3}H-TdR. The times will be governed by the nature of the substance, the activity of metabolising systems and the type of cells. To detect the peak of UDS, ^{3}H-TdR should be added either simultaneously with the test subsrance or within a few minutes after exposure to the test substance. The choice between these two procedures will be influenced by possible interactions between test substance and ^{3}H-TdR. In order to discriminate between UDS and semi-conservative DNA replication, the latter can be inhibited, for example, by the use of an arginine-deficient medium, low serum content or by hydroxyurea in the culture medium.

LSC measurements of UDS: Prior to treatment with test substance, entry of cells into S-phase should be blocked as described above; cells should then be exposed to test chemical as described for autoradiography. At the end of the incubation period, DNA should be extracted from the cells and the total DNA content, and the extent of ^{3}H-TdR, incorporation determined.

It should be noted that, where human lymphocytes are used in the above techniques, the suppression of semi-conservative DNA replication is unnecessary in unstimulated cultures.

Analysis

Autoradiographic determinations

In determining UDS in cells in culture, S-phase nuclei are not counted. At least 50 cells per concentration should be counted. Slides should be coded before counting. Several widely separated random fields should be counted on each slide. The amount of ^{3}H-TdR incorporation in the cytoplasm should be determined by counting three nucleus-sized areas in the cyptoplasm of each cell counted.

LSC determinations

An adequate number of cultures should be used at each concentration and in the controls in LSC UDS determinations.

All results should be confirmed in an independent experiment.

2. DATA

Data should be presented in tabular form.

2.1. AUTORADIOGRAPHIC DETERMINATIONS

The extent of ^{3}H-TdR incorporation in the cytoplasm and the number of grains found over the cell nucleus should be recorded separately.

Mean, median and mode may be used to describe the distribution of the extent of ^{3}H-TdR incorporation in the cytoplasm and the number of grains per nucleus.

2.2. LSC DETERMINATIONS

For LSC determinations, ^{3}H-TdR incorporation should be reported as dpm/μg DNA. The mean dpm/μg DNA with standard deviation may be used to describe the distribution of incorporation.

Data should be evaluated using appropriate statistical methods.

3. REPORTING

3.1. TEST REPORT

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

– cells used, density and passage number at time of treatment, number of cell cultures,

– methods used for maintenance of cell cultures including medium, temperature and CO_2 concentration,

– test substance, vehicle, concentrations and rationale for selection of concentrations used in the assay,

– details of metabolic activation systems,

– treatment schedule,

– positive and negative controls,

– autoradiographic technique used,

– procedures used to block entry of cells into S-phase,

– procedures used for DNA extraction and determination of total DNA content in LSC determination,

– dose/response relationship, where possible,

– statistical evaluation,

– discussion of results,

– interpretation of results.

3.2. VALUATION AND INTERPRETATION

See General Introduction Part B.

4. REFERENCES

See General Introduction Part B.

B. 19. SISTER CHROMATID EXCHANGE ASSAY *IN VITRO*

1. METHOD

1.1. INTRODUCTION

See General Introduction Part B.

1.2. DEFINITION

See General Introduction Part B.

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLE OF THE TEST METHOD

The Sister Chromatid Exchange (SCE) assay is a short-term test for the detection of reciprocal exchanges of DNA between two sister chromatids of a duplicating chromosome. SCEs represent the interchange of DNA replication products at apparently homologous loci. The exchange process presumably involves DNA breakage and reunion, although little is known about its molecular basis. Detection of SCEs requires some means of differentially labelling sister chromatids and this can be achieved by incorporation of bromodeoxyuridine (BrdU) into chromosomal DNA for two cell cycles.

Mammalian cells *in vitro* are exposed to the test chemical with and without a mammalian exogenous metabolic activation system, if appropriate, and cultured for two rounds of replication in BrdU-containing medium. After treatment with a spindle inhibitor (e.g. colchicine) to accumulate cells in a metaphase-like stage of mitosis (c-metaphase), cells are harvested and chromosome preparations are made.

1.5. QUALITY CRITERIA

None.

1.6. DESCRIPTION OF THE TEST METHOD

1.6.1. Preparations

– Primary cultures, (human lymphocytes) or established cell lines (e.g. Chinese hamster ovary cells) may be used in the assay. Cell lines should be checked for *Mycoplasma* contamination,

– Appropriate culture media and incubation conditions (e.g. temperature, culture vessels, CO_2 concentration and humidity) should be used,

– Test substances may be prepared in culture media or dissolved or suspended in appropriate vehicles prior to treatment of the cells. The final concentration of a vehicle in the culture system should not significantly affect, cell viability or growth rate and effects on SCE frequency should be monitored by a solvent control,

– Cells should be exposed to the test substance both in the presence and absence of an exogenous mammalian metabolic activation system. Alternatively, where cell types with intrinsic metabolic activity are used, the rate and nature of the activity should be appropriate to the chemical class being tested.

1.6.2. Test conditions

Number of cultures

At least duplicate cultures should be used for each experimental point.

Use of negative and positive controls

Positive controls, using both a direct acting compound and a compound requiring metabolic activation should be included in each experiment; a vehicle control should also be used.

The following are examples of substances which might be used as positive controls:

– direct acting compound:

– ethylmethanesulphonate,

– indirect acting compound:

– cyclophosphamide.

When appropriate, an additional positive control of the same chemical class as the chemical under test may be included.

Exposure concentrations

At least three adequately spaced concentrations of the test substance should be used. The highest concentration should give rise to a significant toxic effect but must still allow adequate cell replication to occur. Relatively water-insoluble substances should be tested up to the limit of solubility using appropriate procedures. For freely water-soluble non-toxic substances the upper test substance concentration should be determined on a case-by-case basis.

1.6.3. Procedure

Preparation of cultures

Established cell lines are generated from stock cultures (e.g. by trypsinization or by shaking off), seeded in culture vessels at appropriate density and incubated at 37 °C. For monolayer cultures, the number of cells per culture vessel should be adjusted so that the cultures are not much more than 50% confluent at the time of harvest. Alternatively, cells may be used in suspension culture. Human lymphocyte cultures are set up from heparinized blood, using appropriate techniques, and incubated at 37 °C.

Treatment

Cells in an exponential stage of growth are exposed to the test substance for a suitable period of time; in most cases one to two hours may be effective, but the treatment time may be extended up to two complete cell cycles in certain cases. Cells without sufficient instrinsic metabolic activity should be exposed to the test chemical in the presence and absence of an appropriate metabolic activation system. At the end of the exposure period, cells are washed free of test substance and cultured for two rounds of replication in the presence of BrdU. As an alternative procedure cells may be exposed simultaneously to the test chemical and BrdU for the complete culture time of two cell cycles.

Human lymphocyte cultures are treated while they are in a semisynchronous condition.

Cells are analysed in their second post-treatment division, ensuring that the most sensitive cell cycle stages have been exposed to the chemical. All cultures to which BrdU is added should be handled in darkness or in dim light from incandescent lamps up to the harvesting of cells in order to minimize photolysis of BrdU-containing DNA.

Harvesting of cells

Cell cultures are treated with a spindle inhibitor (e.g. colchicine) one to four hours prior to harvesting. Each culture is harvested and processed separately for the preparation of chromosomes.

Chromosome preparation and staining

Chromosome preparations are made by standard cytogenetic techniques. Staining of slides to show SCEs can be performed by several techniques, (e.g. the fluorescence plus Giemsa method).

Analysis

The number of cells analysed should be based on the spontaneous control frequency of SCE. Usually, at least 25 well-spread metaphases per culture are analysed for SCEs. Slides are coded before analysis. In human lymphocytes only metaphases containing 46 centromeres are analysed. In established cell lines only metaphases containing ± 2 centromeres of the modal number are analysed. It should be stated whether or not centromeric switch of label is, scored as an SCE. The results should be confirmed in an independent experiment.

2. DATA

Data should be presented in tabular form. The number of SCEs for each metaphase and the number of SCEs per chromosome for each metaphase should be listed separately for all treated and control cultures.

The data should be evaluated using appropriate statistical methods.

3. REPORTING

3.1. TEST REPORT

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

– cells used, methods of maintenance of cell culture,

– test conditions: composition of media, CO_2 concentration, concentration of test substance, vehicle used, incubation temperature, treatment time, spindle inhibitor used, its concentration and the duration of treatment with it, type of mammalian activation system used, positive and negative controls,

– number of cell cultures per experimental point,

– details of the technique used for slide preparation,

– number of metaphases analysed (data given separately for each culture),

– mean number of SCE per cell and per chromosome (data given separately for each culture),

– criteria for scoring SCE,

– rationale for dose selection,

– dose-response relationship, if applicable,

– statistical evaluation,

– discussion of results,

– interpretation of results.

3.2. EVALUATION AND INTERPRETATION

See General Introduction Part B.

4. REFERENCES

See General Introduction Part B.

B.20. SEX-LINKED RECESSIVE LETHAL TEST IN *DROSOPHILA MELANOGASTER*

1. METHOD

1.1. INTRODUCTION

See General Introduction Part B.

1.2. DEFINITION

See General Introduction Part B.

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLES OF THE TEST METHOD

The sex-linked recessive lethal (SLRL) test using *Drosophila melanogaster* detects the occurrence of mutations, both point mutations and small deletions, in the germ line of the insect. This test is a forward mutation assay capable of screening for mutations at about 800 loci on the X-chromosome; this represents about 80% of all X-chromosal loci. The X-chromosome represents approximately one-fifth of the entire haploid genome.

Mutations in the X-chromosome of *Drosophila melanogaster* are phenotypically expressed in males carrying the mutant gene. When the mutation is lethal in the hemizygous condition, its presence is inferred from the absence of one class of male offspring out of the two that are normally produced by a heterozygous female. The SLRL test takes advantage of these facts by means of specially marked and arranged chromosomes.

1.5. QUALITY CRITERIA

None.

1.6. DESCRIPTION OF THE TEST METHOD

Preparations

Stocks

Males of a well-defined wild-type stock and females of the Muller-5 stock may be used. Other appropriately marked female stocks with multiple inverted X-chromosomes may also be used.

Test substance

Test substances should be dissolved in water. Substances which are insoluble in water may be dissolved or suspended in appropriate vehicles (e.g. a mixture of ethanol and Tween-60 or 80), then diluted in water or saline prior to administration. Dimethylsulphoxide (DMSO) should be avoided as a vehicle.

Number of animals

The test should be designed with a predetermined sensitivity and power. The spontaneous mutant frequency observed in the appropriate control will influence strongly the number of treated chromosomes that must be analysed.

Route of administration

Exposure may be oral, by injection or by exposure to gases or vapours. Feeding of the test substance may be done in sugar solution. When necessary, substances may be dissolved in a 0,7% NaCl solution and injected into the thorax or abdomen.

Use of negative and positive controls

Negative (vehicle) and positive controls should be included. However, if appropriate laboratory historical control data are available, no concurrent controls are needed.

Exposure levels

Three exposure levels should be used. For a preliminary assessment one exposure level of the test substance may be used, that exposure level being either the maximum tolerated concentration or that producing some indication of toxicity. For non-toxic substances exposure to the maximum practicable concentration should be used.

Procedure

Wild-type males (three to five days old) are treated with the test substance and mated individually to an excess of virgin females from the Muller-5 stock or from another appropriately marked (with multiple inverted X-chromosomes) stock. The females are replaced with fresh virgins every two to three days to cover the entire germ cell cycle. The offspring of these females are scored for lethal effects corresponding to the effects on mature sperm, mid or late-stage spermatids, early spermatids, spermatocytes and spermatogonia at the time of treatment.

Heterozygous F_1 females from the above crosses are allowed to mate individually (i.e. one female per vial) with their brothers. In the F_2 generation, each culture is scored for the absence of wild-type males. If a culture appears to have arisen from an F_1 female carrying a lethal in the parental X-chromosome (i.e. no males with the treated chromosome are observed) daughters of that female with the same genotype should be tested to ascertain whether the lethality is repeated in the next generation.

2. DATA

Data should be tabulated to show the number of X-chromosomes tested, the number of non-fertile males and the number of lethal chromosomes at each exposure concentration and for each mating period for each male treated. Numbers of clusters of different sizes per male should be reported. These results should be confirmed in a separate experiment.

Appropriate statistical methods should be used in evaluation sex-linked recessive lethal tests. Clustering of recessive lethals originating from one male should be considered and evaluated in an appropriate statistical manner.

3. REPORTING

3.1. TEST REPORT

The test report shall, if possible, contain the following information;

– stock: Drosophila stocks or strains used, age of insects, number of males treated, number of sterile males, number of F2 cultures established, number of F2 cultures without progeny, number of chromosomes carrying a lethal detected at each germ cell stage,

– criteria for establishing the size of treated groups,

– test conditions. detailed description of treatment and sampling schedule, exposure levels, toxicity data, negative (solvent) and positive controls, if appropriate,

– criteria for scoring lethal mutations,

– exposure/effect relationship where possible,

– evaluation of data,

– discussion of results,

– interpretation of results.

3.2. EVALUATION AND INTERPRETATION

See General Introduction Part B.

4. REFERENCES

See General Introduction Part B.

B. 21. *IN VITRO* MAMMALIAN CELL TRANSFORMATION TESTS

1. METHOD

1.1. INTRODUCTION

See General Introduction Part B.

1.2. DEFINITION

See General Introduction Part B.

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLE OF THE TEST METHOD

Mammalian cell culture systems may be used to detect phenotypic changes in vitro induced by chemical substances associated with malignant transformation *in vivo*. Widely used cells include C3H10$T_{1/2}$, 3T3, SHE, Fischer rat and the tests rely on changes in cell morphology, focus formation or changes in anchorage dependence in semi-solid agar. Less widely used systems exist which detect other physiological or morphological changes in cells following exposure to carcinogenic chemicals. None of the *in vitro* test endpoints has an established mechanistic link with cancer. Some of the test systems are capable of detecting tumour promotors. Cytotoxicity may be determined by measuring the effect of the test material on colony-forming abilities (cloning efficiency) or growth rates of the cultures. The measurement of cytotoxicity is to establish that exposure to the test chemical has been toxicologically relevant but cannot be used to calculate transformation: frequency in all assays since some may involve prolonged incubation and/or replating.

1.5. QUALITY CRITERIA

None.

1.6. DESCRIPTION OF THE TEST METHOD

Preparations

Cells

A variety of cell lines or primary cells are available depending on the transformation test being used. The investigator must ensure that the cells in the test being performed exhibit the appropriate phenotypic change after exposure to known carcinogens and that the test, in the investigator's laboratory, is of proven and documented validity and reliability.

Medium

Media and experimental conditions should be used that are appropriate to the transformation assay in use.

Test substance

Test substances may be prepared in culture media or dissolved or suspended in appropriate vehicles prior to treatment of the cells. The final concentration of the vehicle in the culture system should not affect cell viability, growth rate or transformation incidence.

Metabolic activation

Cells should be exposed to the test substance both in the presence and absence of an appropriate metabolic activation system. Alternatively, when cell types are used that possess intrinsic metabolic activity, the nature of the activity should be known to be appropriate to the chemical class being tested.

Test conditions

Use of negative and positive controls

Positive controls, using both a direct-acting compound and a compound requiring metabolic activation should be included in each experiment; a negative (vehicle) control should also be used.

The following are examples of substances which might be used as positive controls:

– Direct-acting chemicals:

– Ethylmethanesulphonate,

– β-propiolactone,

– Compounds requiring metabolic activation:

– 2-acetylaminofluorene,

– 4-dimethylaminoazobenzene,

– 7,12-dimethylbenzanthracene.

When appropriate, an additional positive control of the same chemical class as the compound under test should be included.

Exposure concentrations

Several concentrations of the test substance should be used. These concentrations should yield a concentration-related toxic effect, the highest concentration producing a low level of survival and the survival in the lowest concentration being approximately the same as that in the negative control. Relatively water-insoluble substances should be tested up to the limit of solubility using appropriate procedures. For freely water-soluble non-toxic substances the upper test substance concentration should be determined on a case-by-case basis.

Procedure

Cells should be exposed for a suitable period of time depending on the test system in use, and this may involve re-dosing accompanied by a change of medium (and if necessary, fresh metabolic activation mixture) if exposure is prolonged. Cells without sufficient intrinsic metabolic activity should be exposed to the test substance in the presence and absence of an appropriate metabolic activation system. At the end of the exposure period, cells are washed free of test substance and cultured under conditions appropriate for the appearance of the transformed phenotype being monitored and the incidence of transformation determined. All results are confirmed in an independent experiment.

2. DATA

Data should be presented in tabular form and may take a variety of forms according to the assay being used e.g. plate counts, positive plates or numbers of transformed cells. Where appropriate, survival should be expressed as a percentage of control levels and transformation frequency expressed as the number of transformants per number of survivors. Data should be evaluated using appropriate statistical methods.

3. REPORTING

3.1. TEST REPORT

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

– cell type used, number of cell cultures, methods for maintenance of cell cultures,

– test conditions: concentration of test substance, vehicle used, incubation time, duration and frequency of treatment, cell density during treatment, type of exogenous metabolic activation system used, positive and negative controls, specification of phenotype being monitored, selective system used (if appropriate), rational for dose selection,

– method used to enumerate viable and transformed cells,

– statistical evaluation,

– discussion of results,

– interpretation of results.

3.2 EVALUATION AND INTERPRETATION

See General Introduction Part B.

4. REFERENCES

See General Introduction Part B.

B.22. RODENT DOMINANT LETHAL TEST

1. METHOD

1.1. INTRODUCTION

See General Introduction Part B.

1.2. DEFINITION

See General Introduction Part B.

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLE OF THE TEST METHOD

Dominant lethal effects cause embryonic or foetal death. Induction of dominant lethals by exposure to a chemical substance indicates that the substance has affected germinal tissue of the test species. It is generally accepted that dominant lethals are due to chromosomal damage (structural and numerical anomalies). Embryonic death if females are treated may also be the result of toxic effects.

Generally, male animals are exposed to the test compound and mated to untreated virgin females. The various germ cell stages can be tested separately by the use of sequential mating intervals. The increase of dead implants per female in the treated group over the dead implants per female in the control group reflects the post-implantational loss. Pre-implantational loss can be estimated based on corpora lutea counts or by comparing the total implants per female in treated and control groups. The total dominant lethal effect is the sum of pre- and post-implantational loss. The calculation of the total dominant lethal effect is based on comparison of the live implants per female in the test group to the live implants per female in the control group. A reduction in the number of implants at certain intervals may be the result of cell killing (i.e. of spermatocytes and/or spermatogonia).

1.5. QUALITY CRITERIA

None.

1.6. DESCRIPTION OF THE TEST METHOD

Preparations

When possible, test substances should be dissolved or suspended in isotonic saline. Chemicals insoluble in water may be dissolved or suspended in appropriate vehicles. The vehicle used

should neither interfere with the test chemical nor produce toxic effects. Fresh preparations of the test chemical should be employed.

Test conditions

Route of administration

The test compound should generally be administered only once. Based on toxicological information a repeated treatment schedule can be employed. The usual routes of administration are oral intubation or intraperitoneal injection. Other routes of administration may be appropriate.

Experimental animals

Rats or mice are recommended as the test species. Healthy fully sexually mature animals are randomized and assigned to treatment and control groups.

Number and sex

An adequate number of treated males should be used, taking into account the spontaneous variation of the biological character being evaluated. The number chosen should be based on the pre-determined sensitivity of detection and power of significance. For example in a typical test, the number of males in each dose group should be sufficient to provide between 30 and 50 pregnant females per mating interval.

Use of negative and positive controls

Generally concurrent positive and negative (vehicle) controls should be included in each experiment. When acceptable positive control results are available from experiments conducted recently in the same laboratory these results can be used instead of a concurrent positive control. Positive control substances should be used at an appropriate low dose (e.g. MMS, intraperitoneally, at 10 mg/kilogram) to demonstrate the test sensitivity.

Dose levels

Normally, three dose levels should be used. The high dose should produce signs of toxicity or reduced fertility in the treated animals. In certain cases a single high dose level may be sufficient.

Limit test

Non-toxic substances should be tested at 5 g/kilogram on a single administration or at 1 g/kilogram/day on repeated administration.

Procedure

Several treatment schedules are available. Single administration of the test substance is the most widely used. Other treatment schedules may be used.

Individual males are mated sequentially to one or two untreated virgin females at appropriate intervals after treatment. Females should be left with the males for at least the duration of one oestrous cycle or until mating has occurred as determined by the presence of sperm in the vagina or by the presence of a vaginal plug.

The number of matings following treatment is governed by the treatment schedule and should ensure that all germ cell stages are sampled after treatment.

Females are sacrificed in the second half of pregnancy and uterine contents are examined to determine the number of dead and live implants. The ovaries may be examined to determine the number of corpora lutea.

2. DATA

Data should be tabulated to show the number of males, the number of pregnant females, and the number of non-pregnant females. Results of each mating, including the identity of each male and female, should be reported individually. For each female, week of mating, dose level received by the males, the frequencies of live implants and of dead implants should be recorded.

The calculation of the total dominant lethal effect is based on comparison of the live implants per female in the test group to the live implants per female in the control group. The ratio of dead to live implants from the treated group compared to the same ratio from the control group is analysed to indicate the post-implantation loss.

If the data are recorded as early and late deaths, the tables should make that clear. If pre-implantation loss is estimated, it should be reported. Pre-implantation loss can be calculated as a discrepancy between the number of corpora lutea and the number of implants or as a reduction in the average number of implants per uterus in comparison with control matings.

Data are evaluated using appropriate statistical methods.

3. REPORTING

3.1. TEST REPORT

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

– species, strain, age and weights of animals used, number of animals of each sex in experimental and control groups,

– test substance, vehicle, dose levels tested and rationale for dose selection, negarive and positive controls, toxicity data,

– route and treatment schedule,

– mating schedule,

– method used to determine that mating has occurred,

– time of sacrifice,

– criteria for scoring dominant lethals,

– dose/response relationship, if applicable,

– statistical evaluation,

– discussion of results,

– interpretation of results.

3.2. EVALUATION AND INTERPRETATION

See General Introduction Part B.

4. REFERENCES

See General Introduction Part B.

B.23. MAMMALIAN SPERMATOGONIAL CHROMOSOME ABERRATION TEST

1. METHOD

This method is a replicate of the OECD TG 483, Mammalian Spermatogonial Chromosome Aberration Test (1997).

1.1 INTRODUCTION

The purpose of the *in vivo* mammalian spermatogonial chromosome aberration test is to identify those substances that cause structural chromosome aberrations in mammalian spermatogonial cells (1)(2)(3)(4)(5). Structural aberrations may be of two types, chromosome or chromatid. With the majority of chemical mutagens, induced aberrations are of the chromatid type, but chromosome-type aberrations also occur. This method is not designed to measure numerical aberrations and is not routinely used for that purpose. Chromosome mutations and related events are the cause of many human genetic diseases.

This test measures chromosome events in spermatogonial germ cells and is, therefore, expected to be predictive of induction of inheritable mutations in germ cells.

Rodents are routinely used in this test. This *in vivo* cytogenetic test detects chromosome aberrations in spermatogonial mitoses. Other target cells are not the subject of this method.

To detect chromatid-type aberrations in spermatogonial cells, the first mitotic cell division following treatment should be examined before these lesions are lost in subsequent cell divisions. Additional information from treated spermatogonial stem cells can be obtained by meiotic chromosome analysis for chromosome-type aberrations at diakinesis-metaphase I when the treated cells become spermatocytes.

This *in vivo* test is designed to investigate whether somatic cell mutagens are also active in germ cells. In addition, the spermatogonial test is relevant to assessing mutagenicity hazard in that it allows consideration of factors of *in vivo* metabolism, pharmacokinetics and DNA-repair processes.

A number of generations of spermatogonia are present in the testis with a spectrum of sensitivity to chemical treatment. Thus, the aberrations detected represent an aggregate response of treated spermatogonial cell populations, with the more numerous differentiated spermatogonial cells predominating. Depending on their position within the testis, different generations of spermatogonia may or may not be exposed to the general circulation, because of the physical and physiological Sertoli cell barrier and the blood-testis barrier.

If there is evidence that the test substance, or a reactive metabolite, will not reach the target tissue, it is not appropriate to use this test.

See also General Introduction Part B.

1.2 DEFINITIONS

Chromatid-type aberration: structural chromosome damage expressed as breakage of single chromatids or breakage and reunion between chromatids.

Chromosome-type aberration: structural chromosome damage expressed as breakage, or breakage and reunion, of both chromatids at an identical site.

Gap: an achromatic lesion smaller than the width of one chromatid, and with minimum misalignment of the chromatids.

Numerical aberration: a change in the number of chromosomes from the normal number characteristic of the animals utilised.

Polyploidy: a multiple of the haploid chromosome number (*n*) other than the diploid number (i.e. 3*n*, 4*n* and so on).

Structural aberration: a change in chromosome structure detectable by microscopic examination of the metaphase stage of cell division, observed as deletions, intrachanges or interchanges.

1.3 PRINCIPLE OF THE TEST METHOD

Animals are exposed to the test substance by an appropriate route of exposure and are sacrificed at appropriate times after treatment. Prior to sacrifice, animals are treated with a metaphase-arresting substance (e.g. colchicine or Colcemid®). Chromosome preparations are then made from germ cells and stained, and metaphase cells are analysed for chromosome aberrations.

1.4 DESCRIPTION OF THE TEST METHOD

1.4.1 Preparations

1.4.1.1 Selection of animal species

Male Chinese hamsters and mice are commonly used. However, males of other appropriate mammalian species may be used. Commonly used laboratory strains of young healthy adult animals should be employed. At the commencement of the study the weight variation of animals should be minimal and not exceed ± 20% of the mean weight.

1.4.1.2 Housing and feeding conditions

General conditions referred in the General Introduction to Part B are applied although the aim for humidity should be 50-60%.

1.4.1.3 Preparation of the animals

Healthy young adult males 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. The animals are acclimated to the laboratory conditions for at least five days prior to the start of the study.

1.4.1.4 Preparation of doses

Solid test substances should be dissolved or suspended in appropriate solvents or vehicles and diluted, if appropriate, prior to dosing of the animals. Liquid test substances may be dosed directly or diluted prior to dosing. Fresh preparations of the test substance should be employed unless stability data demonstrate the acceptability of storage.

1.4.2 Test conditions

1.4.2.1 Solvent/vehicle

The solvent/vehicle should not produce toxic effects at the dose levels used and should not be suspected of chemical reaction with the test substance. If other than well-known solvents/vehicles are used, their inclusion should be supported by data indicating their compatibility. It is recommended that wherever possible, the use of an aqueous solvent/vehicle should be considered first.

1.4.2.2 Controls

Concurrent positive and negative (solvent/vehicle) controls should be included in each test. Except for treatment with the test substance, animals in the control groups should be handled in an identical manner to animals in the treated groups.

Positive controls should produce structural chromosome aberrations *in vivo* in spermatogonial cells when administered at exposure levels expected to give a detectable increase over background.

Positive control doses should be chosen so that the effects are clear but do not immediately reveal the identity of the coded slides to the reader. It is acceptable that the positive control be administered by a route different from the test substance and sampled at only a single time. In addition, the use of chemical class-related positive control chemicals may be considered, when available. Examples of positive control substances include:

Substance	**CAS No.**	**EINECS No.**
Cyclophosphamide Cyclophosphamide monohydrate	50-18-0 6055-19-2	200-015-4
Cyclohexylamine	108-91-8	203-629-0
Mitomycin C	50-07-7	200-008-6
Monomeric acrylamide	79-06-1	201-173-7
Triethylenemelamine	51-18-3	200-083-5

Negative controls, treated with solvent or vehicle alone, and otherwise treated in the same way as the treatment groups, should be included for every sampling time, unless acceptable inter-animal variability and frequency of cells with chromosome aberrations are demonstrated by historical control data. In addition, untreated controls should also be used unless there are

historical or published control data demonstrating that no deleterious or mutagenic effects are induced by the chosen solvent/vehicle.

1.5 PROCEDURE

1.5.1 Number of animals

Each treated and control group must include at least 5 analysable males.

1.5.2 Treatment schedule

Test substances are preferably administered once or twice (i.e. as a single treatment or as two treatments). Test substances may also be administered as a split dose, i.e. two treatments on the same day separated by no more than a few hours, to facilitate administering a large volume of material. Other dose regimens should be scientifically justified.

In the highest dose group two sampling times after treatment are used. Since cell cycle kinetics can be influenced by the test substance, one early and one late sampling time are used around 24 and 48 hours after treatment. For doses other than the highest dose, a sampling time of 24 hours or 1.5 cell cycle length after treatment should be taken, unless another sampling time is known to be more appropriate for detection of effects (6).

In addition, other sampling times may be used. For example in the case of chemicals which may induce chromosome lagging, or may exert S-independent effects, earlier sampling times may be appropriate (1).

The appropriateness of a repeated treatment schedule needs to be identified on a case-by-case basis. Following a repeated treatment schedule the animals should then be sacrificed 24 hours (1.5 cell cycle length) after the last treatment. Additional sampling times may be used where appropriate.

Prior to sacrifice, animals are injected intraperitoneally with an appropriate dose of a metaphase arresting substance (e.g. Colcemid® or colchicine). Animals are sampled at an appropriate interval thereafter. For mice this interval is approximately 3 - 5 hours, for Chinese hamsters this interval is approximately 4 - 5 hours.

1.5.3 Dose levels

If a range finding study is performed because there are no suitable data available, it should be performed in the same laboratory, using the same species, strain and treatment regimen to be used in the main study (7). If there is toxicity, three dose levels are used for the first sampling time. These dose levels should cover a range from the maximum to little or no toxicity. At the later sampling time only the highest dose needs to be used. The highest dose is defined as the dose producing signs of toxicity such that higher dose levels, based on the same dosing regimen, would be expected to produce lethality.

Substances with specific biological activities at low non-toxic doses (such as hormones and mitogens) may be exceptions to the dose-setting criteria and should be evaluated on a case-by-case basis. The highest dose may also be defined as a dose that produces some indication of toxicity in the spermatogonial cells (e.g. a reduction in the ratio of spermatogonial mitoses to first and second meiotic metaphases; this reduction should not exceed 50%).

1.5.4 Limit test

If a test at one dose level of at least 2000 mg/kg body weight/day using a single treatment, or as two treatments on the same day, produces no observable toxic effects, and if genotoxicity would not be expected based upon data from structurally related substances, then a full study using three dose levels may not be considered necessary. Expected human exposure may indicate the need for a higher dose level to be used in the limit test.

1.5.5 Administration of doses

The test substance is usually administered by gavage using a stomach tube or a suitable intubation cannula, or by intraperitoneal injection. Other routes of exposure may be acceptable where they can be justified. The maximum volume of liquid that can be administered by gavage or injection at one time depends on the size of the test animal. The volume should not exceed 2 ml/100g body weight. The use of volumes higher than these must be justified. 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.

1.5.6 Chromosome preparation

Immediately after sacrifice, cell suspensions are obtained from one or both testes, exposed to hypotonic solution and fixed. The cells are then spread on slides and stained.

1.5.7 Analysis

For each animal at least 100 well-spread metaphase should be analysed (i.e. a minimum of 500 metaphases per group). This number could be reduced when high numbers of aberrations are observed. All slides, including those of positive and negative controls, should be independently coded before microscopic analysis. Since fixation procedures often result in the breakage of a proportion of metaphases with loss of chromosomes, the cells scored should contain a number of centromeres equal to the number $2n \pm 2$.

2. DATA

2.1 TREATMENT OF RESULTS

Individual animal data should be presented in a tabular form. The experimental unit is the animal. For each individual animal the number of cells with structural chromosome aberrations and the number of chromosome aberrations per cell should be evaluated. Different types of structural chromosome aberrations should be listed with their numbers and frequencies for treated and control groups. Gaps are recorded separately and reported but generally not included in the total aberration frequency.

If mitosis as well as meiosis is observed, the ratio of spermatogonial mitoses to first and second meiotic metaphases should be determined as a measure of cytotoxicity for all treated and negative control animals in a total sample of 100 dividing cells per animal to establish a possible cytotoxic effect. If only mitosis is observed, the mitosis index should be determined in at least 1000 cells for each animal.

2.2 EVALUATION AND INTERPRETATION OF RESULTS

There are several criteria for determining a positive result, such as a dose-related increase in the relative number of cells with chromosome aberrations or a clear increase in the number of cells with aberrations in a single dose at a single sampling time. Biological relevance of the results should be considered first. Statistical methods may be used as an aid in evaluating the test results (8). Statistical significance should not be the only determining factor for a positive response. Equivocal results should be clarified by further testing preferably using a modification of experimental conditions.

A test substance for which the results do not meet the above criteria is considered non-mutagenic in this test.

Although most experiments will give clearly positive or negative results, in rare cases the data set will preclude making a definite judgement about the activity of the test substance. Results may remain equivocal or questionable regardless of the number of times the experiment is repeated.

Positive results from the *in vivo* spermatogonial chromosome aberration test indicate that the test substance induces structural chromosome aberrations in the germ cells of the species tested. Negative results indicate that, under the test conditions, the test substance does not induce chromosome aberrations in the germ cells of the species tested.

The likelihood that the test substance or its metabolites reach the target tissue should be discussed.

3. REPORTING

TEST REPORT

The test report must include the following information:

Solvent/Vehicle:

– justification for choice of vehicle;

– solubility and stability of the test substance in solvent/vehicle, if known;

Test animals:

– species/strain used;

– number and age of animals;

– source, housing conditions, diet, etc.;

– individual weight of the animals at the start of the test, including body weight range, mean and standard deviation for each group;

Test conditions :

– data from range finding study, if conducted;

– rationale for dose level selection;

– rationale for route of administration;

– details of test substance preparation;

– details of the administration of the test substance;

– rationale for sacrifice times;

– 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;

– detailed description of treatment and sampling schedules;

– methods for measurement of toxicity;

– identity of metaphase arresting substance, its concentration and duration of treatment;

– methods of slide preparation;

– criteria for scoring aberrations;

– number of cells analysed per animal;

– criteria for considering studies as positive, negative or equivocal;

Results:

– signs of toxicity;

– mitotic index;

– ratio of spermatogonial mitoses cells to first and second meiotic metaphases;

– type and number of aberrations, given separately for each animal;

– total number of aberrations per group;

– number of cells with aberrations per group;

– dose-response relationship, if possible;

– statistical analyses, if any;

– concurrent negative control data;

– historical negative control data with ranges, means and standard deviations;

– concurrent positive control data;

– changes in ploidy, if seen.

Discussion of results.

Conclusions.

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B. 24. MOUSE SPOT TEST

1. METHOD

1.1. INTRODUCTION

See General Introduction Part E.

1.2. DEFINITION

See General Introduction Part E.

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLE OF THE TEST METHOD

This is an *in vivo* test in mice in which developing embryos are exposed to the chemicals. The target cells in the developing embryos are melanoblasts, and the target genes are those which control the pigmentation of the coat hairs. The developing embryos are heterozygous for a number of these coat colour genes. A mutation in, or loss of (by a variety of genetic events), the dominant allele of such a gene in a melanoblast results in the expression of the recessive phenotype in its descendant cells, constituting a spot of changed colour in the coat of the resulting mouse. The number of offspring with these spots, mutations, are scored and their frequency is compared with that among offspring resulting from embryos treated with the solvent only. The mouse spot test detects presumed somatic mutations in foetal cells.

1.5. QUALITY CRITERIA

None.

1.6. DESCRIPTION OF THE TEST METHOD

Preparations

When possible, test substances are dissolved or suspended in isotonic saline. Chemicals insoluble in water are dissolved or suspended in appropriate vehicles. The vehicle used should neither interfere with the test chemical nor produce toxic effects. Fresh preparations of the test chemical should be used.

Experimental animals

Mice of the T strain (nonagouti, a/a; chinchilla, pink eye, $c^{ch}p/c^{ch}p$; brown, b/b; dilute, short ear, d se/d se; piebald spotting, s/s) are mated either with the HT strain (pallid, nonagouti, brachypody, pa a bp/pa a bp; leaden fuzzy, ln fz/ln fz; pearl pe/pe) or with C57BL (nonagouti,

a/a). Other appropriate crosses such as between NMRI (nonagouti, a/a; albino, c/c) and DBA (nonagouti, a/a; brown, b/b; dilute d/d) may be used provided they produce nonagouti offspring.

Number and sex

Sufficient pregnant females are treated to provide an appropriate number of surviving offspring for each dose level used. The appropriate sample size in governed by the number of spots observed in the treated mice and the scale of the control data. A negative result is acceptable only when at least 300 offspring from females treated with the highest dose have been scored.

Use of negative and positive controls

Concurrent control data from mice treated with the vehicle only (negative controls) should be available. Historical control data from the same laboratory may be pooled to increase the sensitivity of the test provided they are homogeneous. Positive control data recently obtained in the same laboratory from treatment with a chemical known to show mutagenicity by this test should be available if no mutagenicity of the test chemical is detected.

Route of administration

The usual routes of administration are oral intubation or intraperitoneal injection of the pregnant females. Treatment by inhalation or other routes of administration are used when appropriate.

Dose levels

At least two dose levels are used including one showing signs of toxicity or reduced litter size. For non-toxic chemicals exposure to the maximum practicable dose should be used.

Procedure

A single treatment is normally given on day 8,9 or 10 of pregnancy, counting as day 1 the day on which the vaginal plug is first dbserved. These days correspond to 7,25 , 8,25 and 9,25 days after conception. Successive treatments over these days may be used.

Analysis

The offspring are coded and scored for spots between three and four weeks after birth. Three classes of spots are distinguished:

(a) white spots within 5 mm of the mid-ventralline which are presumed to result from cell killing (WMVS);

(b) yellow, agouti-like, spots associated with mammae, genitalia, throat, axillary and inguinal areas and on the mid-forehead, which are presumed to result from misdifferentiation (MDS); and

(c) pigmented and white spots randomly distributed on the coat which are presumed to result from somatic mutations (RS).

All three classes are scored but only the last, RS, is of genetic relevance. Problems of distinguishing between MDS and RS may be solved by fluorescence microscopy of sample hairs.

Obvious gross morphological abnormalities of the offspring should be noted.

2. DATA

The data are presented as the total number of offspring scored and the number having one or more presumed somatic mutation spots. Treatment and negative control data are compared by appropriate methods. Data are also presented on a per-litter basis.

3. REPORTING

3.1. TEST REPORT

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

– the strains used in the cross,

– the number of pregnant females in the experimental and control groups,

– the average litter size in the experimental and control groups at birth and at weaning,

– the dose level(s) of the test chemical,

– the solvent used,

– the day of pregnancy of which treatment was given,

– the route of treatment,

– the total number of offspring scored, and the number with WMVS, MDS and RS in the experimental and control groups,

– gross morphological abnormalities,

– dose/response relationship of RS when possible,

– statistical evaluation,

– discussion of results,

– interpretation of results.

3.2. EVALUATION AND INTERPRETATION

See General Introduction Part B.

4. REFERENCES

See General Introduction Part B.

B. 25. MOUSE HERITABLE TRANSLOCATION

1. METHOD

1.1. INTRODUCTION

See General Introduction Part B.

1.2. DEFINITION

See General Introduction Part B.

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLE OF THE TEST METHOD

The mouse heritable translocation test detects structural and numerical chromosome changes in mammalian germ cells as recovered in first generation progeny. The types of chromosome changes detected are reciprocal translocations and, if female progeny are included, X-chromosome loss. Carriers of translocations and XO-females show reduced fertility which is used to select F_1 progeny for cytogenetic analysis. Complete sterility is caused by certain types of translocations (X-autosome and c-t type). Translocations are cytogenetically observed in meiotic cells at diakinesis- metaphase I of male individuals, either F_1 males or male offspring of F_1 females. The XO-females are cytogenetically identified by the presence of only 39 chromosomes in bone marrow mitoses.

1.5. QUALITY CRITERIA

None.

1.6. DESCRIPTION OF THE TEST METHOD

Preparations

The test chemicals are dissolved in isotonic saline. If insoluble they are dissolved or suspended in appropriate vehicles. Freshly prepared solutions of the test compound are employed. If a vehicle is used to facilitate dosing, it must not interfere with the test compound or produce toxic effects.

Route of administration

Routes of administration are usually oral intubation ot intraperitoneal injection. Other routes of administration may be appropriate.

Experimental animals

For the ease of breeding and cytological verification these experiments are performed with mice. No specific mouse strain is required. However, the average litter-size of the strain should be greater than eight and be relatively constant.

Healthy sexually mature animals are used.

Number of animals

The number of animals necessary depends upon the spontaneous translocation frequency and the minimal rate of induction required for a positive result.

The test is usually performed by analyses of male F_1 progeny. At least 500 male F_1 progeny should be tested per dose group. If female F_1 progeny are included, 300 males and 300 females are required.

Use of negative and positive controls

Adequate control data, derived from concurrent and historic control should be available. When acceptable positive control results are available from experiments conducted recently in the same laboratory these results can be used instead of a concurrent positive control.

Dose levels

One dose level is tested, usually the highest dose associated with the production of minimal toxic effects, but without affecting reproductive behaviour or survival. To establish a dose/response relationship two additional lower doses are required. For non-toxic chemicals exposure to the maximum practicable dose should be used.

Procedure

Treatment and mating

Two treatment schedules are available. Single administration of the test substance is most widely used. Administration of the test substance on seven days per week for 35 days may also be used. The number of matings following treatment is governed by the treatment schedule and should ensure that all treated germ cell stages are sampled. At the end of the mating period females are caged individually. When females give birth, the date, litter size and sex of progeny are recorded. All male progeny are weaned and all female progeny are discarded unless they are included in the experiment.

Testing for translocation heterozygosity

One of two possible methods is used:

– Fertility testing of F_1 progeny and subsequent verification of possible translocation carriers by cytogenetic analysis,

– Cytogenetic analysis of all male F_1 progeny without prior selection by fertility testing.

(a) Fertility testing

Reduced fertility of an F_1 individual can be established by litter size observation and/or analysis of uterine contents of female mates.

Criteria for determining normal and reduced fertility must be established for the mouse strain used.

Litter size observation: F_1 males to be tested are caged individually with females either from the same experiment or from the colony. Cages are inspected daily beginning 18 days after mating. Litter size and sex of the F_2 progeny are recorded at birth and litters are discarded thereafter. If female F_1 progeny are tested the F_2 progeny of small litters are kept for further testing. Female translocation carriers are verified by cytogenetic analysis of a translocation in any of their male offspring. XO-females are recognized by the change in sex ratio among their progeny from 1:1 to 1:2 males *vs.* females. In a sequential procedure, normal F_1 animals are eliminated from further testing if the first F_2 litter reaches or exceeds a predetermined normal value, otherwise a second or third F_2 litter is observed.

F_1 animals that cannot be classified as normal after observation of up to three F_2 litters are either tested further by analysis of uterine contents of female mates or directly subjected to cytogenetic analysis.

Analysis of uterine contents: The reduction in litter size of translocation carriers is due to embryonic death so that a high number of dead implants is indicative of the presence of a translocation in the animal under test. F_1 males to be tested are mated to two to three females each. Conception is established by daily inspection for vaginal plugs in the morning. Females are sacrificed 14 to 16 days later and living and dead implants in their uteri are recorded.

(b) Cytogenetic analysis

Testes preparations are made by the air-drying technique. Translocation carriers are identified by the presence of multivalent configurations at diakinesis-metaphase I in primary spermatocytes. Observation of at least two cells with multivalent association constitutes the required evidence that the tested animal is a translocation carrier.

If no breeding selection has been performed all F_1 males are inspected cytogenetically. A minimum of 25 diakinesis-metaphase I cells per male must be scored microscopically. Examination of mitotic metaphases, in spermatogonia or bone-marrow, is required in F_1 males with small testes and meiotic breakdown before diakinesis or from F_1 female XO suspects. The presence of an unusually long and/or short chromosome in each of 10 cells is evidence for a particular male sterile translocation (c-t type). Some X-autosome translocations that cause male sterility may only be identified by banding analysis of mitotic chromosomes. The presence of 39 chromosomes in all of 10 mitoses is evidence for an XO condition in a female.

2. DATA

Data are presented in tabular form.

The mean litter size and sex ratio from parental matings at birth and weaning are reported for each mating interval.

For fertility assessment of F_1 animals, the mean litter size of all normal matings and the individual litter sizes of F_1 translocation carriers are presented. For analysis of uterine

contents, the mean number of living and dead implants of normal matings and the individual numbers of living and dead implants for each mating of F_1 translocation carriers are reported.

For cytogenetic analysis of diakinesis-metaphase I, the numbers of types of multivalent configurations and the total number of cells are listed for each translocation carrier.

For sterile F_1 individuals, the total number of matings and the duration of the mating period are reported. Testes weights and cytogenetic analysis details are given.

For XO females, the mean litter size, sex ratio of F_1 progeny and cytogenetic analysis results are reported.

Where possible F_1 translocation carriers are preselected by fertility tests, the tables have to include information on how many of these were confirmed translocation heterozygotes.

Data from negative controls and the positive control experiments are reported.

3. REPORTING

3.1. TEST REPORT

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

– strain of mice, age of animals, weights of treated animals,

– numbers of parental animals of each sex in experimental and control groups,

– test conditions, detailed description of treatment, dose levels, solvents, mating schedule,

– number and sex of offspring per female, number and sex of offspring raised for translocation analysis,

– time and criteria of translocation analysis,

– number and detailed description of translocation carriers, including breeding data and uterine content data, if applicable;

– cytogenetic procedures and details of microscopic analysis, preferably with pictures,

– statistical evaluation,

– discussion of results,

– interpretation of results.

3.2. EVALUATION AND INTERPRETATION

See General Introduction Part B.

4. REFERENCES

See General Introduction Part B.

B.26. SUB-CHRONIC ORAL TOXICITY TEST
REPEATED DOSE 90 - DAY ORAL TOXICITY STUDY IN RODENTS

1. METHOD

This sub-chronic oral toxicity test method is a replicate of the OECD TG 408 (1998).

1.1. INTRODUCTION

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 prolonged period of time covering post-weaning maturation and growth well into 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.

The method places additional emphasis on neurological endpoints and gives an indication of immunological and reproductive effects. The need for careful clinical observations of the animals, so as to obtain as much information as possible, is also stressed. This study should allow for the identification of chemicals with the potential to cause neurotoxic, immunological or reproductive organ effects, which may warrant further in-depth investigation.

See also General Introduction Part B.

1.2. DEFINITIONS

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), or as constant dietary concentrations (ppm).

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

NOAEL: is the abbreviation for no-observed-adverse-effect level and is the highest dose level where no adverse treatment-related findings are observed.

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 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.

1.4. DESCRIPTION OF THE TEST METHOD

1.4.1. Preparations of animals

Healthy animals, which have been acclimated to laboratory conditions for at least 5 days and have not been subjected to previous experimental procedures, should be used. 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.

1.4.2. Preparations of doses

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 test material.

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.

1.4.3. Test conditions

1.4.3.1. Experimental animals

The preferred species is the rat, although other rodent species, e.g., the mouse, 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 ± 20 % of the mean weight of each sex. Where the study is conducted as a preliminary to a long term chronic toxicity study, animals from the same strain and source should be used in both studies.

1.4.3.2. Number and sex

At least 20 animals (ten female and ten 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. Based on previous knowledge of the chemical or a close analogue, consideration should be given to including an additional satellite group of ten animals (five per sex) in the control and in the 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.

1.4.3.3. Dose levels

At least three dose levels and a concurrent control shall be used, except where a limit test is conducted
(see 1.4.3.4). 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 substance 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.

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.

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.

1.4.3.4. Limit test

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 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.

1.5. PROCEDURE

1.5.1. Administration of doses

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. The volume should not exceed 1 ml/100g body weight, except in the case of aqueous solutions where 2 ml/100g 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**.**

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; 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 90-day study is used as a preliminary to a long term chronic toxicity study, a similar diet should be used in both studies.

1.5.2. Observations

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.

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 are inspected for signs of morbidity and mortality.

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 outside the home cage, preferably in a standard arena and at similar times on each occasion. 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 observation conditions are minimal. 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, 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 bizarre behaviour (e.g., self-mutilation, walking backwards) should also be recorded (1).

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.

Towards the end of the exposure period and in any case not earlier than in week 11, sensory reactivity to stimuli of different types (1) (e.g., auditory, visual and proprioceptive stimuli) (2), (3), (4), assessment of grip strength (5) and motor activity assessment (6) should be conducted. Further details of the procedures that could be followed are given in the respective references. However, alternative procedures than those referenced could also be used.

Functional observations conducted towards the end of the study may be omitted when data on functional observations are available from other studies and the daily clinical observations did not reveal any functional deficits.

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.

1.5.2.1. Body weight and food/water consumption

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.

1.5.2.2. Haematology and Clinical Biochemistry

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.

The following haematological examinations should be made at the end of the test period and when any interim blood samples may have been collected: haematocrit, haemoglobin concentration, erythrocyte count, total and differential leukocyte count, platelet count and a measure of blood clotting time/potential.

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 each animal just prior to or as part of the procedure for killing the animals (apart from those found moribund and/or intercurrently killed). In a similar manner to haematological investigations, interim sampling for clinical biochemical tests may be performed. Overnight fasting of the animals prior to blood sampling is recommended[(2)]. Determinations in plasma or serum should include sodium, potassium, glucose, total cholesterol, urea, blood urea nitrogen, creatinine, total protein and albumin, and more than 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, which may provide useful information under certain circumstances, may also be included.

Optionally, the following urinalysis 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, phosphorus, fasting triglycerides, specific hormones, methaemoglobin and cholinesterase. These need to be identified for chemicals 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 from a given substance.

If historical baseline data are inadequate, consideration should be given as to whether haematological and clinical biochemistry variables need to be determined before dosing commences; it is generally not recommended that this data be generated before treatment (7).

1.5.2.3. 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

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[2] 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 of the study.

and abdominal cavities and their contents. The liver, kidneys, adrenals, testes, epididymides, uterus, ovaries, thymus, spleen, brain and heart of all animals (apart from those found moribund and/or intercurrently killed) 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 medulla/pons), spinal cord (at three levels: cervical, mid-thoracic and lumbar), pituitary, thyroid, parathyroid, thymus, oesophagus, salivary glands, stomach, small and large intestines (including Peyer's patches), liver, pancreas, kidneys, adrenals, spleen, heart, trachea and lungs (preserved by inflation with fixative and then immersion), aorta, gonads, uterus, accessory sex organs, female mammary gland, prostate, urinary bladder, gall bladder (mouse), 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), skin and eyes (if changes were observed during ophthalmological examinations). 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.

1.5.2.4. Histopathology

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.

1.5.2.5. All gross lesions should 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 AND REPORTING

2.1. 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 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.

2.2. TEST REPORT

The test report must include the following information:

2.2.1. Test substance:

– physical nature, purity and physico-chemical properties;

– identification data;

– vehicle (if appropriate): justification for choice of vehicle, if other than water.

2.2.2. Test species:

– species and strain used;

– number, age and sex of animals;

– source, housing conditions, diet etc.;

– individual weights of animals at the start of the test.

2.2.3. 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.

2.2.4. Results:

– body weight and 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);

– results of ophthalmological examination;

– sensory activity, grip strength and motor activity assessments (when available);

– 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.

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B.27. SUB-CHRONIC ORAL TOXICITY TEST REPEATED DOSE 90 - DAY ORAL TOXICITY STUDY IN NON-RODENTS

1. METHOD

This sub-chronic oral toxicity test method is a replicate of the OECD TG 409 (1998).

1.1. INTRODUCTION

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.

The test method 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.

See also General Introduction Part B.

1.2. DEFINITIONS

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), or as constant dietary concentrations (ppm).

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

NOAEL: is the abbreviation for no-observed-adverse-effect level and is the highest dose level where no adverse treatment-related findings are observed.

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 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.

1.4. DESCRIPTION OF THE TEST METHOD

1.4.1. Selection of animal species

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.

1.4.2. Preparation of animals

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.

1.4.3. Preparations of doses

The test substance 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.

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.

1.5. PROCEDURE

1.5.1. Number and sex of animals

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 substance 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.

1.5.2. Dosage

At least three dose levels and a concurrent control shall be used, except where a limit test is conducted (see 1.5.3). 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.

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.

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.

1.5.3. Limit test

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 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.

1.5.4. Administration of doses

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.

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.

1.5.5. Observations

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.

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.

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.

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.

1.5.5.1. Body weight and food/water consumption

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.

1.5.5.2. Haematology and Clinical Biochemistry

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.

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.

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.

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).

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.

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

1.5.5.3. 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 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.

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.

1.5.5.4. Histopathology

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.

All gross lesions should 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 AND REPORTING

2.1. 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 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.

2.2. TEST REPORT

The test report must include the following information:

2.2.1. Test substance:

– physical nature, purity and physico-chemical properties;

– identification data;

– vehicle (if appropriate): justification for choice of vehicle, if other than water.

2.2.2. Test species:

– species and strain used;

– number, age and sex of animals;

– source, housing conditions, diet etc.;

– individual weights of animals at the start of the test.

2.2.3. 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.

2.2.4. 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.

B. 28 SUB-CHRONIC DERMAL TOXICITY STUDY 90-DAY REPEATED DERMAL DOSE STUDY USING RODENT SPECIES

1. METHOD

1.1. INTRODUCTION

See General Introduction Part B.

1.2. DEFINITIONS

See General Introduction Part B.

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLE OF THE TEST METHOD

The test substance is applied daily to the skin in graduated doses to several groups of experimental animals, one dose per group for a period of 90 days. During the period of application the animals are observed daily to detect signs of toxicity. Animals which die during the test are necropsied, and at the conclusion of the test surviving animals are necropsied.

1.5. QUALITY CRITERIA

None.

1.6. DESCRIPTION OF THE TEST METHOD

1.6.1. Preparations

The animals are kept under the experimental housing and feeding conditions for at least five days prior to the test. Before the test healthy young animals are randomized and assigned to the treated and control groups. Shortly before testing fur is clipped from the dorsal area of the trunk of the test animals. Shaving may be employed but it should be carried out approximately 24 hours before the test. Repeat clipping or shaving is usually needed at approximately weekly intervals. When clipping or shaving the fur, care must be taken to avoid abrading the skin. Not less than 10% of the body surface area should be clear for the application of the test substance. The weight of the animal should be taken into account when deciding on the area to be cleared and on the dimensions of the covering. When testing solids, which may be pulverized if appropriate, the test substance should be moistened sufficiently with water or, where necessary, a suitable vehicle to ensure good contact with the skin. Liquid

test substances are generally used undiluted. Daily application on a five to seven-day per week basis is used.

1.6.2. Test conditions

1.6.2.1. Experimental animals

The adult rat, rabbit or guinea pig may be used. Other species may be used but their use would require justification. At the commencement of the test the range of the weight variation should be ± 20% of the mean weight. Where a sub-chronic dermal study is conducted as a preliminary to a long-term study, the same species and strain should be used in both studies.

1.6.2.2. Number and sex

At least 20 animals (10 female and 10 male) with healthy skin should be used at each dose level. 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. In addition, a satellite group of 20 animals (10 animals per sex) may be treated with the high-dose level for 90 days and observed for reversibility, persistence, or delayed occurrence of toxic effects for 28 days post-treatment.

1.6.2.3. Dose levels

At least three dose levels are required with a controle or a vehicle control if a vehicle is used. The exposure period should be at least six hours per day. The application of the test substance should be made at similar times each day, and the amount of substance applied adjusted at intervals (weekly or bi-weekly) to maintain a constant dose level in terms of animal body weight. Except for treatment with the test substance, animals in the control group should be handled in an identical manner to the test group subjects. Where a vehicle is used to facilitate dosing, the vehicle control group should be dosed in the same way as the treated groups, and receive the same amount as that received by the highest dose level group. The highest dose level should result in toxic effects but produce no, or few, fatalities. 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 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.

If application of the test substance produces severe skin irritation the concentrations should be reduced and this may result in a reduction in, or absence of, other toxic effects al: the high-dose level. If the skin has been badly damaged it may be necessary to terminate the study and undertake a new study at lower concentrations.

1.6.3. Limit test

If a preliminary study at a dose level of 1000 mg/kilograms, or a higher dose level related to possible human exposure where this is known, produces no toxic effects, further testing may not be considered necessary.

1.6.4. Observation period

The experimental animals should be observed daily for signs of toxicity. The time of death and the time at which signs of toxicity appear and disappear should be recorded.

1.6.5. Procedure

Animals should be caged individually. The animals are treated with the test substance, ideally on seven days per week, for a period of 90 days.

Animals in any satellite groups scheduled for follow-up observations should be kept for a further 28 days without treatment to detect recovery from, or persistence of, toxic effects. Exposure time should be six hours per day.

The test substance should be applied uniformly over an area which is approximately 10% of the total body surface area. With highly toxic substances, the surface area covered may be less but as much of the area should be covered with as thin and uniform a film as possible.

During exposure the test substance is held in contact with the skin with a porous gauze dressing and non-irritating tape. The test site should be further covered in a suitable manner to retain the gauze dressing and test substance and ensure that the animals cannot ingest the test substance. Restrainers may be used to prevent the ingestion of the test substance but complete immobilization is not a recommended method.

At the end of the exposure period, residual test substance should be removed where practicable using water or some other appropriate method of cleansing the skin.

All the animals should be observed daily and signs of toxicity recorded, including the time of onset, their degree and duration. Cageside observations should include changes in skin and fur, eyes and mucous membranes, as well as respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behavior pattern. Measurements should be made of food consumption weekly and the animals weighed weekly. Regular observations 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 study period all survivors in the non-satellite treatment groups are necropsied. Moribund animals should be removed and necropsied when noticed.

The following examinations are customarily made on all animals including the controls:

(a) Ophthalmological examination, using an ophthalmoscope or equivalent suitable equipment, should be made prior to exposure to 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 appropriate to the species), sercum glutamic pyruvic transaminase ([3]), serum glutamic oxaloacetic transaminase ([4]), ornithine decarboxylase, gamma glutamyl transpeptidase, urea nitrogen, albumin, 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 and 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 haem a to logical and clinical biochemistry parameters before dosing commences.

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 must 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, any thymic tissue, (trachea), lungs, heart, aorta, salivary glands, liver, spleen, kidneys, adrenals, pancreas, gonads, uterus, accessory genital organs, gall bladder (if present), oesophagus, stomach, duodenum, jejunum, ileum, caecum, colon, rectum, urinary bladder, representative lymph node, (female mammary gland), (thigh musculature), peripheral nerve, (eyes), (sternum with bone marrow), (femur - including articular surface), (spinal cord at three levels - cervical, mid-thoracic 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.

Histopathological examination

(a) Full histopathology should be carried out on normal and treated skin and on organs and tissues of 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) Where rats are used, 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. Further histopathological examination may not be required routinely on the animals in these

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[3] Now known as serum alanine aminotransferase.

[4] Now known as serum aspartate aminotransferase.

groups, but must always be carried out in organs which show 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 other treated groups.

2. DATA

Data should be summarized 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 type of lesions and the percentage of animals displaying each type of lesion. Results should be evaluated by an appropriate statistical method. Any recognized statistical method may be used.

3. REPORTING

3.1. TEST REPORT

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

– species, strain, source, environmental conditions, diet,

– test conditions,

– dose levels (including vehicle, if used) and concentrations,

– toxic response data by sex and dose,

– no-effect level, where possible,

– time of death during the study or whether animals survived to termination,

– description of toxic or other effects,

– the time of observation of each abnormal sign and its subsequent course,

– food and bodyweight data,

– ophthalmological findings,

– haematological tests employed and all results,

– clinical biochemistry tests employed and all results (including results of any urinalysis),

– necropsy findings,

– a detailed description of all histopathological findings,

– statistical treatment of results where possible,

– discussion of the results,

– interpretation of the results.

3.2. EVALUATION AND INTERPRETATION

See General Introduction Part B.

4. REFERENCES

See General Introduction Part B.

B.29 SUB-CHRONIC INHALATION TOXICITY STUDY 90-DAY REPEATED INHALATION DOSE STUDY USING RODENT SPECIES

1. METHOD

1.1. INTRODUCTION

See General Introduction Part B.

1.2. DEFINITIONS

See General Introduction Part B.

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLE OF THE TEST METHOD

Several groups of experimental animals are exponed daily for a defined period to the test substance in graduated concentrations, one concentration being used per group, for a period of 90 days. Where a vehicle is used to help generate an appropriate concentration of the test substance in the atmosphere, a vehicle control group should be used. During the period of administration the animals are observed daily to detect signs of toxicity. Animals which die during the test are necropsied and at the conclusion of the test surviving animals are necropsied.

1.5. QUALITY CRITERIA

None.

1.6. DESCRIPTION OF THE TEST METHOD

1.6.1. Preparations

The animals are kept under the experimental housing and feeding conditions for at least five days prior to the experiment. Before the test, healthy young aimals are randomized and assigned to the treatment and control groups. Where necessary, a suitable vehicle may be added to the test substance to help generate an appropriate concentration of the substance in the atmosphere. If a vehicle or other additives are used to facilitate dosing, they should be known not to produce toxic effects. Historical data can be used if appropriate.

1.6.2. Test conditions

Experimental animals

Unless there are contra-indications, the rat is the preferred species. Commonly used laboratory strains of young healthy animals should be employed. At the commencement of the study the range of weight variation of animals used should not exceed ± 20% of the appropriate mean value. Where a subchronic inhalation 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 for each exposure concentration. 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 sacrified before the completion of the study. In addition, a satellite group of 20 animals (10 animals per sex) may be treated with the high concentration level for 90 days and observed for reversibility, persistence, or delayed occurrence of toxic effects for 28 days post treatment.

Exposure concentrations

At least three concentrations are required, with a control or a vehicle control (corresponding to the concentration of vehicle at the highest level) if a vehicle is 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 concentration should result in toxic effects but no, or few, fatalities. Where there is a usable estimation of human exposure the lowest level should exceed this. Ideally, the intermediate concentration should produce minimal observable toxic effects. If more than one intermediate concentration is used the concentrations 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 to permit a meaningful evaluation of the results.

Exposure time

The duration of daily exposure should be six hours after equilibration of the chamber concentrations. Other durations may be used to meet specific requirements.

Equipment

The animals should be tested in inhalation equipment designed to sustain a dynamic air flow of at least 12 air changes per hour to ensure an adequate oxygen content and an evenly distributed exposure atmosphere. Where a chamber is used its design should minimize crowding of the test animals and maximize their exposure by inhalation to the test substance. As a general rule, to ensure stability of a chamber atmosphere the total volume of the test animals should not exceed 5% of the volume of the test chamber. Oro-nasal, head only, or whole body individual chamber exposure may be used; the first two will minimize uptake by other routes.

Observation period

The experimental animals should be observed daily for signs of toxicity during the entire treatment and recovery period. The time of death and the time at which signs of toxicity appear and disappear should be recorded.

1.6.3. Procedure

The animals are exposed to the test substance daily, five to seven days per week, for a period of 90 days. Animals in any satellite groups scheduled for follow-up observations should be kept for a further 28 days without treatment to detect recovery from, or persistence of, toxic effects. The temperature at which the test is performed should be maintained at 22 ± 3 °C. Ideally, the relative humidity should be maintained between 30% and 70%, but in certain instances (e.g. tests of aerosols) this may not be practicable. Food and water should be withheld during exposure.

A dynamic inhalation system with a suitable analytical concentration control system should be used. To establish suitable exposure concentrations a trial test is recommended. The air flow should be adjusted to ensure that conditions throughout the exposure chamber are homogeneous. The system should ensure that stable exposure conditions are achieved as rapidly as possible.

Measurements or monitoring should be made of:

(a) the rare of air flow (continuously);

(b) the actual concentration of the test substance measured in the breathing zone. During the daily exposure period the concentration should not vary by more than ± 15% of the mean value. However, in the case of dusts and aerosols, this level of control may not be achievable and a wider range would then be acceptable. During the total duration of the study, the day-to-day concentrations should be held as constant as practicable. During the development of the generating system, particle-size analysis should be performed to establish the stability of aerosol concentrations. During exposure, analysis should be conducted as often as necessary to determine the consistency of particle-size distribution;

(c) temperature and humidity;

(d) during and following exposure, observations are made and recorded systematically; individual records should be maintained for each animal. All the animals should be observed daily and signs of toxicity recorded including the time of onset, their degree and duration. Cageside observations should include: changes in the skin and fur, eyes, mucous membranes, respiratory, circulatory, autonomic and central nervous systems; somatomotor activity and behaviour pattern. Measurements should be made of food consumption 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 exposure period all surviving animals are necropsied. Moribund animals should be removed and necropsied when noticed.

The following examinations are customarily made on all animals including the controls:

(a) ophthalmological examination, using an ophthalmoscope or equivalent suitable equipment, should be made prior to the exposure to 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 appropriate to the species), serum glutamic pyruvic transaminase ([5]), serum glutamic oxaloacetic transaminase ([6]), ornithine decarboxylase, gamma glutamyl transpeptidase, urea nitrogen, albumin, 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 and 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.

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, lungs -which should be removed intact, weighed and treated with a suitable fixative to ensure that lung structure is maintained (perfusion with the fixative is considered to be an effective procedure), nasopharyngeal tissues, brain -including sections of medulla/ pons, cerebellar cortex and cerebral cortex, pituitary, thyroid/parathyroid, any thymic tissue, trachea, lungs, heart, aorta, salivary glands, liver, spleen, kidneys, adrenals, pancreas, gonads, uterus (accessory genital organs), (skin), gall bladder (if present), oesophagus, stomach, duodenum, jejunum, ileum, caecum, colon, rectum, urinary bladder, representative lymph node, (female mammary gland), (thigh musculature), peripheral nerve, (eyes), sternum with bone marrow, (femur, including articular surface), and (spinal cord at three levels - cervical, mid-thoracic and lumbar). The tissues mentioned between brackets need only be examined if indicated by signs of toxicity, or target organ involvement.

Histopathological examination

(a) Full histopathology should be carried out on the respiratory tract and other organs and tissues of all animals in the control and high-dose groups.

(b) All gross lesions should be examined.

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5 Now known as serum alanine aminotransferase.

6 Now known as serum aspartate aminotransferase.

(c) Target organs in other dose groups should be examined.

(d) Lungs of animals in the low - and intermediate-dose group should also be subjected to histopathological examination; since this can provide a convenient assessment of the state of health of the animals. Further histopathological examination may not be required routinely on the animals in these groups but must always be carried out on organs which show 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 other treated groups.

2. DATA

Data should be summarized 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. Results should be evaluated by an appropriate statistical method. Any recognized statistical method may be used.

3. REPORTING

3.1. TEST REPORT

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

– species, strain, source, environmental conditions, diet,

– test conditions:

Description of exposure apparatus: including design, type, dimensions, source of air, system for generating particulates and aerosols, method of conditioning air, treatment of exhaust air and the method of housing animals in a test chamber then this is used. The equipment for measuring temperature, humidity and, where appropriate, stability of aerosol concentrations or particle size, should be described.

Exposure data: these should be tabulated and presented with mean values and a measure of variability

(e.g. standard deviation) and should include:

(a) air flow rates through the inhalation equipment;

(b) temperature and humidity of air;

(c) nominal concentrations (total amount of test substance fed into the inhalation equipment divided by the volume of air);

(d) nature of vehicle, if used;

(e) actual concentrations in test breathing zone;

(f) median particle sizes (where appropriate),

- toxic response data by sex and concentration,
- no-effect level when possible,
- time of death during the study or whether animals surived to termination,
- description of toxic or other effects,
- the time of observation of each abnormal sign and its subsequent course,
- food and bodyweight data,
- ophthalmological findings,
- haematological tests employed and results,
- clinical biochemistry tests employed and results (including results of any urinalysis),
- necropsy findings,
- a detailed description of all histopathological findings,
- statistical treatment of results where appropriate,
- discussion of the results,
- interpretation of the results.

3.2. EVALUATION AND INTERPRETATION

See General Introduction Part B.

4. REFERENCES

See General Introduction Part B.

B.30 CHRONIC TOXICITY TEST

1. METHOD

1.1 INTRODUCTION

See General Introduction Part B.

1.2. DEFINITIONS

See General Introduction Part B.

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLE OF THE TEST METHOD

The test substance is administered normally seven days per week, by an appropriate route, to several groups of experimental animals, one dose per group, for a major portion of their life span. During and after exposure to the test substance, the experimental animals are observed daily to detect signs of toxicity.

1.5 QUALITY CRITERIA

None.

1.6. DESCRIPTION OF THE TEST METHOD

1.6.1. Preparations

The animals are kept under the experimental housing and feeding conditions for at least five days prior to the test. Before the test healthy young animals are randomized and assigned to the treated and control groups.

1.6.2. Test conditions

Experimental animals

The preferred species is the rat. Based upon the results of previously conducted studies other species (rodent or non-rodent) may be used. Commonly used laboratory strains of young healthy animals should be employed and dosing should begin as soon as possible after weaning.

At the commencement of the study the weight variation in the animals used should not exceed ± 20% of the mean value. Where a sub-chronic oral study is conducted as a preliminary to a long-term study, the same species/breed and strain should be used in both studies.

Number and sex

For rodents at least 40 animals (20 female and 20 male) should be used at each dose level and concurrent control 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.

For non-rodents a smaller number of animals, but at least four per sex per group, is acceptable.

Dose levels and frequency of exposure

At least three dose levels should be used in addition to the concurrent control group. The highest dose level should elicit definite signs of toxicity without causing excessive lethality. The lowest dose level should not produce and evidence of toxicity .

The intermediate dose(s) should be established in a mid-range between the high and low doses.

The selection of dose levels should take into account data from preceding toxicity tests and studies.

Frequency of exposure is normally daily. If the chemical is administered in the drinking water or mixed in the diet it should be continuously available.

Controls

A concurrent control group which is identical in every respect to the treated groups, except for exposure to the test substance, should be used.

In special circumstances, such as in inhalation studies involving aerosols or the use of an emulsifier of uncharacterized biological activity in oral studies, a concurrent negative control group should also be used. The negative control group is treated in the same manner as the test groups except that the animals are not exposed to the test substance or any vehicle.

Route of administration

The two main routes of administration are oral and inhalation. The choice of the route of administration depends upon the physical and chemical characteristics of the test substance and the likely route of exposure in humans.

The use of the dermal route presents considerable practical problems. Chronic systemic toxicity resulting from percutaneous absorption can normal1y be inferred form the results of another oral test and a knowledge of the extent of percutaneous absorption derived from preceding percutaneous toxicity tests.

Oral studies

Where the test substance is absorbed from the gastrointestinal tract, and if the ingestion route is one by which humans may be exposed, the oral route of administration is preferred unless there are contra-indications.

The animals may receive the test substance in the diet, dissolved in drinking water or given by capsule.

Ideally, daily dosing on a seven-day per week basis should be used because dosing on a five-day per week basis may permit recovery or withdrawal toxicity in the non-dosing period and thus affect the result and subsequent evaluation. However, based primarily on practical considerations, dosing on a five-day per week basis is considered to be acceptable.

Inhalation studies

Because inhalation studies present technical problems of greater complexity than the other routes of administration, more detailed guidance on this mode of administration is given here. It should also be noted that intratracheal instillation may constitute a valid alternative in specific situations.

Long-term exposures are usually patterned on projected human exposure, giving the animals either a daily exposure of six hours after equilibration of chamber concentrations, for five days a week (intermittent exposure), or, relevant to possible environmental exposure, 22 to 24 hours of exposure per day for seven days a week (continuous exposure), with about an hour for feeding the animals daily at a similar time and maintaining the chamber. In both cases, the animals are usually exposed to fixed concentrations of test substance. A major difference between intermittent and continuous exposure is that with the former there is a 17 to 18 hour period in which animals may recover from the effects of each daily exposure, with an even longer recovery period during weekends.

The choice of intermittent or continuous exposure depends on the objectives of the study and on the human exposure that is to be simulated. However, certain technical difficulties must be considered. For example, the advantages of continuous exposure for simulating environmental conditions may be offset by the necessity for watering and feeding during exposure, and by the need for more complicated (and reliable) aerosol and vapour , generation and monitoring techniques.

Exposure chambers

The animals should be tested in inhalation chambers designed to sustain a dynamic flow of at least 12 air changes per hour to assure adequate oxygen content and an evenly distributed exposure atmosphere. Control and exposure chambers should be identical in construction and design to ensure exposure conditions comparable in al1 respects except for exposure to the test substances. Slight negative pressure inside the chamber is generally maintained to prevent leakage of the test substance into the surrounding area. The chambers should minimize the crowding of test animals. As a general rule, to ensure the stability of the chamber atmosphere, the total volume of the test animals should not exceed 5% of the volume of the chamber .

Measurements or monitoring should be made of:

(i) Air flow: the rate of air flow through the chamber should preferably be monitored continuously;

(ii) Concentration: during the daily exposure period the concentration of the test substance should not vary more than ± 15% of the mean value;

(iii) Temperature and humidity: for rodents, the temperature should be maintained at 22 ± 2°C, and the humidity within the chamber at 30 to 70% , except when water is used to suspend the test substance in the chamber atmosphere. Preferably both should be monitored continuously;

(iv) Particle size measurements: particle-size distribution should be determined in chamber atmospheres involving liquid or solid aerosols. The aerosol particles should be of respirable size for the test animal used. Samples of the chamber atmospheres should be taken in the breathing zone of the animals. The air sample should be representative of the distribution of the particles to which the animals are exposed and should account, on a gravimetric basis, for all of the suspended aerosol even when much of the aerosol is not respirable. Particle size analyses should be carried out frequently during the development of the generating system to ensure the stability of the aerosol and thereafter as often as necessary during the exposures to determine adequately the consistency of the particle distribution to which the animals have been exposed.

Duration of study

The duration of the period of administration should be at least 12 months.

1.6.3. Procedure

Observations

A careful clinical examination should be made at least once each day. Additional observations should be made daily with appropriate actions taken to minimize loss of animals to the study, for example necropsy or refrigeration of those animals found dead and isolation or sacrifice of weak or moribund animals. Careful observations should be made to detect onset and progression of all toxic effects as well as to minimize loss due to disease, autolysis or cannibalism.

Clinical signs, including neurological and ocular changes as well as mortality, should be recorded for all animals. Time of onset and progression of toxic conditions, including suspected tumours, should be recorded.

Bodyweights should be recorded individually for all animals once a week during the first 13 weeks of the test period and at least once every four weeks thereafter. Food intake should be determined weekly during the first 13 weeks of the study, and then at approximately three-monthly intervals unless health status or body weight changes dictate otherwise.

Haematological examination

Haematological examination (e.g. haemoglobin content; packet cell volume, total red blood cells, total white - blood cells, platelets or other measures of clotting potential) should be performed at three months, six months, and thereafter at approximately six-month intervals and at termination on blood samples collected form all non-rodents and from 10 rats/sex of all groups. If possible, samples should be from the same rats at each interval. In addition, a pre-test sample should be collected from non-rodents.

If clinical observations suggest a deterioration in the health of the animals during the study, a differential blood count of the affected animals should be performed.

A differential blood count is performed on samples from the animals in the highest dose group and the controls. Differential blood counts are performed for the next lower group(s) only if there is a major discrepancy between the highest group and the controls, or if indicated by pathological findings.

Urinalysis

Urine samples from all non-rodents and from 10 rats/sex of all groups, if possible from the same rats at the same intervals as haematological examination, should be collected for analysis. The following determinations should be made for either individual animals or on a pooled sample/sex/group for rodents:

– appearance: volume and density for individual animals,

– protein, glucose, ketones, occult blood (semi-quantitatively),

– microscopy of sediment (semi-quantitatively).

Clinical chemistry

At approximately six-monthly intervals and at termination, blood samples are drawn for clinical chemistry measurements from all non-rodents and 10 rats/sex of all groups, if possible, from the same rats at each interval. In addition, a pre-test sample should be collected from non-rodents. Plasma is prepared from these samples and the following determinations are made:

– total protein concentration,

– albumin concentration,

– liver function tests (such as alkaline phosphatase activity, glutamic pyruvic transaminase ([7]) activity and glutamic oxaloacetic transaminase ([8]) activity), gamma glutamyl transpeptldase, ornithine decarboxylase,

– carbohydrate metabolism such as fasting blood glucose,

– kidney function tests such as blood urea nitrogen.

Gross necropsy

Full gross necropsy should be performed on all animals, including those which died during the experiment or were killed having been found in a moribund condition. Prior to sacrifice, samples of blood should be collected from all animals, for differential blood counts. All grossly visible lesions, tumours or lesions suspected of being tumours should be preserved. An attempt should be made to correlate gross observations with the microscopic findings.

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7 Now known as serum alanine aminotransferase.

8 Now known as serum aspartate aminotransferase.

All organs and tissues should be preserved for histopathological examination. This usually concerns the following organs and tissues: brain ([9]) (medullaipons, cerebellar correx, cerebral cortex), pituitary, thyroid (including parathyroid), thymus, lungs (including trachea), heart, aorta, salivary glands, liver ([3]), spleen, kidneys ([3]), adrenals ([3]), oesophagus, stomach, duodenum, jejunum, ileum, caecum, colon, rectum, uterus, urinary bladder, lymph nodes, pancreas, gonads ([3]), accessory genital organs, female mammary gland, skin, musculature, peripheral, nerve, spinal cord (cervical, thoracic, lumbar), sternum with bone marrow and femur (including joint) and eyes. Inflation of lungs and urinary bladder with a fixative is the optimal way to preserve these tissues; inflation of the lungs in inhalation studies is essential for appropriate histopathological examination. In special studies such as inhalation studies, the entire respiratory tract should be studied, including nose, pharynx and larynx.

If other clinical examinations are carried out, the information obtained from these procedures should be available before microscopic examination, because it may give significant guidance to the pathologist.

Histopathology

All visible changes, particularly tumours and other lesions occurring in any organ should be examined microscopically. In addition, the following procedures are recommended:

(a) Microscopic examination of all preserved organs and tissues with complete description of all lesions found in:

1. all animals that died or were killed during the study;

2. all of the high-dose group and controls;

(b) Organs or tissues showing abnormalities caused, or possibly caused, by the test substance are also examined in the lower-dose groups;

(c) Where the result of the test gives evidence of substantial reduction of the animals' normal lifespan or the induction of effects that might affect a toxic response, the next-lower dose level should be examined as described above;

(d) Information on the incidence of lesions normally occurring in the strain of animals used, under the same laboratory conditions, i.e. historical control data, is indispensable for correctly assessing the significance of changes observed in treated animals.

2. DATA

Data should be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing lesions and the percentage of animals displaying each type of lesion. Results should be evaluated by an appropriate statistical method. Any recognized statistical method may be used.

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[9] These organs, from ten animals per sex per group for rodents and all non-rodents, plus thyroid (with parathyroids) for all non-rodents, should be weighed.

3. REPORTING

3.1. TEST REPORT

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

– species, strain, source, environmental conditions, diet,

– test conditions:

3.1.1. Description of exposure apparatus:

Including design, type, dimensions, source of air, system for generating particulates and aerosols, method of conditioning air, treatment of exhaust air and the method of housing animals in a test chamber when this is used. The equipment for measuring temperature, humidity and, where appropriate, stability of aerosol concentration or particle size, should be described.

3.1.2. Exposure data:

These should be tabulated and presented with mean values and a measure of variability (e.g. standard deviation) and should include:

(a) air flow rates through the inhalation equipment;

(b) temperature and humidity of air;

(c) nominal concentrations (total amount of test substance fed into the inhalation equipment divided by the volume of air);

(d) nature of vehicle, if used;

(e) actual concentrations in test breathing zone;

(f) median particle sizes (where appropriate),

– dose levels (including vehicle, if used) and concentrations,

– toxic response data by sex and dose,

– no-effect level,

– time of death during the study or whether animals survived to termination,

– description of toxic and other effects,

– the time of observation of each abnormal sign and its subsequent course,

– food and bodyweight data,

– ophthalmological findings,

– haematological tests employed and all results,

– clinical biochemistry tests employed and all results (including results of any urinalysis),

– necropsy findings,

– a detailed description of all histopathological findings,

– statistical treatment of results where possible,

– discussion of the results,

– interpretation of the results.

3.2. EVALUATION AND INTERPRETATION

See General Introduction Part B.

4. REFERENCES

See General Introduction Part B.

B. 31. TERATOGENICITY STUDY - RODENT AND NON-RODENT

1. METHOD

1.1. INTRODUCTION

See General Introduction Part B.

1.2. DEFINITIONS

See General Introduction Part B.

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLE OF THE TEST METHOD

The test substance is administered in graduated doses, or concentrations, for at least that part of the pregnancy covering the period of organogenesis, to several groups of pregnant experimental animals, one dose being used per group. Shortly before the expected date of delivery, the dam is sacrificed, the uterus removed and the contents examined. This test method covers embryo- and fetotoxicity.

1.5. QUALITY CRITERIA

None.

1.6. DESCRIPTION OF THE TEST METHOD

1.6.1. Preparations

Healthy young adult virgin females of comparable age and size are acclimatized to the laboratory conditions for at least five days prior to the test and are then mated with males of established fertility. Inseminated females are randomized and assigned to treatment groups.

Mating may be accomplished naturally or by artificial insemination. The test substance is administered daily to the females beginning soon after implantation and continuing through the period of organogenesis. One day prior to term, foetuses are delivered by hysterectomy and examined for visceral or skeletal abnormalities, including growth retardation, delayed ossification and intestinal haemorrhages.

1.6.2. Test conditions

Experimental animals

Species commonly used are the rat, mouse, hamster and rabbit. The preferred species are the rat and the rabbit. Commonly used laboratory strains should be employed. The strain should not have low fecundity and should be characterized for its response to teratogens. Animals should be caged individually.

Number and sex

At least 20 pregnant rats, mice or hamsters or 12 pregnant rabbits are required at each dose level. The objective is to ensure that sufficient litters and pups are produced to permit an evaluation of the teratogenic potential of the substance.

Dose levels

At least three dose levels and a control should be used. When the test substance is administered in a vehicle, a vehicle control group should also be used. If a vehicle is used its toxicological properties should be understood; it should not be teratogenic or have effects on reproduction. Except for treatment with the test substance, animals in the control group(s) should be handled in an identical manner to the test group subjects. Unless limited by the physical/chemical nature or biological properties of the substance, the highest dose level should ideally induce some overt maternal toxicity such as slight weight loss, but not more than 10% maternal deaths. The low-dose level should not induce observable effects attributable to the test substance. The intermediate dose(s) should be spaced geometrically between the high- and low-dose levels.

Limit test

In the case of substances of low toxicity, if a dose level of at least 1000 mg/kilogram produces no evidence of embryotoxicity or teratogenicity, studies at other dose levels may not be considered necessary.

Exposure time

Day 0 in the test is the day on which vaginal plug and/or sperm are observed (where feasible). The dose period should cover the period of major organogenesis. This may betaken as days 6 to 15 for the rat and mouse, 6 to 14 for hamster, or 6 to 18 for rabbit. If day 0 is based on observation of mating or artificial insemination, the times stated should be adjusted by adding one day. Alternatively, the period of dosing may be extended to approximately one day before the expected delivery date.

Observation period

A careful clinical examination should be made at least once each day. Additional observations should be made daily with appropriate actions taken to minimize loss of animals to the study.

1.6.3. Procedure

The test substance is administered orally by gavage. The test substance should be administered at approximately the same time each day.

The female test animals are treated with the test substance daily throughout the appropriate treatment period. The dose may be based on the weight of the females at the start of the substance administration, or, alternatively, in view of the rapid weight gain which takes place during pregnancy, the animals may be weighed periodically and the dose based on the most

recent weight determination. Signs of toxicity should be recorded as they are observed, including the time of onset, the degree and duration. Females showing signs of abortion or premature delivery should be sacrificed and subjected to thorough macroscopic examination. The post-treatment observation period should continue until approximately one day prior to term; the objective is to cover most of the pregnancy period but to avoid complicating the interpretation of results which could arise following natural birth. Cageside observations should include, but not be limited to, changes in skin and fur, eyes and mucous membranes, as well as respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behaviour pattern. Measurements should be made of food consumption weekly. Animals should be weighed weekly.

Necropsy

At death during, or at the end of, the study the dam should be examined macroscopically for any structural abnormalities or pathological changes which may have influenced the pregnancy. Immediately after death, the uterus should be removed and the contents examined for embryonic or foetal deaths and the number of live foetuses. It is usually possible to estimate the time of death in utero where this has occurred. In rats and rabbits the number of corpora lutea may be determined. The sex of the foetuses should be determined and they should be weighed individually, the weights recorded, and the mean foetal weight derived. Following removal each foetus should be examined externally. For rats, mice and hamsters, one-third to one-half of each litter should be prepared and examined for skeletal anomalies and the remaining part of each litter should be prepared and examined for soft tissue anomalies using appropriate methods. For rabbits, each foetus should be examined by careful dissection for visceral abnormalities and then examined for skeletal anomalies.

2. DATA

Data should be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number which became pregnant, the number and percentages of live foetuses and foetuses with any soft tissue or skeletal abnormalities and their relation to specific litters. Results should be evaluated by an appropriate statistical method. Any recognized statistical method may be used.

3. REPORTING

3.1. TEST REPORT

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

– species, strain, source, environmental conditions, diet,

– test conditions,

– dose levels (including vehicle, if used) and concentrations,

– toxic response data by dose,

– no-effect level (where possible),

– time of death during the study or whether animals survived to termination,
– description of toxic or other effects,
– the time of observation of each abnormal sign and its subsequent course,
– food and bodyweight data,
– duration of pregnancy and litter data (including historical data),
– foetal data (live/dead, sex, soft tissue and skeletal defects),
– litter data (live/dead, sex, soft tissue and skeletal defects for each litter),
– statistical treatment of results,
– discussion of the results,
– interpretation of the results.

3.2. EVALUATION AND INTERPRETATION

See General Introduction Part B.

4. REFERENCES

See General Introduction Part B.

B.32 CARCINOGENICITY TEST

1. METHOD

1.1. INTRODUCTION

See General Introduction Part B.

1.2. DEFINITIONS

See General Introduction Part B.

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLE OF THE TEST METHOD

The test substance is administered normally seven days per week, by an appropriate route, to several groups of experimental animals, one dose per group, for a major portion of their lifespan. During and after exposure to the test substance, the experimental animals are observed daily to detect signs of toxicity, particularly the development of tumours.

1.5. QUALITY CRITERIA

None.

1.6. DESCRIPTION OF THE TEST METHOD

The animals are kept under the experimental housing and feeding conditions for at least five days prior to the test. Before the test healthy young animals are randomized and assigned to the treated and control groups.

1.6.1. Experimental animals

Based upon the results of previously conducted studies other species (rodent or non-rodent) may be used. Commonly used laboratory strains of young healthy animals should be employed and dosing should begin as soon as possible after weaning.

At the commencement of the study the weight variation in the animals used should not exceed ± 20% of the mean value. Where a sub-chronic oral study is conducted as a preliminary to a long-term study, the same species/breed and strain should be used in both studies.

1.6.2. Number and sex

For rodents at least 100 animals (50 female and 50 male) should be used at each dose level and concurrent control 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.

1.6.3. Dose levels and frequency of exposure

At least three dose levels should be used in addition to the concurrent control group. The highest dose level should elicit signs of minimal toxicity, such as a slight depression of bodyweight gain (less than 10%), without substantially altering the normallifespan due to effects other than tumours.

The lowest dose level should not interfere with normal growth, development and longevity of the animal or produce any indication of toxicity. In general, this should not be lower than 10% of the high dose.

The intermediate dose(s) should be established in a mid-range between the high and low doses.

The selection of dose levels should take into account data from preceding toxicity tests and studies.

Frequency of exposure is normally daily. If the chemical is administered in the drinking water or mixed in the diet it should be continuously available.

1.6.4. Controls

A concurrent control group which is identical in every respect to the treated groups, except for exposure to the test substance, should be used.

In special circumstances, such as in inhalation studies involving aerosols or the use of an emulsifier of uncharacterized biological activity in oral studies, an additional control group which is not exposed to the vehicle should be used.

1.6.5. Route of administration

The three main routes of administration are oral, dermal and inhalation. The choice of the route of administration depends upon the physical and chemical characteristics of the test substance and the likely route of exposure in humans.

1.6.5.1. Oral studies

Where the test substance is absorbed from the gastro-intestinal tract, and if the ingestion route is one by which humans may be exposed, the oral route of administration is preferred, unless there are contra-indications. The animals may receive the test substance in their diet, dissolved in drinking water or given by capsule.

Ideally, daily dosing on a seven-day per week basis should be used because dosing on a five-day per week basis may permit recovery or withdrawal toxicity in the non-dosing period and thus affect the result and subsequent evaluation. However, based primarily on practical considerations, dosing on a five-day per week basis is considered to be acceptable.

1.6.5.2. Dermal studies

Cutaneous exposure by skin painting may be selected to simulate a main route of human exposure and as a model system for induction of skin lesions.

1.6.5.3. Inhalation studies

Because inhalation studies present technical problems of greater complexity than the other routes of administration, more detailed guidance on this mode of administration is given here. It should be noted that intratracheal instillation may constitute a valid alternative in specific situations.

Long-term exposures are usually patterned on projected human exposure, giving the animals either a daily exposure of six hours after equilibration of chamber concentrations, for five days a week (intermittent exposure), or, relevant to possible environmental exposure, 22 to 24 hours of exposure per day for seven days a week (continuous exposure), with about an hour for feeding the animals daily at a similar time and maintaining the chambers. In both cases, the animals are usually exposed to fixed concentrations of test substance.

A major difference between intermittent and continuous exposure is that with the former there is a 17 to 18 hour period in which animals may recover from the effects of each daily exposure with an even longer recovery period during weekends.

The choice of intermittent or continuous exposure depends on the objectives of the study and on the human exposure that is to be simulated. However, certain technical difficulties must be considered. For example, the advantages of continuous exposure for simulating environmental conditions may be offset by the necessity for watering and feeding during exposure and by the need for more complicated (and reliable) aerosol and vapour generation and monitoring techniques.

1.6.6. Exposure chambers

The animals should be tested in inhalation chambers designed to sustain a dynamic flow of at least 12 air changes per hour to assure adequate oxygen content and an evenly distributed exposure atmosphere. Control and exposure chambers should be identical in construction and design to ensure exposure conditions comparable in all respects except for exposure to the test substances. Slight negative pressure inside the chamber is generally maintained to prevent leakage of the test substance into the surrounding area. The chambers should minimize the crowding of test animals. As a general rule, to ensure the stability of the chamber atmosphere, the total volume of the test animals should not exceed 5% of the volume of the chamber.

Measurements or monitoring should be made of:

(i) Air flow: the rate of air flow through the chamber should preferably be monitored continuously;

(ii) Concentration: during the daily exposure period the concentration of the test substance should not vary more than ± 15% of the mean value. During the total duration of this study, the day-to-day concentrations should be held as constant as practicable;

(iii) Temperature and humidity: for rodents, the temperature should be maintained at 22 ± 2 °C and the humidity within the chamber at 30 to 70%, except when water is used to

suspend the test substance in the chamber atmosphere. Preferably both should be monitored continuously;

(iv) Particle size measurements: particle size distribution should be determined in chamber atmospheres involving liquid or solid aerosols. The aerosol particles should be of respirable size for the test animal used. Samples of the chamber atmospheres should be taken in the breathing zone of the animals. The air sample should be representative of the distribution of the particles to which the animals are exposed and should account, on a gravimetric basis, for all of the suspended aerosol even when much of the aerosol is not respirable. Particle size analyses should be carried out frequently during the development of the generating system to ensure the stability of the aerosol and thereafter as often as necessary during the exposures to determine adequately the consistency of the particle distribution to which the animals have been exposed.

1.6.7. Duration of study

The duration of a carcinogenicity test comprises the major portion of the normal lifespan of the test animals. The termination of the test should be at 18 months for mice and hamsters and 24 months for rats; however, for certain strains of animals with greater longevity and/or low spontaneous tumour rate, termination should be at 24 months for mice and hamsters and at 30 months for rats. Alternatively, termination of such an extended study is acceptable when the number of survivors in the lowest dose or control group reaches 25%. When terminating a test in which there is an apparent sex difference in response, each sex should be considered separately. Where only the high-dose group dies prematurely for obvious reasons of toxicity, this need not trigger termination providing toxic manifestations are not causing problems in the other groups. For a negative test result to be acceptable, not more than 10% of any group may be lost from the experiment due to autolysis, cannibalism or management problems and the survival of all groups is not less than 50% at 18 months for mice and hamsters and at 24 months for rats.

1.6.8. Procedure

1.6.8.1. Observations

Daily cageside observations should include changes in skin and fur, eyes and mucous membranes as well as respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behaviour pattern.

Regular observations of the animals is necessary to ensure that, as far as possible, animals are not lost from the study due to causes such as cannibalism, autolysis of tissues or misplacement. Moribund animals should be removed and necropsied when noticed.

Clinical signs and mortality should be recorded for all animals. Special attention must be paid to tumour development: the time of onset; location, dimensions, appearance and progression of each grossly visible or palpable tumour should be recorded.

Measurements should be made of food consumption (and water consumption when the test substance is administered in the drinking water) weekly during the first 13 weeks of the study and then at approximately three-month intervals unless health status or body weight changes dictate otherwise.

Bodyweights should be recorded individually for all animals once a week during the first 13 weeks of the test period and at least once every four weeks thereafter.

1.6.8.2. Clinical examinations

Haematology

If cage side observations suggest a deterioration in health of the animals during the study, a differential blood count of the affected animals should be performed.

At 12 months, 18 months, and prior to sacrifice, a blood smear is obtained from the animals. A differential blood count is performed on samples from the animals in the high-dose group and the controls. If these data, particularly those obtained prior to sacrifice, or data from the pathological examination indicate a need, differential blood counts should be performed on the next-lower group(s) as well.

Gross necropsy

Full gross necropsy should be performed on all animals, including those which died during the experiment or were sacrificed having been found in a moribund condition. All grossly visible tumours or lesions, or lesions suspected of being tumours, should be preserved.

The following organs and tissues should be preserved in suitable media for possible future histopathological examination: brain (including sections of medulla/pons, cerebellar cortex, cerebral cortex), pituitary, thyroid/parathyroid, any thymic tissue, 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, femur (including joint), spinal cord at three levels (cervical, mid-thoracic and lumbar) and eyes.

Inflation of lungs and urinary bladder with a fixative is the optimal way to preserve these tissues; inflation of the lungs in inhalation studies is essential for appropriate histopathological examination. In inhalation studies, the entire respiratory tract should be preserved, including nasal cavity , pharynx and larynx.

Histpathology

(a) Full histpatology should be carried out on the organs and tissues of all animals that died or were sacrificed during the test and all animals in the control and high-dose groups.

(b) All grossly visible tumours or lesions suspected of being tumours should be examined microscopically in all groups.

(c) If there is a significant difference in the incidence of neoplastic lesions in the high-dose and control groups, histopathology should be carried out on that particular organ or tissue in the other groups.

(d) If the survival of the high-dose group is substantially less than the control then the next-lower dose group should be examined fully.

(e) If there is evidence in the high-dose group of the induction of toxic or other effects that might affect a neoplastic response, the next-lower dose level should be examined fully.

2. DATA

Data should be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing tumours detected during the test, the time of detection and the number of animals found to have tumours following sacrifice. Results should be evaluated by an appropriate statistical method. Any recognized statistical method may be used.

3. REPORTING

3.1. TEST REPORT

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

– species, strain, source, environmental conditions, diet,

– test conditions:

3.1.1. Description of exposure apparatus:

including design, type, dimensions, source of air, system for generating particulates and aerosols, method of conditioning air, treatment of exhaust air and the method of housing animals in a test chamber when this is used. The equipment for measuring temperature, humidity and, where appropriate, stability of aerosol) concentration or particle size, should be described.

3.1.2. Exposure data:

These should be tabulated and presented with mean values and a measure of variability (e.g. standard deviation) and should include:

(a) air flow rates through the inhalation equipment;

(b) temperature and humidity of air;

(c) nominal concentrations (total amount of test substance fed into the inhalation equipment divided by the volume of air);

(d) nature of vehicle, if used;

(e) actual concentrations in test breathing zone;

(f) median particle sizes (where appropriate),

– dose levels (including vehicle, if used) and concentrations,

– tumour incidence data by sex, dose and tumour type,

- time of death during the study or whether animals survived to termination,
- toxic response data by sex and dose,
- description of toxic or other effects,
- the time of observation of each abnormal sign and its subsequent course,
- food and bodyweight data,
- haematological tests employed and all results,
- necropsy findings,
- a detailed description of all histopathological findings,
- statistical treatment of results with a description of the methods used,
- discussion of the results,
- interpretation of the result.

3.2. EVALUATION AND INTERPRETATION

See General Introduction Part B.

4. REFERENCES

See General Introduction Part B.

B.33 COMBINED CHRONIC TOXICITY / CARCINOGENICITY TEST

1. METHOD

1.1. INTRODUCTION

See General Introduction Part B.

1.2. DEFINITIONS

See General Introduction Part B.

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLE OF THE TEST METHOD

The objective of a combined chronic toxicity carcinogenicity test is to determine the chronic and carcinogenic effects of a substance in a mammalian species following prolonged exposure.

To this end a carcinogenicity test is supplemented with a least one treated satellite group and a control satellite group. The dose used for the high-dose satellite group may be higher than that used for the high-dose group in the carcinogenicity test. The animals in the carcinogenicity test are examined for general toxicity as well as for carcinogenic response. The animals in the treated satellite group are examined for general toxicity.

The test substance is administered normally seven days per week, by an appropriate route, to several groups of experimental animals, one dose per group, for a major portion of their lifespan. During and after exposure to the test substance, the experimental animals are observed daily to detect signs of toxicity and the development of tumours.

1.5. QUALITY CRITERIA

None.

1.6. DESCRIPTION OF THE TEST METHOD

The animals are kept under the experimental housing and feeding conditions for at least five days prior to the test. Before the test healthy young animals are randomized and assigned to the treated and control groups.

1.6.1. Experimental animals

The preferred species is the rat. Based upon the results of previously conducted tests other species (rodent or non-rodent) may be used. Commonly used laboratory strains of young healthy animals should be employed and dosing should begin as soon as possible after weaning.

At the commencement of the test the weight variation in the animals used should not exceed ± 20% of the mean value. Where a sub-chronic oral test is conducted as a preliminary to a long-term test, the same species and breed/strain should be used in both studies.

1.6.2. Number and sex

For rodents, at least 100 animals (50 female and 50 male) should be used at each dose level and concurrent control 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 treated satellite group(s) for the evaluation of pathology other than tumours should contain 20 animals of each sex, while the satellite control group should contain 10 animals of each sex.

1.6.3. Dose levels and frequency of exposure

For carcinogenicity testing purposes, at least three dose levels should be used in addition to the concurrent control group. The highest dose level should elicit signs of minimal toxicity, such as a slight depression of body weight gain (less than 10%), without substantially altering the normal lifespan due to effects other than tumours.

The lowest dose level should not interfere with normal growth, development and longevity of the animal or produce any indication of toxicity. In general, this should not be lower than 10% of the high dose.

The intermediate dose(s) should be established in a mid-range between the high and low doses.

The selection of dose levels should take into account data from preceding toxicity tests and studies.

For chronic toxicity testing purposes, additional treated groups and a concurrent control satellite group are included in the test. The high dose for treated satellite animals should produce definite signs of toxicity.

Frequency of exposure is normally daily. If the chemical is administered in the drinking water or mixed in the diet, it should be continuously available.

1.6.4. Controls

A concurrent group which is identical in every respect to the treated groups, except for exposure to the test substance; should be used.

In special circumstances, such as in inhalation studies involving aerosols or the use of an emulsifier of uncharacterized biological activity in oral studies, an additional control group which is not exposed to the vehicle should be utilized.

1.6.5. Route of administration

The three main routes of administration are oral, dermal and inhalation. The choice of the route of administration depends upon the physical and chemical characteristics of the test substance and the likely route of exposure in humans.

1.6.5.1. Oral tests

Where the test substance is absorbed from the gastro-intestinal tract and the ingestion route is one by which humans may be exposed, the oral route of administration is preferred, unless there are contra-indications. The animals may receive the test substance in their diet, dissolved in drinking water or given by capsule. Ideally, daily dosing on a seven-day per week basis should be used because dosing on a five-day per week basis may permit recovery or withdrawal toxicity in the non-dosing period and thus affect the result and subsequent evaluation. However, based primarily on practical considerations, dosing on a five-day per week basis is considered to be acceptable.

1.6.5.2. Dermal tests

Cutaneous exposure by skin painting may be selected to simulate a main route of human exposure and as a model system for induction of skin lesions.

1.6.5.3. Inhalation tests

Because inhalation tests present technical problems of greater complexity than the other routes of administration, more detailed guidance on this mode of administration is given here. It should be noted that intratracheal instillation may constitute a valid alternative in specific situations.

Long-term exposures are usually patterned on projected human exposure, giving the animals either a daily exposure of six hours after equilibration of chamber concentrations, for five days a week (intermittent exposure), or, relevant to possible environmental exposure, 22 to 24 hours of exposure per day for seven days a week (continuous exposure), with about an hour for feeding the animals daily at a similar time and maintaining the chambers. In both cases, the animals are usually exposed to fixed concentrations of test substance. A major difference between intermittent and continuous exposure is that, with the former, there is a 17 to 18 hour period in which animals may recover from the effects of each daily exposure, with an even longer recovery period during weekends.

The choice of intermittent or continuous exposure depends on the objectives of the test and on the human exposure that is to be simulated. However, certain technical difficulties must be considered. For example, the advantages of continuous exposure for simulating environmental conditions may be offset by the necessity for watering and feeding during exposure and by the need for more complicated (and reliable) aerosol and vapour generation and monitoring techniques.

1.6.6. Exposure chambers

The animals should be tested in inhalation chambers designed to sustain a dynamic flow of at least 12 air changes per hour to assure adequate oxygen content and an evenly distributed exposure atmosphere. Control and exposure chambers should be identical in construction and design to ensure exposure conditions comparable in all respects except for exposure to the test substances. Slight negative pressure inside the chamber is generally maintained to prevent leakage of the test substance into the surrounding area. The chambers should minimize the crowding of test animals. As a general rule, to ensure the stability of the chamber atmosphere, the total volume of the test animals should not exceed 5% of the volume of the chamber.

Measurements or monitoring should be made of:

(i) Air flow: the rate of air flow through the chamber should preferably be monitored continuously;

(ii) Concentration: during the daily exposure period the concentration should not vary more than ± 15% of the mean value. During the total duration of this study, the day-to-day concentrations should be held as constant as practicable;

(iii) Temperature and humidity: for rodents, the temperature should be maintained at 22 ± 2 °C, and the humidity within the chamber at 30 to 70%, except when water is used to suspend the test substance in the chamber atmosphere. Preferably both should be monitored continuously;

(iv) Particle size measurements: particle size distribution should be determined in chamber atmospheres involving liquid or solid aerosols. The aerosol particles should be of respirable size for the test animal used. Samples of the chamber atmospheres should be taken in the breathing zone of the animals. The air sample should be representative of the distribution of the particles to which the animals are exposed and should account, on a gravimetric basis, for all of the suspended aerosol even when much of the aerosol is not respirable. Particle size analyses should be carried out frequently during the development of the generating system to ensure the stability of the aerosol and thereafter as often as necessary during the exposures to determine adequately the consistency of the particle distribution to which the animals have been exposed.

1.6.7. Duration of test

The duration of the carcinogenicity part of the test comprises the major portion of the normal life span of the test animals. The termination of the test should be at 18 months for mice and hamsters and 24 months for rats; however, for certain strains of animals with greater longevity and/or low spontaneous tumour rate, termination should be at 24 months for mice and hamsters and at 30 months for rats. Alternatively, termination of such an extended test is acceptable when the number of survivors in the lowest dose or control group reaches 25%. When terminating a test in which there is an apparent sex difference in response, each sex should be considered separately. Where only the high-dose group dies prematurely for obvious reasons of toxicity, this need not trigger termination providing toxic manifestations are not causing problems in the other groups. For a negative test result to be acceptable not more than 10% of any group may be lost from the experiment due to autolysis, cannibalism or management problems, and the survival of all groups is not less than 50 at 18 months for mice and hamsters and at 24 months for rats.

The satellite groups of 20 dosed animals per sex and 10 associated control animals per sex used for chronic toxicity testing should be retained in the test for at least 12 months. These animals should be scheduled for sacrifice for an examination of test-substance-related pathology uncomplicated by gerontological changes.

1.6.8. Procedure

1.6.8.1. Observations

Daily cageside observations should be made and should include changes in skin and fur, eyes and mucous membranes as well as respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behaviour pattern.

Clinical examination should be performed at appropriate intervals on animals in the treated satellite group(s).

Regular observations of the animals is necessary to ensure, as far as possible, that animals are not lost from the test due to causes such as cannibalism, autolysis of tissues or misplacment. Moribund animals should be removed and necropsied when noticed.

Clinical signs, including neurological and ocular changes as well as mortality should be recorded for all animals. Special attention must be paid to tumour development: the time of onset, location, dimensions, appearance and progression of each grossly visible or palpable tumour should be recorded; the time of onset and progression of toxic conditions should be recorded.

Measurements should be made of food consumption (and water consumption when the test substance is administered in the drinking water) weekly during the first 13 weeks of the study and then at approximately three-month intervals unless health status or body weight changes dictate otherwise.

Bodyweights should be recorded individually for all animals once a week during the first 13 weeks of the test period and at least once every four weeks thereafter.

1.6.8.2. Clinical examinations

Haematology

Haematological examination (e.g. haemoglobin content, packed cell volume, total red blood cells, total white blood cells, platelets, or other measures of clotting potential) should be performed at three months, six months and at approximately six-month intervals thereafter, and at termination on blood samples collected from 10 rats/sex of all groups. If possible, samples should be from the same rats at each interval.

If cageside observations suggest a deterioration in the health of the animals during the study, a differential blood count of the affected animals should be performed.

A differential blood count is performed on samples of those animals in the highest dose group and the controls. Differential blood counts are performed for the next lower group(s) only if there is a major discrepancy between the highest group and the controls, or if indicated by pathological findings.

Urinalysis

Urine samples from 10 rats/sex of all groups, if possible from the same rats at the same intervals as haematological examination, should be collected for analysis. The following determinations should be made from either individual animals or on a pooled sample/sex/group of rodents:

– appearance: volume and density for individual animals,

– protein, glucose, ketones, occult blood (semi-quantitatively),

– microscopy of sediment (semi-quantitatively).

Clinical chemistry

At approximately six-monthly intervals, and at termination, blood samples are drawn for clinical chemistry measurements from all non-rodents and 10 rats/sex of all groups, if possible, from the same rats at each interval. In addition, a pre-test sample should be collected from non-rodents. Plasma is prepared from these samples and the following determinations are made:

– total protein concentration,

– albumin concentration,

– liver function tests (such as alkaline phosphatase activity, glutamic pyruvic transaminase ([10]) activity and glutamic oxaloacetic transaminase ([11]) activity), gamma glutamyl transpeptidase, ornithine decarboxylase,

– carbohydrate metabolism such as fasting blood glucose,

– kidney function tests such as blood urea nitrogen.

Gross necropsy

Full gross necropsy should be performed in all animals, including those which died during the experiment or were sacrificed having been found in a moribund condition. Prior to sacrifice, samples of blood should be collected from all animals for differential blood counts. All grossly visible tumours or lesions suspected of being tumours should be preserved. An attempt should be made to correlate gross observations with the microsopic findings.

All organs and tissues should be preserved for histopathological examination. This usually concerns the following organs and tissues: brain ([12]) (medulla/pons, cerebellar cortex, cerebral cortex); pituitary, thyroid (including parathyroid), thymus, lungs (including trachea), heart, aorta, salivary glands, liver ([1]), spleen, kidneys ([1]), adrenals ([1]), oesophagus, stomach, duodenum, jejunum, ileum, caecum, colon, rectum, urinary bladder, lymph nodes, pancreas, gonads ([1]), accessory genital organs; female mammary gland, skin, musculature, peripheral nerve, spinal cord (cervical, thoracic, lumbar), sternum with bone marrow and femur (including joint) and eyes.

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[10] Now known as serum alanine aminotransferase.

[11] Now known as serum aspartate aminotransferase.

[12] These organs, from 10 animals per sex per groups for rodents, should be weighed.

Although inflation of lungs and urinary bladder with a fixative is the optimal way to preserve these tissues, inflation of the lungs in inhalation studies is a necessary requirement for appropriate histopathological examination. In special studies such as inhalation studies, the entire respiratory tract should be studied, including nose, pharynx and larynx.

If other clinical examinations are carried out, the information obtained from these procedures should be available before microsopic examination, because it may give significant guidance to the pathologist.

Histopathology

For the chronic toxicity testing portion:

Detailed examination should be made of all preserved organs of all animals of the satellite high-dose and control groups. Where test-substance-related pathology is found in the high-dose satellite group, target organs of all other animals in any other treated satellite group should be subjected to full and detailed histological examination as well as those of the heated groups in the carcinogenicity testing portion of the study at its termination.

For the carcinogenicity testing portion:

(a) Full histopathology should be carried out on the organs and tissues of all animals that died or were sacrificed during the test, and of all animals in the control and high-dose groups;

(b) All grossly visible tumours or lesions suspected of being tumours in all groups occurring in any organ should be examined microscopically;

(c) If there is a significant difference in the incidence of neoplastic lesions in the high-dose and control groups, histopathology should be carried out on that particular organ or tissue in the other groups;

(d) If the survival of the high-dose group is substantially less than the control then the next-lower dose group should be examined fully;

(e) If there is evidence in the high-dose group of the induction of toxic or other effects that might affect a neoplastic response, the next-lower dose level should be examined fully.

2. DATA

Data should be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing tumours or toxic effects detected during the test, the time of detection and the number of animals found to have tumours following sacrifice. Results should be evaluated by an appropriate statistical method. Any recognized statistical method may be used.

3. REPORTING

3.1. TEST REPORT

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

– species, strain source, environmental conditions, diet,

– test conditions:

3.1.1. Description of exposure apparatus:

including design, type, dimensions, source of air, system for generating particulates and aerosols, method of conditioning air, treatment of exhaust air and the method of housing animals in a test chamber when this is used. The equipment for measuring temperature, humidity and, where appropriate, stability of aerosol concentration or particle size, should be described.

3.1.2. Exposure data:

These should be tabulated and presented with mean values and a measure of variability (e.g. standard deviation), and should include:

(a) air flow rates through the inhalation equipment;

(b) temperature and humidity of air;

(c) nominal concentrations (total amount of test substance fed into the inhalation equipment divided by the volume of air);

(d) nature of vehicle, if used;

(e) actual concentrations in test breathing zone;

(f) median particle sizes (where appropriate),

– dose levels (including vehicle, if used) and concentrations,

– tumour incidence data by sex, dose and tumour type;

– time of death during the study or whether animals survived to termination, including satellite group,

– toxic response data by sex and dose,

– description of toxic or other effects,

– the time of observation of each abnormal sign and its subsequent course,

– ophthalmological findings,

– food and bodyweight data,

- haematological tests employed and all results,
- clinical biochemistry test employed and all results (including any urinalysis),
- necropsy findings,
- a detailed description of all histopathological findings,
- statistical treatment of results with a description of the methods used,
- discussion of the results,
- interpretation of the results.

3.2. EVALUATION AND INTERPRETATION

See General Introduction Part B.

4. REFERENCES

See General Introduction Part B.

B. 34. ONE-GENERATION REPRODUCTION TOXICITY TEST

1. METHOD

1.1. INTRODUCTION

See General Introduction Part B.

1.2. DEFINITIONS

See General Introduction Part B.

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLE OF THE TEST METHOD

The test substance is administered in graduated doses to several groups of males and females. Males should be dosed during growth and for at least one complete spermatogenic cycle (approximately 56 days in the mouse and 70 days in the rat) in order to elicit any adverse effects on spermatogenesis by the test substance.

Females of the parental (P) generation should be dosed for at least two complete oestrous cycles in order to e adverse effects on oestrus by the test substance. The animals are then mated. The test substance is administered to both sexes during the mating period and thereafter only to females during pregnancy and for the duration of the nursing period.

For administration by inhalation the method will require modification.

1.5. QUALITY CRITERIA

None.

1.6. DESCRIPTION OF THE TEST METHOD

1.6.1. Preparations

Before the test, healthy young adult animals are randomized and assigned to the treated and control groups. The animals are kept under the experimental housing and feeding conditions for at least five days prior to the test.

It is recommended that the test substance be administered in the diet or drinking water. Other routes of administration are also acceptable. All animals should be dosed by the same method during the appropriate experimental period. If a vehicle or other additives are used to facilitate dosing, they should be known not to produce toxic effects.

Dosing should be on a seven-day per week basis.

1.6.2. Experimental animals

Selection of species

The rat or mouse are the preferred species. Healthy animals, not subjected to previous experimental procedures, should be used. Strains with low fecundity should not be used. The test animals should be characterized as to species, strain, sex, weight and/or age.

For an adequate assessment of fertility, both males and females should be studied. All test and control animals should be weaned before dosing begins.

Number and sex

Each treated and control group should contain a sufficient number of animals to yield about 20 pregnant females at or near term.

The objective is to produce enough pregnancies and offspring to assure a meaningful evaluation of the potential of the substance to affect fertility, pregnancy and maternal behaviour in P generation animals and suckling, growth and development of the F_1 offspring from conception to weaning.

1.6.3. Test conditions

Food and water should be provided ad libitum. Near parturition, pregnant females should be caged separately in delivery or maternity cages and may be provided with nesting materials.

1.6.3.1. Dose levels

At least three treated groups and a control group should be used. If a vehicle is used in administering the test substance, the control group should receive the vehicle in the highest volume used. If a test substance causes reduced dietary intake or utilization, then the use of a paired fed control group may be considered necessary. Ideally, unless limited by the physical/chemical nature or biological effects of the test substance, the highest dose level should induce toxicity but not mortality in the parental (P) animals. The intermediate dose(s) should induce minimal toxic effects attributable to the test substance, and the low dose should not induce any observable adverse effects on the parents or offspring. When administered by gavage or capsule the dosage given to each animal should be based on the individual animal's body weight and adjusted weekly for changes in body weight. For females during pregnancy, dosages may be based on the body weight at day 0 or 6 of the pregnancy, if desired.

1.6.3.2. Limit test

In the case of substances of low toxicity, if a dose level of at least 1000 mg/kilogram produces no evidence of interference with reproductive performance, studies at other dose levels may not be considered necessary. If a preliminary study at the high-dose level, with definite evidence of maternal toxicity, shows no adverse effects on fertility, studies at other dose levels may not be considered necessary.

1.6.3.3. Performance of the test

Experimental schedules

Daily dosing of the parental (P) males should begin when they are about five to nine weeks of age, after they have been weaned and acclimatized for at least five days. In rats, dosing is continued for 10 weeks prior to the mating period (for mice, eight weeks). Males should be killed and examined either at the end of the mating period or, alternatively, males may be retained on the test diet for the possible production of a second litter and should be killed and examined at some time before the end of the study. For parental (P) females dosing should begin after at least five days of acclimatization and continue for at least two weeks prior to mating. Daily dosing of the p females should continue throughout the three-week mating period, pregnancy and up to the weaning of the F_1 offspring. Consideration should be given to modification of the dosing schedule based on other available information on the test substance, such as induction of metabolism or bioaccumulation.

Mating procedure

Either 1:1 (one male to one female) or 1:2 (one male to two females) mating may be used in reproduction toxicity studies.

Based on 1:1 mating, one female should be placed with the same male until pregnancy occurs or three weeks have elapsed. Each morning the females should be examined for presence of sperm or vaginal plugs. Day 0 of pregnancy is defined as the day a vaginal plug or sperm is found.

Those pairs that fail to mate should be evaluated to determine the cause of the apparent infertility. This may involve such procedures as providing additional opportunities to mate with other proven sires or dams, microscopic examination of the reproductive organs, and examination of the oestrous cycle or spermatogenesis.

Litter sizes

Animals dosed during the fertility study are allowed to litter normally and rear their progency to the stage of weaning without standardization of litters.

Where standardization is done, the following procedure is suggested. Between day 1 and day 4 after birth, the size of each litter may be adjusted by eliminating extra pups by selection to yield, as nearly as possible, four males and four females per litter. Whenever the number of male or female pups prevents having four of each sex per litter, partial adjustment (for example, five males and three females) is acceptable. Adjustments are not applicable for litters of less than eight pups.

1.6.4. Observations

Throughout the test period, each animal should be observed at least once daily. Pertinent behavioural changes, signs of difficult or prolonged parturition, and all signs of toxicity, including mortality, should be recorded. During pre-mating and mating periods, food consumption may be measured daily. After parturition and during lactation, food consumption measurements (and water consumption measurements when the test substance is administered in the drinking water) should be made on the same day as the weighing of the litter. P males and females should be weighed on the first day of dosing and weekly thereafter. These observations should be reported individually for each adult animal.

The duration of gestation should be calculated from day 0 of pregnancy. Each litter should be examined as soon as possible after delivery to establish the number and sex of pups, still births, live births and the presence of gross anomalies.

Dead pups and pups sacrificed at day 4 should be preserved and studied for possible defects. Live pups should be counted and litters weighed on the morning after birth and on days 4 and 7 and weekly thereafter until the termination of the study, when animals should be weighed individually. Physical or behavioural abnormalities observed in the dams or offspring should be recorded.

1.6.5. Pathology

1.6.5.1. Necropsy

At the time of sacrifice or death during the study the animals of the P generation should be examined macroscopically for any structural abnormalities or pathological changes, with special attention being paid to the organs of the reproductive system. Dead or moribund pups should be examined for defects.

1.6.5.2. Histopathology

The ovaries, uterus, cervix, vagina, testes, epididymes, seminal vesicles, prostate, coagulating gland, pituitary gland and target organ(s) of all P animals should be preserved for microscopic examination. In the event that these organs have not been examined in other multiple-dose studies, they should be microscopically examined in all high-dose and control animals and animals which die during the study where practicable.

Organs showing abnormalities in these animals should then be examined in all other P animals. In these instances, microscopic examination should be made of all tissues showing gross pathological changes. As suggested under mating procedures, reproductive organs of animals suspected of infertility may be subjected to microscopic examination.

2. DATA

Data may be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of fertile males, the number of pregnant females, the types of changes and the percentage of animals displaying each type of change.

When possible, numerical results should be evaluated by an appropriate statistical method. Any generally accepted statistical method may be used.

3. REPORTING

3.1. TEST REPORT

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

– species/strain used;

– toxic response data by sex and dose, including fertility, gestation and viability,

– time of death during the study or whether animals survived to time of scheduled sacrifice or to termination of the study,

– table presenting the weights of each litter, the mean: pup weights and the individual weights of the pups at termination,

– toxic or other effects on reproduction, offspring and postnatal growth,

– the day of observation of each abnormal sign and its subsequent course,

– bodyweight data for P animals,

– necropsy findings,

– a detailed description of all microscopic findings,

– statistical treatment of results, where appropriate,

– discussion of the results,

– interpretation of the results.

3.2. EVALUATION AND INTERPRETATION

See General Introduction Part B.

4. REFERENCES

See General Introduction Part B.

B. 35. TWO-GENERATION REPRODUCTION TOXICITY TEST

1. METHOD

1.1. INTRODUCTION

See General Introduction Part B.

1.2. DEFINITIONS

See General Introduction Part B.

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLE OF THE TEST METHOD

The test substance is administered in graduated doses to several groups of males and females. Males of the parental (P) generation should be dosed during growth and for at least one complete spermatogenic cycle: (approximately 56 days in the mouse and 70 days in the rat) in order to elicit any adverse effects on spermatogenesis by the test substance.

Females of the parental (P) generation should be dosed for at least two complete oestrous cycles in order to elicit any adverse effects on oestrus by the test substance. The animals are then mated. The test substance is administered to both sexes during the mating period and thereafter only to females during pregnancy and for the duration of the nursing period. At weaning the administration of the substance is continued to F_1 offspring during their growth into adulthood, mating and production of an F_2 generation, until the F_2 generation is weaned. For administration by inhalation the method will require modification.

1.5. QUALITIY CRITERIA

None.

1.6. DESCRIPTION OF THE TEST METHOD

Preparations

Before the test, healthy animals are randomized and assigned to the treated and control groups. The parental (P) animals are kept under the experimental housing and feeding conditions for at least five days prior to the test. It is recommended that the test substance be administered in the diet or drinking water. Other routes of administration are also acceptable. All animals should be dosed by the same method during the entire experimental period. If a vehicle or other additive is used to facilitate dosing, they should be known not to produce toxic effects. Dosing should be on a seven-day per week basis.

Experimental animals: selection of species

The rat or mouse are the preferred species.

Healthy P animals, not subjected to previous experimental procedures, should be used. Strains with low fecundity should not be used. The test animals should be characterized as to species, strain, sex, weight and/or age.

For an adequate assessment of fertility, both males and females should be studied. All test and control animals should be weaned before dosing begins.

Number and sex

Each treated and control group should contain a sufficient number of animals to yield about 20 pregnant females at or near term. The objective is to produce enough pregnancies and offspring to assure a meaningful evaluation of the potential of the substance to affect fertility, pregnancy and maternal behaviour and suckling, growth and development of the F_1 offspring from conception to maturity, and the development of their offspring (F_2) to weaning.

Test conditions

Food and water should be provided ad libitum. Near parturition, pregnant females should be caged separately in delivery or maternity cages and may be provided with nesting materials.

Dose levels

At least three treatment groups and a control group should be used. If a vehicle is used in administering the test substance, the control group should receive the vehicle in the highest volume used. If a test substance causes reduced dietary intake or utilization, then the use of a paired fed control group may be considered necessary. Ideally, unless limited by the physical/chemical nature or biological effects of the test substance, the highest dose level should induce toxicity but not mortality in the parental (P) animals. The intermediate dose(s) should induce minimal toxic effects attributable to the test substance, and the low dose should not induce any observable adverse effects on the parents or offspring. When administered by gavage or capsule the dosage given to each animal should be based on the individual animal's body weight and adjusted weekly. For females during pregnancy, the dosage may be based on the body weight at day 0 or 6 of the pregnancy, if desired.

Limit test

In the case of substances of low toxicity, if a dose level of a least 1000 mg/kilogram produces no evidence of interference with reproductive performance, studies at other dose levels may not be considered necessary. If a preliminary study at the high-dose level, with definite evidence of maternal toxicity, shows no adverse effects on fertility, studies at other dose levels may not be considered necessary.

Performance of the test

Experimental schedules

Daily dosing of the parental (P) males should begin when they are about five to nine weeks old, after they have been weaned and acclimatized for at least five days. In rats dosing is continued for 10 weeks prior to the mating period (for mice, eight weeks). Males should be

killed and examined either at the end of the mating period or, alternatively, males may be retained on the test diet for the possible production of a second litter and should be killed and examined at some time before the end of the experiment.

For parental (P) females, dosing should begin after at least five days of acclimatization and continue for at least two weeks prior to mating. Daily dosing of the P females should continue throughout the three-week mating period, pregnancy and up to the weaning of the F_1 offspring. Consideration should be given to modifications of the dosing schedule based on other available information on the test substance, such as induction of metabolism or bioaccumulation.

Dosing of the F_1 animals begins at weaning and ends when they are sacrificed.

Mating procedure

Either 1:1 (one male to one female) or 1:2 (one male to two females) matings may be used in reproduction toxicity studies.

Based on 1:1 mating, one female should be placed with the same male until pregnancy occurs or three weeks have elapsed. Each morning the females should be examined for presence of sperm or vaginal plugs. Day 0 of pregnancy is defined as the day a vaginal plug or sperm is found. Taking into account spermiogenesis the F_1 offspring should not be mated until they are aged at least 11 weeks for mice, 13 weeks for rats. For mating the F_1 offspring, one male and one female are randomly selected from each litter for cross-mating with a pup of another litter of the same dose group to produce the F_2 generation. F_1 males and females not selected for mating are killed at weaning.

Those pairs that fail to mate should be evaluated to determine the cause of the apparent infertility. This may involve such procedures as additional opportunities to mate with other proven sires or dams, microscopic examination of the reproductive organs, and examination of the oestrous cycles or spermatogenesis.

Litter sizes

Animals dosed during the fertility study are allowed 1:0 litter normally and rear their progeny to the stage of weaning without standardization of litters.

Where standardization is done, the following procedure is suggested. Between days 1 and 4 after birth, the size of each litter may be adjusted by eliminating extra pups by selection to yield, as nearly as possible, four males and four females per litter. Whenever the number of male or female pups prevents having four of each sex per litter, partial adjustment (for example, five males and three females) is acceptable. Adjustments are not applicable for litters of less than eight pups. Adjustments of the F_2 litters are conducted in the same manner.

Observations

Throughout the test period, each animal should be observed at least once daily. Pertinent behavioural changes, signs of difficult or prolonged parturition and all signs of toxicity, including mortality, should be recorded. During pre-mating and mating periods, food consumption may be measured weekly. Optionally, during pregnancy, food consumption may be measured daily. After parturition and during lactation, food consumption measurements should be made on the same day as the weighing of litters. Parental animals (P and F_1) should

be weighed on the first day of dosing and weekly thereafter. These observations should be reported individually for each adult animal.

The duration of gestation should be calculated from day 0 of pregnancy. Each litter should be examined as soon as possible after delivery to establish the number and sex of pups, still births, live births and the presence of gross anomalies.

Dead pups and pups killed at day 4 should be preserved and studied for possible defects. Live pups should be counted and litters weighed on the morning after birth and on days 4 and 7 and weekly thereafter until the termination of the study, when animals should be weighed individually. Physical or behavioural abnormalities observed in the dams or offspring should be recorded.

Pathology

Necropsy

All P and F_1 adult animals should be killed when they are no longer necessary to assess reproductive effects. F_1 offspring not selected for mating and all F_2 offspring should be killed when weaned.

At the time of sacrifice or death during the study all parental animals (P and F_1) should be examined microscopically for any structural abnormalities or pathological changes, with special attention paid to the organs of the reproductive system. Dead or moribund pups should be examined for defects.

Histopathology

The ovaries, uterus, cervix, vagina, testes, epididymes, seminal vesicles, coagulating gland, prostate, pituitary gland and target organ(s) of all P and F_1 animals should be preserved for microscopic examination. In the event that these organs have not been examined in other multiple-dose studies, they should be examined microscopically in all high-dose and control P and F_1 animals selected for mating and, where practicable, in animals which die during the study. Organs showing abnormalities in these animals should then be examined in animals from the other dose groups. In these instances, microscopic examination should be made of all tissues showing gross pathological changes. As suggested under mating procedures, reproductive organs of animals suspected of infertility may be subjected to microsocpic examination.

2. DATA

Treatment of results

Data may be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of pregnant animals, the type of changes and the percentage of animals displaying each type of change.

When possible, numerical results should be evaluated by an appropriate statistical method. Any generally accepted statistical method may be used.

3. REPORTING

3.1. TEST REPORT

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

– species/strain used,

– toxic response data by sex and dose, including fertility, gestation and viability indices,

– time of death during the study or whether animals survived to termination of the study,

– table presenting the weights of each litter, the mean pup weight and the individual weight of the pups at termination,

– toxic or other effects on reproduction, offspring and postnatal growth,

– the day of observation of each abnormal sign and its subsequent course,

– bodyweight data for P and F_1 animals selected for mating,

– necropsy findings,

– a detailed description of all microscopic findings,

– statistical treatment of results, where appropriate,

– discussion of the results,

– interpretation of the results.

3.2. EVALUATION AND INTERPRETATION

See General Introduction Part B.

4. REFERENCES

See General Introduction Part B.

B. 36. TOXICOKINETICS

1. METHOD

1.1. INTRODUCTION

See General Introduction Part B.

1.2. DEFINITIONS

See General Introduction Part B.

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLE OF THE TEST METHOD

The test substance is administered by an appropriate route. Depending on the purpose of the study, the substance may be administered in single or repeated doses over defined periods to one or several groups of experimental animals. Subsequently, depending on the type of study, the substance and/or metabolites are determined in body fluids, tissues and/or excreta.

Studies may be done with 'unlabelled' or 'labelled' forms of the test substance. Where a label is used it should be positioned in the substance in such a way to provide the most information about the fate of the compound.

1.5. QUALITY CRITERIA

None.

1.6. DESCRIPTION OF THE TEST METHOD

Preparations

Healthy young adult animals are acclimatized to the laboratory conditions for at least five days prior to the test. Before the test, animals are randomized and assigned to the treatment groups. In special situations, very young, pregnant or pre-treated animals may be used.

Test conditions

Experimental animals

Toxicokinetic studies may be carried out in one or more appropriate animal species and should take account of the species used or intended to be used in other toxicological studies

on the same test substance. Where rodents are used in a test the weight variation should not exceed ± 20% of the mean weight.

Number and sex

For absorption and excretion studies, there should be four animals in each dose group initially. Sex preference is not mandatory, but under some circumstances both sexes may need to be studied. If there are sex differences in response, then four animals of each sex should be tested. In the case of studies with non-rodents fewer animals may be used.

When tissue distribution is being studied, the initial group size should take into account both the number of animals to be sacrificed at each time point and the number of time points to be examined.

When metabolism is being studied, the group size is related to the needs of the study.

For multiple-dose and multiple-time-point studies, the group size should take into account the number of time points and planned sacrifice(s), but may not be smaller than two animals. The group size should be sufficient to provide an acceptable characterization of uptake, plateau and depletion (as appropriate) of the test substance and/or metabolites.

Dose levels

In the case of single-dose administration, at least two dose levels should be used. There should be a low dose at which no toxic effects are observed and a high dose at which there might be changes in toxicokinetic parameters or at which toxic effects occur.

In the case of repeated-dose administration the low dose is usually sufficient, but under certain circumstances a high dose may also be necessary.

Route of administration

Toxicokinetic studies should be performed using the same route and, where appropriate, the same vehicle as that used or intended to be used in the other toxicity studies. The test substance is usually administered orally by gavage or in the diet, applied to the skin, or administered by inhalation for defined periods to groups of experimental animals. Intravenous administration of the test substance may be useful in determining relative absorption by other routes. In addition, useful information may be provided on the pattern of distribution soon after the intravenous administration of a substance.

The possibility of interference of the vehicle with the rest substance should be taken into consideration. Attention should be given to differences in absorption between the administration of the test substance by gavage and in the diet and the need for an accurate determination of dose particularly when the test substance is given in the diet.

Observation period

All the animals should be observed daily and signs of toxicity and other relevant clinical features recorded, including time of onset, degree and duration.

Procedure

After weighing test animals, the test substance is administered by an appropriate route. If considered relevant, animals may be fasted before the test substance is administered.

Absorption

The rate and extent of absorption of the administered substance can be evaluated using various methods, with and without reference groups ([13]), for example by:

– determination of the amount of test substance and/or metabolites in excreta, such as urine, bile, faeces, exhaled air and that remaining in the carcase,

– comparison of the biological response (e.g. acute toxicity studies) between test and control and/or reference groups,

– comparison of the amount of renally excreted substance and/or metabolite in test and reference groups,

– determination of the area under the plasma-level/time curve of the lest substance and/or metabolites and comparison with data from a reference group.

Distribution

Two approaches are available at present, one or both of which may be used for analysis of distribution patterns:

– useful qualitative information is obtained using whole body autoradiographic techniques,

– quantitative information is obtained by sacrificing animals at different times after exposure and determining the concentration and amount of the test substance and/or metabolites in tissues and organs.

Excretion

In excretion studies, urine, faeces and expired air and, in certain circumstances, bile are collected. The amount of test substance and/or metabolites in these excreta should be measured several times after exposure, either until about 95% of the administered dose has been excreted or for seven days, whichever comes first.

In special cases, the excretion of the test substance in the milk of lactating test animals may need to be considered.

Metabolism

To determine the extent and pattern of metabolism, biological samples should be analysed by suitable techniques. Structures of metabolites should be elucidated and appropriate metabolic pathways proposed where there is a need to answer questions arising from previous toxicological studies. It may be helpful to perform studies *in vitro* to obtain information on metabolic pathways.

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[13] In this method a reference group is one in which the test substance is administered by another route that ensures complete bioavailability of the dose.

Further information on the relationship of metabolism to toxicity may be obtained from biochemical studies, such as the determination of effects on metabolizing enzyme systems, depletion of endogenous non-protein sulphydryl compounds and binding of the substance with macromolecules.

2. DATA

According to the type of study performed, data should be summarized in tabular form supported by graphical presentation whenever appropriate. For each test group, mean and statistical variations of measurements in relation to time, dosage, tissues and organs should be shown when appropriate. The extent of absorption and the amount and rates of excretion should be determined by appropriate methods. When metabolism studies are performed, the structure of identified metabolites should be given and possible metabolic pathways presented.

3. REPORTING

3.1. TEST REPORT

According to the type of study performed, the test report shall, if possible, contain the following

– information:

– species, strain, source, environmental conditions, diet,

– characterization of labelled materials, when used,

– dosage levels and intervals used,

– route(s) of administration and any vehicles used;

– toxic and other effects observed,

– methods for determination of test substance and/or metabolites in biological samples, including

– expired air,

– tabulation of measurements by sex, dose, regimen, time, tissues and organs,

– presentation of the extent of absorption and excretion with time,

– methods for the characterization and identification of metabolites in biological samples,

– methods for biochemical measurements related to metabolism,

– proposed pathways for metabolism,

– discussion of the results,

– interpretation of the results.

3.2. EVALUATION AND INTERPRETATION

See General Introduction Part B.

4. REFERENCES

See General Introduction Part B.

B. 37. DELAYED NEUROTOXICITY OF ORGANOPHOSPHORUS SUBSTANCES FOLLOWING ACUTE EXPOSURE

1. METHOD

1.1. INTRODUCTION

In the assessment and evaluation of the toxic effects of substances, it is important to consider the potential of certain classes of substances to cause specific types of neurotoxicity that might not be detected in other toxicity studies. Certain organophosphorus substances have been observed to cause delayed neurotoxicity and should be considered as candidates for evaluation.

In vitro screening tests could be employed to identify those substances which may cause delayed polyneuropathy; however, negative findings from *in vitro* studies do not provide evidence that the test substance is not a neurotoxicant.

See General Introduction Part B.

1.2. DEFINITIONS

Organophopsphorus substances include uncharged organophosphorus esters, thioesters or anhydrides of organophosphoric, organophosphonic or organophosphoramidic acids or of related phosphorothioic, phosphonothioic or phosphorothioamidic acids, or other substances that may cause the delayed neurotoxicity sometimes seen in this class of substances.

Delayed neurotoxicity is a syndrome associated with prolonged delayed onset of ataxia, distal axonopathies in spinal cord and peripheral nerve, and inhibition and aging of neuropathy target esterase (NTE) in neural tissue.

1.3. REFERENCE SUBSTANCES

A reference substance may be tested with a positive control group as a means of demonstrating that under the laboratory test conditions, the response of the tested species has not changed significantly.

An example of a widely used neurotoxicant is tri–o–tolyl phosphate (CAS 78-30-8, EINECS 201-103-5, CAS nomenclature : phosphoric acid, tris(2–methylphenyl)ester), also known as tris–o–cresylphosphate.

1.4. PRINCIPLE OF THE TEST METHOD

The test substance is administered orally in a single dose to domestic hens which have been protected from acute cholinergic effects, when appropriate. The animals are observed for 21 days for behavioural abnormalities, ataxia, and paralysis. Biochemical measurements, in particular neuropathy target esterase inhibition (NTE), are undertaken on hens randomly selected from each group, normally 24 and 48 hours after dosing. Twenty-one days after

exposure, the remainder of the hens are killed and histopathological examination of selected neural tissues is undertaken.

1.5. DESCRIPTION OF THE TEST METHOD

1.5.1. Preparations

Healthy young adult hens free from interfering viral diseases and medication and without abnormalities of gait should be randomized and assigned to treatment and control groups and acclimatized to the laboratory conditions for at least 5 days prior to the start of the study.

Cages or enclosures which are large enough to permit free mobility of the hens, and easy observation of gait should be used.

Dosing with the test substance should normally be by the oral route using gavage, gelatine capsules, or a comparable method. Liquids may be given undiluted or dissolved in an appropriate vehicle such as corn oil; solids should be dissolved if possible since large doses of solids in gelatine capsules may not be absorbed efficiently. For non-aqueous vehicles the toxic characteristics of the vehicle should be known, and if not known should be determined before the test.

1.5.2. Test conditions

1.5.2.1. Test animals

The young adult domestic laying hen (*Gallus gallus domestícus*), aged 8 to 12 months, is recommended. Standard size breeds and strains should be employed and the hens normally should have been reared under conditions which permitted free mobility.

1.5.2.2. Number and sex

In addition to the treatment group, both a vehicle control group and a positive control group should be used. The vehicle control group should be treated in a manner identical to the treatment group, except that administration of the test substance is omitted.

Sufficient number of hens should be utilized in each group of birds so that at least six birds can be killed for biochemical determination (three at each of two time points) and six can survive the 21 day observation period for pathology.

The positive control group may be run concurrently or be a recent historical control group. It should contain at least six hens, treated with a known delayed neurotoxicant, three hens for biochemistry and three hens for pathology. Periodic updating of historical data is recommended. New positive control data should be developed when some essential element (e.g. strain, feed, housing conditions) of the conduct of the test has been changed by the performing laboratory.

1.5.2.3. Dose levels

A preliminary study using an appropriate number of hens and dose levels groups should be performed to establish the level to be used in the main study. Some lethality is typically necessary in this preliminary study to define an adequate main study dose. However, to prevent death due to acute cholinergic effects, atropine or another protective agent, known to

not interfere with delayed neurotoxic responses, may be used. A variety of test methods may be used to estimate the maximum non-lethal dose of test substances (See method B.1bis). Historical data in the hen or other toxicological information may also be helpful in dose selection.

The dose level of the test substance in the main study should be as high as possible taking into account the results of the preliminary dose selection study and the upper limit dose of 2,000 mg/kg body weight. Any mortality which might occur should not interfere with the survival of sufficient animals for biochemistry (six) and histology (six) at 21 days. Atropine or another protective agent, known to not interfere with delayed neurotoxic responses, should be used to prevent death due to acute cholinergic effects.

1.5.2.4. Limit test

If a test at a dose level of at least 2,000 mg/kg body weight/day, 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 study using a higher dose may not be considered necessary. The limit test applies except when human exposure indicates the need for a higher dose level to be used.

1.5.3. Observation period

Observation period should be 21 days.

1.5.4. Procedure

After administration of a protective agent to prevent death due to acute cholinergic effect, the test substance is administered in a single dose.

General observation

Observations should start immediately after exposure. All hens should be carefully observed several times during the first 2 days and thereafter at least once daily for a period of 21 days or until scheduled kill. All signs of toxicity should be recorded, including the time of onset, type, severity and duration of behavioural abnormalities. Ataxia should be measured on an ordinal grading scale consisting of at least four levels, and paralysis should be noted. At least twice a week the hens selected for pathology should be taken outside the cages and subjected to a period of forced motor activity, such as ladder climbing, in order to facilitate the observation of minimal toxic effects. Moribund animals and animals in severe distress or pain should be removed when noticed, humanely killed and necropsied.

Body weight

All hens should be weighed just prior to administration of the test substance and at least once a week thereafter.

Biochemistry

Six hens randomly selected from each of the treatment and vehicle control groups, and three hens from the positive control group (when this group is run concurrently), should be killed within a few days after dosing, and the brain and lumbar spinal cord prepared and assayed for neuropathy target esterase inhibition activity. In addition, it may also be useful to prepare and assay sciatic nerve tissue for neuropathy target esterase inhibition activity. Normally, three

birds of the control and each treatment group are killed after 24 hours and three at 48 hours, whereas the three hens of the positive controls should be killed at 24 hours. If observation of clinical signs of intoxication (this can often be assessed by observation of the time of onset of cholinergic signs) indicates that the toxic agent may be disposed of very slowly then it may be preferable to sample tissue from three birds at each of two times between 24 and as late as 72 hours after dosing.

Analyses of acetylcholinesterase (AChE) may also be performed on these samples, if deemed appropriate. However, spontaneous reactivation of AChE may occur *in vivo*, and so lead to underestimation of the potency of the substance as an AChE inhibitor.

Gross necropsy

Gross necropsy of all animals (scheduled killed and killed when moribund) should include observation of the appearance of the brain and spinal cord.

Histopathological examination

Neural tissue from animals surviving the observation period and not used for biochemical studies should be subjected to microscopic examination. Tissues should be fixed *in situ*, using perfusion techniques. Sections should include cerebellum (mid-longitudinal level), medulla oblongata, spinal cord, and peripheral nerves. The spinal cord sections should be taken from the upper cervical segment, the mid-thoracic and the lumbo-sacral regions. Sections of the distal region of the tibial nerve and its branches to the gastrocnemial muscle and of the sciatic nerve should be taken. Sections should be stained with appropriate myelin and axon-specific stains.

2. DATA

Negative results on the endpoints selected in this method (biochemistry, histopathology and behavioural observation) would not normally require further testing for delayed neurotoxicity. Equivocal or inconclusive results for these endpoints may require further evaluation.

Individual data should be provided. Additionally, all data should be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing lesions, behavioural or biochemical effects, the types and severity of these lesions or effects, and the percentage of animals displaying each type and severity of lesion or effect.

The findings of this study should be evaluated in terms of the incidence, severity, and correlation of behavioural, biochemical and histopathological effects and any other observed effects in the treated and control groups.

Numerical results should be evaluated by appropriate and generally acceptable statistical methods. The statistical methods used should be selected during the design of the study.

3. REPORTING

TEST REPORT

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

3.1. Test animals:

– strain used;

– number and age of animals;

– source, housing conditions, etc.;

– individual weights of animals at the start of the test.

3.2. Test conditions :

– details of test substance preparation, stability and homogeneity, where appropriate;

– justification for choice of vehicle;

– details of the administration of the test substance;

– details of food and water quality;

– rationale for dose selection;

– specification of doses administered, including details of the vehicle, volume and physical form of the material administered;

– identity and details of the administration of any protective agent.

3.3. Results :

– body weight data;

– toxic response data by group, including mortality;

– nature, severity and duration of clinic observations (whether reversible or not);

– a detailed description of biochemical methods and findings;

– necropsy findings;

– a detailed description of all histopathological findings;

– statistical treatment of results, where appropriate.

Discussion of results.

Conclusions.

4. REFERENCES

This method is analogius to OECD TG 418.

B. 38. DELAYED NEUROTOXICITY OF ORGANOPHOSPHORUS SUBSTANCES 28 DAY REPEATED DOSE STUDY

1. METHOD

1.1. INTRODUCTION

In the assessment and evaluation of the toxic effects of substances, it is important to consider the potential of certain classes of substances to cause specific types of neurotoxicity that might not be detected in other toxicity studies. Certain organophosphorus substances have been observed to cause delayed neurotoxicity and should be considered as candidates for evaluation.

In vitro screening tests could be employed to identify those substances which may cause delayed polyneuropathy; however, negative findings from *in vitro* studies do not provide evidence that the test substance is not a neurotoxicant.

This 28-day delayed neurotoxicity test provides information on possible health hazards likely to arise from repeated exposures over a limited period of time. It will provide information on dose response and can provide an estimate of a no-observed-adverse effect level which can be of use for establishing safety criteria for exposure.

See also General Introduction Part B.

1.2. DEFINITIONS

Organophosphorus substances include uncharged organophosphorus esters, thioesters or anhydrides of organophosphoric, organophosphonic or organophosphoramidic acids or of related phosphorothioic, phosphonothioic or phosphorothioamidic acids or other substances that may cause the delayed neurotoxicity sometimes seen in this class of substances.

Delayed neurotoxicity is a syndrome associated with prolonged delayed onset of ataxia, distal axonopathies in spinal cord and peripheral nerve, and inhibition and ageing of neuropathy target esterase (NTE) in neural tissue.

1.3. PRINCIPLE OF THE TEST METHOD

Daily doses of the test substance are administered orally to domestic hens for 28 days. The animals are observed at least daily for behavioural abnormalities, ataxia and paralysis until 14 days after the last dose. Biochemical measurements, in particular neuropathy target esterase inhibition (NTE), are undertaken, on hens randomly selected from each group, normally 24 and 48 hours after the last dose. Two weeks after the last dose, the remainder of the hens are killed and histopathological examination of selected neural tissues is undertaken.

1.4. DESCRIPTION OF THE TEST METHOD

1.4.1. Preparations

Healthy young adult hens free from interfering viral diseases and medication, and without abnormalities of gait should be randomized and assigned to treatment and control groups and acclimatized to the laboratory conditions for at least 5 days prior to the start of the study.

Cages or enclosures which are large enough to permit free mobility of the hens and easy observation of gait should be used.

Oral dosing each day, 7 days per week, should be carried out, preferably by gavage or administration of gelatine capsules. Liquids may be given undiluted or dissolved in an appropriate vehicle such as corn oil; solids should be dissolved if possible since large doses of solids in gelatine capsules may not be absorbed efficiently. For non-aqueous vehicles the toxic characteristics of the vehicle should be known, and if not known should be determined before the test.

1.4.2. Test conditions

Test animals

The young adult domestic laying hen (Gallus gallus domesticus), aged 8 to 12 months, is recommended. Standard size, breeds and strains should be employed and the hens normally should have been reared under conditions which permitted free mobility.

Number and sex

Generally at least three treatment groups and a vehicle control group should be used. The vehicle control group should be treated in a manner identical to the treatment group, except that administration of the test substance is omitted.

Sufficient number of hens should be utilized in each group of birds so that at least six birds can be killed for biochemical determinations (three at each of two timepoints) and six birds can survive the 14-day post-treatment observation period for pathology.

Dose levels

Dose levels should be selected taking into account the results from an acute test on delayed neurotoxicity and any other existing toxicity or kinetic data available for the test compound. The highest dose level should be chosen with the aim of inducing toxic effects, preferably delayed neurotoxicity, but not death nor obvious suffering. Thereafter, a descending sequence of dose levels should be selected with a view to demonstrate any dose-related response and no-observed-adverse effects at the lowest dose level.

Limit test

If a test at a dose level of at least 1000 mg/kg body weight/day, 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 study using a higher dose may not be considered necessary. The limit test applies except when expected human exposure indicates the need for a higher dose level to be used.

Observation period

All the animals should be observed at least daily during the exposure period and 14 days after, unless scheduled necropsy.

1.4.3. Procedure

Animals are dosed with the test substance on seven days per week for a period of 28 days.

General observations

Observations should start immediately after treatment begins. All hens should be carefully observed at least once daily on each of the 28 days of treatment, and for 14 days after dosing or until scheduled kill. All signs of toxicity should be recorded including their time of onset, type, severity and duration. Observations should include, but not be limited to, behavioural abnormalities. Ataxia should be measured on an ordinal grading scale consisting of at least four levels, and paralysis should be noted. At least twice a week the hens should be taken outside the cages and subjected to a period of forced motor activity, such as ladder climbing, in order to facilitate the observation of minimal toxic effects. Moribund animals in severe distress or pain should be removed when noticed, humanely killed and necropsied.

Body weight

All hens should be weighed just prior to the first administration of the test substance and at least once a week thereafter.

Biochemistry

Six hens randomly selected from each of the treatment and vehicle control groups should be killed within a few days after the last dose, and the brain and lumbar spinal cord prepared and assayed for neuropathy target esterase (NTE) inhibition activity. In addition, it may also be useful to prepare and assay sciatic nerve tissue for neuropathy target esterase (NTE) inhibition activity. Normally, three birds of the control and each treatment group are killed after 24 hours and three at 48 hours after the last dose. If data from the acute study or other studies (e.g. toxicokinetics) indicate that other times of killing after final dosing are preferable then these times should be used and the rationale documented.

Analyses of acetylcholinesterase (AChE) may also be performed on these samples, if deemed appropriate. However, spontaneous reactivation of AChE may occur *in vivo*, and so lead to underestimation of the potency of the substance as an AChE inhibitor.

Gross necropsy

Gross necropsy of all animals (scheduled killed and killed when moribund) should include observation of the appearance of the brain and spinal cord.

Histopathological examination

Neural tissue from animals surviving the observation period and not used for biochemical studies should be subjected to microscopic examination. Tissues should be fixed *in situ*, using perfusion techniques. Sections should include cerebellum (mid longitudinal level), medulla oblongata, spinal cord and peripheral nerves. The spinal cord sections should be taken from the upper cervical segment, the mid-thoracic and the lumbo-sacral regions. Sections of the

distal region of the tibial nerve and its branches to the gastrocnemial muscle and of the sciatic nerve should be taken. Sections should be stained with appropriate myelin and axon-specific stains. Initially, microscopic examination should be carried out on the preserved tissues of all animals in the control and high dose group. When there is evidence of effects in the high dose group, microscopic examination should also be carried out in hens from the intermediate and low dose groups.

2. DATA

Negative results on the endpoints selected in this method (biochemistry, histopathology and behavioural observation) would not normally require further testing for delayed neurotoxicity . Equivocal or inconclusive results for these endpoints may require further evaluation.

Individual data should be provided. Additionally, all data should be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing lesions, behavioural or biochemical effects, the types and severity of these lesions or effects, and the percentage of animals displaying each type and severity of lesion or effect.

The findings of this study should be evaluated in terms of the incidence, severity, and correlation of behavioural, biochemical and histopathological effects and any other observed effects in each of the treated and control groups.

Numerical results should be evaluated by appropriate and generally acceptable statistical methods. 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 :

3.1 Test animals :

– strain used;

– number and age of animals;

– source, housing conditions, etc.;

– individual weights of animals at the start of the test.

3.2 Test conditions :

– details of test substance preparation, stability and homogeneity, where appropriate;

– justification for choice of vehicle;

– details of the administration of the test substance;

– details of food and water quality;

– rationale for dose selection;

– specification of doses administered, including details of the vehicle, volume and physical form of the material administered;

– rationale for choosing other times for biochemical determination, if other than 24 and 48 h.

3.3. Results :

– body weight data;

– toxic response data by dose level, including mortality;

– no-observed adverse effect level;

– nature, severity and duration of clinic observations (whether reversible or not);

– a detailed description of biochemical methods and findings;

– necropsy findings;

– a detailed description of all histopathological findings;

– statistical treatment of results, where appropriate.

Discussion of results.

Conclusions.

4 REFERENCES

This method is analogous to OECD TG 419.

B.39. UNSCHEDULED DNA SYNTHESIS (UDS) TEST WITH MAMMALIAN LIVER CELLS *IN VIVO*

1. METHOD

This method is a replicate of the OECD TG 486, Unscheduled DNA Synthesis (UDS) Test with Mammalian Liver Cells *In Vivo* (1997).

1.1 INTRODUCTION

The purpose of the unscheduled DNA Synthesis (UDS) test with mammalian liver cells i*n vivo* is to identify test substances that induce DNA repair in liver cells of treated animals (see 1,2,3,4).

This *in vivo* test provides a method for investigating genotoxic effects of chemicals in the liver. The end-point measured is indicative of DNA damage and subsequent repair in liver cells. The liver is usually the major site of metabolism of absorbed compounds. It is thus an appropriate site to measure DNA damage *in vivo*.

If there is evidence that the test substance will not reach the target tissue, it is not appropriate to use this test.

The end-point of unscheduled DNA synthesis (UDS) is measured by determining the uptake of labelled nucleosides in cells that are not undergoing scheduled (S-phase) DNA synthesis. The most widely used technique is the determination of the uptake of tritium-labelled thymidine (^{3}H-TdR) by autoradiography. Rat livers are preferably used for *in vivo* UDS tests. Tissues other than the livers may be used, but are not the subject of this method.

The detection of a UDS response is dependent on the number of DNA bases excised and replaced at the site of the damage. Therefore, the UDS test is particularly valuable to detect substance-induced “longpatch repair” (20-30 bases). In contrast, “shortpatch repair” (1-3 bases) is detected with much lower sensitivity. Furthermore, mutagenic events may result because of non-repair, misrepair or misreplication of DNA lesions. The extent of the UDS response gives no indication of the fidelity of the repair process. In addition, it is possible that a mutagen reacts with DNA but the DNA damage is not repaired via an excision repair process. The lack of specific information on mutagenic activity provided by the UDS test is compensated for by the potential sensitivity of this endpoint because it is measured in the whole genome.

See also General Introduction Part B.

1.2 DEFINITIONS

Cells in repair: a net nuclear grain (NNG) higher than a preset value, to be justified at the laboratory conducting the test.

Net nuclear grains (NNG): quantitative measure for UDS activity of cells in autoradiographic UDS tests, calculated by subtracting the average number of cytoplasmic grains in nucleus-equivalent cytoplasmic areas (CG) from the number of nuclear grains (NG): NNG = NG -

CG. NNG counts are calculated for individual cells and then pooled for cells in a culture, in parallel cultures, etc.

Unscheduled DNA Synthesis (UDS): DNA repair synthesis after excision and removal of a stretch of DNA containing a region of damage induced by chemical substances or physical agents.

1.3 PRINCIPLE OF THE TEST METHOD

The UDS test with mammalian liver cells *in vivo* indicates DNA repair synthesis after excision and removal of a stretch of DNA containing a region of damage induced by chemical substances or physical agents. The test is usually based on the incorporation of ^{3}H-TdR into the DNA of liver cells which have a low frequency of cells in the S-phase of the cell cycle. The uptake of ^{3}H-TdR is usually determined by autoradiography, since this technique is not as susceptible to interference from S-phrase cells as, for example, liquid scintillation counting.

1.4 DESCRIPTION OF THE METHOD

1.4.1 Preparations

1.4.1.1 Selection of animal species

Rats are commonly used, although any appropriate mammalian species may be used. Commonly used laboratory strains of young healthy adult animals should be employed. At the commencement of the study the weight variation of animals should be minimal and not exceed ± 20% of the mean weight for each sex.

1.4.1.2 Housing and feeding conditions

General conditions referred in the General Introduction to Part B are applied although the aim for humidity should be 50-60%.

1.4.1.3 Preparation of the animals

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.

1.4.1.4 Test substance/Preparation

Solid test substances should be dissolved or suspended in appropriate solvents or vehicles and diluted, if appropriate, prior to dosing of the animals. Liquid test substances may be dosed directly or diluted prior to dosing. Fresh preparations of the test substance should be employed unless stability data demonstrate the acceptability of storage.

1.4.2 Test conditions

1.4.2.1 Solvent/vehicle

The solvent/vehicle should not produce toxic effects at the dose levels used, and should not be suspected of chemical reaction with the test substance. If other than well-known

solvents/vehicles are used, their inclusion should be supported with data indicating their compatibility. It is recommended that wherever possible, the use of an aqueous solvent/vehicle should be considered first.

1.4.2.2 Controls

Concurrent positive and negative controls (solvent/vehicle) should be included in each independently performed part of the experiment. Except for treatment with the test substance, animals in the control group should be handled in an identical manner to the animals in the treated groups.

Positive controls should be substances known to produce UDS when administered at exposure levels expected to give a detectable increase over background. Positive controls needing metabolic activation should be used at doses eliciting a moderate response (4). The doses may be chosen so that the effects are clear but do not immediately reveal the identity of the coded slides to the reader. Examples of positive control substances include:

Sampling Times	Substance	CAS No.	EINECS No.
Early sampling times (2-4 hours)	N-Nitrosodimethylamine	62-75-9	200-249-8
Late sampling times (12-16 hours)	N-2-Fluorenylacetamide (2-AAF)	53-96-3	200-188-6

Other appropriate positive control substances may be used. It is acceptable that the positive control should be administered by a route different from the test substance.

1.5 PROCEDURE

1.5.1 Number and sex of animals

An adequate number of animals should be used, to take account of natural biological variation in test response. The number of animals should be at least 3 analysable animals per group. Where a significant historical database has been accumulated, only 1 or 2 animals are required for the concurrent negative and positive control groups.

If at the time of the study there are data available from studies in the same species and using the same route of exposure that demonstrate that there are no substantial differences in toxicity between sexes, then testing in a single sex, preferably males, will be sufficient. Where human exposure to chemicals may be sex-specific, as for example with some pharmaceutical agents, the test should be performed with animals of the appropriate sex.

1.5.2 Treatment schedule

Test substances are generally administered as a single treatment.

1.5.3 Dose levels

Normally, at least two dose levels are used. The highest dose is defined as the dose producing signs of toxicity such that higher dose levels, based on the same dosing regimen, would be expected to produce lethality. In general, the lower dose should be 50% to 25% of the high dose.

Substances with specific biological activities at low non-toxic doses (such as hormones and mitogens) may be exceptions to the dose-setting criteria and should be evaluated on a case-by-case basis. If a range finding study is performed because there are no suitable data available, it should be performed in the same laboratory, using the same species, strain, sex, and treatment regimen to be used in the main study.

The highest dose may also be defined as a dose that produces some indication of toxicity in the liver (e.g. pyknotic nuclei).

1.5.4 Limit test

If a test at one dose level of at least 2000 mg/kg body weight, applied in a single treatment, or in two treatments on the same day, produces no observable toxic effects, and if genotoxicity would not be expected, based upon data from structurally related substances, then a full study may not be necessary. Expected human exposure may indicate the need for a higher dose level to be used in the limit test.

1.5.5 Administration of doses

The test substance is usually administered by gavage using a stomach tube or a suitable intubation cannula. Other routes of exposure may be acceptable where they can be justified. However, the intraperitoneal route is not recommended as it could expose the liver directly to the test substance rather than via the circulatory system. The maximum volume of liquid that can be administered by gavage or injection at one time depends on the size of the test animal. The volume should not exceed 2 ml/100g body weight. The use of volumes higher than these must be justified. 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.

1.5.6 Preparation of liver cells

Liver cell are prepared from treated animals normally 12-16 hours after dosing. An additional earlier sampling time (normally 2-4 hours post-treatment) is generally necessary unless there is a clear positive response at 12-16 hours. However, alternative sampling times may be used when justified on the basis of toxicokinetic data.

Short-term cultures of mammalian liver cells are usually established by perfusing the liver *in situ* with collagenase and allowing freshly dissociated liver cells to attach themselves to a suitable surface. Liver cells from negative control animals should have a viability (5) of at least 50 percent.

1.5.7 Determination of UDS

Freshly isolated mammalian liver cells are incubated usually with medium containing ^{3}H-TdR for an appropriate length of time, e.g. 3-8 hours. At the end of the incubation period, medium should be removed from the cells, which may then be incubated with medium containing

excess unlabelled thymidine to diminish unincorporated radioactivity ("cold chase"). The cells are then rinsed, fixed and dried. For more prolonged incubation times, cold chase may not be necessary. Slides are dipped in autoradiographic emulsion, exposed in the dark (e.g. refrigerated for 7-14 days), developed, stained, and exposed silver grains are counted. Two to three slides are prepared from each animal.

1.5.8 Analysis

The slide preparations should contain sufficient cells of normal morphology to permit a meaningful assessment of UDS. Preparations are examined microscopically for signs of overt cytotoxicity (e.g. pyknosis, reduced levels of radiolabelling).

Slides should be coded before grain counting. Normally 100 cells are scored from each animal from at least two slides; the scoring of less than 100 cells/animal should be justified. Grain counts are not scored for S-phase nuclei, but the proportion of S-phase cells may be recorded.

The amount of ^{3}H-TdR incorporation in the nuclei and the cytoplasm of morphologically normal cells, as evidenced by the deposition of silver grains, should be determined by suitable methods.

Grain counts are determined over the nuclei (nuclear grains, NG) and nucleus equivalent areas over the cytoplasm (cytoplasmic grains, CG). CG counts are measured by either taking the most heavily labelled area of cytoplasm, or by taking an average of two to three random cytoplasmic grain counts adjacent to the nucleus. Other counting methods (e.g. whole cell counting) may be used if they can be justified (6).

2. DATA

2.1 TREATMENT OF RESULTS

Individual slide and animal data should be provided. Additionally, all data should be summarised in tabular form. Net nuclear grain (NNG) counts should be calculated for each cell, for each animal and for each dose and time by subtracting CG counts from NG counts. If "cells in repair" are counted, the criteria for defining "cells in repair" should be justified and based on historical or concurrent negative control data. Numerical results may be evaluated by statistical methods. If used, statistical tests should be selected and justified prior to conducting the study.

2.2 EVALUATION AND INTERPRETATION OF RESULTS

Examples of criteria for positive/negative responses include:

positive	(i)	NNG values above a pre-set threshold which is justified on the basis of laboratory historical data;
or	(ii)	NNG values significantly greater than concurrent control;
negative	(i)	NNG values within/below historical control threshold;

or (ii) NNG values not significantly greater than concurrent control.

The biological relevance of data should be considered: i.e. parameters such as inter-animal variation, dose-response relationship and cytotoxicity should be taken into account. Statistical methods may be used as an aid in evaluating the test results. However, statistical significance should not be the only determining factor for a positive response.

Although most experiments will give clearly positive or negative results, in rare cases the data set will preclude making a definite judgement about the activity of the test substance. Results may remain equivocal or questionable regardless of the number of times the experiment is repeated.

A positive result from the UDS test with mammalian liver cells *in vivo* indicate that a test substance induces DNA damage in mammalian liver cells *in vivo* that can be repaired by unscheduled DNA synthesis *in vitro*. A negative result indicates that, under the test conditions, the test substance does not induce DNA damage that is detectable by this test.

The likelihood that the test substance reaches the general circulation or specifically the target tissue (e.g. systemic toxicity) should be discussed.

3. REPORTING

TEST REPORT

The test report must include the following information :

Solvent/Vehicle:

– justification for choice of vehicle;

– solubility and stability of the test substance in solvent/vehicle, if known.

Test animals:

– species/strain used;

– number, age and sex of animals;

– source, housing conditions, diet, etc.;

– individual weight of the animals at the start of the test, including body weight range, mean and standard deviation for each group;

Test conditions :

– positive and negative vehicle/solvent controls;

– data from range-finding study, if conducted;

– rationale for dose level selection;

– details of test substance preparation;

– details of the administration of the test substance;

– rationale for route of administration;

– methods for verifying that test agent reached the general circulation or target tissue, if applicable;

– 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;

– detailed description of treatment and sampling schedules;

– methods for measurement of toxicity;

– method of liver cell preparation and culture;

– autoradiographic technique used;

– number of slides prepared and numbers of cells scored;

– evaluation criteria;

– criteria for considering studies as positive, negative or equivocal;

Results:

– individual slide, animal and group mean values for nuclear grains, cytoplasmic grains, and net nuclear grains;

– dose-response relationship, if available;

– statistical evaluation if any;

– signs of toxicity;

– concurrent negative (solvent/vehicle) and positive control data;

– historical negative (solvent/vehicle) and positive control data with range, means and standard deviations;

– number of “cells in repair” if determined;

– number of S-phase cells if determined;

– viability of the cells.

Discussion of results.

Conclusions.

4. REFERENCES

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B.40. SKIN CORROSION

1. METHOD

1.1 INTRODUCTION

Two *in vitro* tests for skin corrosivity, the rat skin transcutaneous electrical resistance (TER) assay and a test employing a human skin model, have been endorsed as scientifically valid by the European Centre for the Validation of Alternative Methods (ECVAM, Joint Research Centre, European Commission) (1)(2)(3). The ECVAM validation study demonstrated that both tests were able to reliably discriminate between known skin corrosives and non-corrosives. Furthermore, the test protocol based on a human skin model enabled correct distinction between degrees of corrosive effects (known severe skin corrosives, R35, and other skin corrosives, R34) (2). Descriptions and procedures for both tests are given; the choice of which test to use depends on the specific requirements and preferences of the user.

See also General Introduction Part B.

1.2 DEFINITIONS

Skin corrosion: the production of irreversible tissue damage in the skin following the application of a test material.

1.3 REFERENCE SUBSTANCES

None specified, but see points 1.5.3.4 and 1.7.2.3.

1.4 PRINCIPLE OF THE TEST METHOD - RAT SKIN TER ASSAY

The test material is applied for up to 24 hours to the epidermal surfaces of skin discs taken from the pelts of humanely killed young rats. Corrosive materials are identified by their ability to produce a loss of normal stratum corneum integrity and barrier function, which is measured as a reduction in the inherent TER below a threshold level (5kΩ) (4)(5). Irritant and non-irritant materials do not reduce the TER below the threshold level. A dye-binding step can be incorporated into the test procedure for surfactants and neutral organics (for definition see reference (6)) to reduce the number of false positive results obtained specifically with these chemical types (2) (7).

1.5 DESCRIPTION OF THE TEST METHOD - RAT SKIN TER ASSAY

1.5.1 Animals

Young (20-23 days) rats (Wistar or a comparable strain) are required for the preparation of skin discs. The dorsal and flank hair is carefully removed with small animal clippers. The animals are then washed by careful wiping, whilst submerging the area in antibiotic solution (containing, for example, streptomycin, penicillin, chloramphenicol and amphotericin at

concentrations effective in inhibiting bacterial growth). Animals are washed with antibiotics again on the third or fourth day after the first wash, and are used within 3 days (animals must not be older than 31 days for pelt preparation).

1.5.2 Preparation of the skin discs

Animals are humanely killed. The dorsal skin of each animal is then removed and stripped of excess fat by carefully peeling it away from the skin. The pelt is placed over the end of a PTFE (polytetrafluoroethylene) tube, ensuring that the epidermal surface is in contact with the tube. A rubber 'O' ring is press-fitted over the end of the tube to hold the skin in place and excess tissue is trimmed away. Tube and 'O' ring dimensions are shown in Figure 1. The rubber 'O' ring is then carefully sealed to the end of the PTFE tube with petroleum jelly. The tube is supported by a spring clip inside a receptor chamber containing magnesium sulphate solution (154mM) (Figure 2).

1.5.3 Test procedure

1.5.3.1 Application of the test material

Liquid test substances (150µl) are applied to the epidermal surface inside the tube (Figure 2). When testing solid materials, a sufficient amount of the solid is applied to the disc to ensure that the whole surface of the epidermis is covered. Deionised water (150µl) is then added on top of the solid and the tubes are gently agitated. Test substances should have maximum contact with the skin. For some solids this may be achieved by warming up to 30°C to melt the test substance, or by grinding to produce a granular material or powder.

Three skin discs are used for each test substance. Test substances are applied for 24 hours (see also 1.5.3.4). The test substance is removed by washing with a jet of tap water at up to 30°C until no further material can be removed. The removal of test substances which have solidified in the tube can be facilitated by jet washing with warm water at approximately 30°C.

1.5.3.2 TER measurements

The TER is measured by using a low-voltage, alternating current databridge (e.g. AIM 401 or 6401, or equivalent). Prior to measuring the electrical resistance, the surface tension of the skin is reduced by adding a sufficient volume of 70% ethanol to cover the epidermis. After a few seconds the ethanol is removed by inverting the tube, and the tissue is then hydrated by the addition of 3ml magnesium sulphate solution (154mM). The databridge electrodes are placed on either side of the skin disc to take the resistance measurement in kΩ/skin disc (Figure 2). Electrode dimensions and the length of the electrode exposed below the crocodile clips are shown in Figure 1. The inner (thick) electrode clip is rested on the top of the PTFE tube during resistance measurement, to ensure that a consistent length of electrode is submerged in the magnesium sulphate solution. The outer (thin) electrode is positioned inside the receptor chamber so that it rests on the bottom of the chamber. The distance between the bottom of the spring clip and the bottom of the PTFE tube is maintained as a constant (Figure 1), since this distance affects the resistance value obtained.

Note that if the measured resistance value is greater than 20kΩ, this may be due to the test substance coating the epidermal surface of the skin disc. Removal of this coating can be attempted, for example, by sealing the PTFE tube with a gloved thumb and shaking it for approximately 10 seconds; the magnesium sulphate solution is discarded and the resistance measurement is repeated with fresh magnesium sulphate.

The mean TER results are accepted on condition that concurrent positive and negative control values fall within the acceptable ranges for the method. The suggested control substances and their associated acceptable resistance ranges for the methodology and apparatus described are:

Control	Substance	Resistance range (kΩ)
Positive	10M Hydrochloric acid (36%)	0.5 - 1.0
Negative	Distilled water	10 - 25

1.5.3.3 Modified procedure for surfactants and neutral organics

If the TER values of test substances which are either surfactants or neutral organics are less than or equal to 5kΩ, an assessment of dye penetration can be carried out on the tissues. This procedure will determine whether the results are false positives (2).

1.5.3.3.1 Sulforhodamine B dye application and removal

Following initial treatment with the test substance, 150µl of a 10% (w/v) dilution of sulforhodamine B dye in distilled water is applied to the epidermal surface of each skin disc for 2 hours. The skin discs are then jet washed with tap water at up to room temperature for approximately 10 seconds to remove any excess/unbound dye. Each skin disc is carefully removed from the PTFE tube and placed in a vial (e.g. a 20ml glass scintillation vial) containing deionised water (8ml). The vials are agitated gently for 5 minutes to remove any further excess/unbound dye. This rinsing procedure is then repeated, after which the skin discs are removed and placed into vials containing 5ml of 30% (w/v) sodium dodecyl sulphate (SDS) in distilled water and are incubated overnight at 60°C. After incubation, each skin disc is removed and discarded and the remaining solution is centrifuged for 8 minutes at 21°C (relative centrifugal force ~175). A 1ml sample of the supernatant is then diluted 1 in 5 (v/v) [i.e. 1ml + 4ml] with 30% (w/v) SDS in distilled water. The optical density (OD) of the solution is measured at approximately 565nm.

1.5.3.3.2 Calculation of dye content

The sulforhodamine B dye content per disc is calculated from the OD values (sulforhodamine B dye molar extinction coefficient at 565nm = 8.7×10^4; molecular weight = 580). The sulforhodamine B dye content is determined for each skin disc and a mean dye content is then calculated for the replicates. The mean dye binding results are accepted on condition that concurrent control values fall within the acceptable ranges for the method. Suggested acceptable dye content ranges for the control substances for the methodology and apparatus described are:

Control	Substance	Dye content range (µg/disc)
Positive	10M Hydrochloric acid (36%)	40 - 100
Negative	Distilled water	15 - 35

1.5.3.4 Additional information

Test substances can also be applied to the skin discs for shorter periods (e.g. 2 hours) to identify those materials which are severely corrosive. However, in the validation study, the TER assay was found to overestimate the corrosive potential of several test chemicals following their application to the skin discs for 2 hours (2), although it enabled the correct identification of corrosives and non-corrosives after a 24-hour application.

The properties and dimensions of the test apparatus and the experimental procedure used may influence the TER values obtained. The 5kΩ corrosive threshold was developed from data obtained with the specific apparatus and procedure described in this method. Different threshold and control values may apply if the test conditions are altered significantly. Therefore, it is recommended that the methodology and resistance threshold value are calibrated by testing a series of reference standards chosen from the chemicals used in the validation study (3).

1.6 PRINCIPLE OF THE TEST METHOD - HUMAN SKIN MODEL ASSAY

The test material is applied topically for up to 4 hours to a three-dimensional human skin model, comprising a reconstructed epidermis with a functional stratum corneum. Corrosive materials are identified by their ability to produce a decrease in cell viability (as determined, for example, by using the MTT reduction assay) below defined threshold levels at specified exposure periods. The principle of the assay is in accordance with the hypothesis that chemicals which are corrosive are those which are able to penetrate the stratum corneum (by diffusion or erosion) and are sufficiently cytotoxic to cause cell death in the underlying cell layers.

1.7 DESCRIPTION OF THE TEST METHOD - HUMAN SKIN MODEL ASSAY

1.7.1 Human skin models

Human skin models can come from various sources, but they must meet certain criteria. The model must have a functional stratum corneum with an underlying layer of living cells. The barrier function of the stratum corneum must be adequate. This can be shown by demonstrating the model's resistance to cytotoxicity following the application of substances which are known to be cytotoxic to cells, but which do not normally pass through the stratum corneum. The model must be shown to give reproducible results under defined experimental conditions.

The viability of the living cells in the model must be sufficiently high to discriminate well between the positive and negative control substances. Cell viability (for example, as measured by the amount of MTT reduction, i.e. an OD value) following exposure to the negative control substance must fall within acceptable limits for the particular model. Similarly, cell viability values with the positive control substance (relative to those for the negative control) must fall within specified limits. Most importantly, the prediction model used must have been shown to meet international validation standards.

1.7.2 Test procedure

1.7.2.1 Application of the test material

For liquid materials, sufficient test substance must be applied to cover the skin surface (a minimum of 25µl/cm²). For solid materials, sufficient test substance must be applied to cover the skin, and it should then be moistened to ensure good contact with the skin; where appropriate, solids should be ground to a powder before application. The application method must be shown to be adequate for a wide range of chemical types (e.g. see reference 2). At the end of the exposure period, the test material must be carefully washed from the skin surface with saline solution.

1.7.2.2 Cell viability measurements

Any quantitative, validated, method can be used to measure cell viability. The most frequently used assay is MTT reduction, which has been shown to give accurate and reproducible results in various laboratories (2). The skin disc is placed in an MTT solution of 0.3mg/ml at 20-28°C for 3 hours. The precipitated blue formazan product is then extracted (solvent extraction) and the concentration of the formazan is measured by determining the OD at a wavelength between 545 and 595 nm.

1.7.2.3 Additional information

The skin model used, and the exact protocol of exposure time and washing procedures, etc. will have a major impact on the cell viability results. It is recommended that the methodology and prediction model are calibrated by testing a series of reference standards chosen from the chemicals used in the ECVAM validation study (3). It is critical that the method used has been shown to be reproducible within and between laboratories for a wide range of chemicals, in accordance with international standards. As a minimum, the method should meet the criteria for scientific validity defined previously (2), and the results of such a validation study must be published in a peer-reviewed scientific journal.

2. DATA

2.1 TREATMENT OF RESULTS

2.1.1 Rat skin TER assay

Resistance values (kΩ) for the test material, positive and negative controls, and any standard reference chemicals should be reported in tabular form, including data for replicates/repeat experiments, mean values and the classification derived.

2.1.2 Human skin model assay

OD values and calculated percentage cell viability data for the test material, positive and negative controls, and any standard reference chemicals should be reported in tabular form, including data for replicates/repeat experiments, mean values and the classification derived.

2.2 EVALUATION AND INTERPRETATION OF RESULTS

2.2.1 Rat skin TER assay

If the mean TER value obtained for the test substance is greater than 5kΩ, then it is non-corrosive. If the TER value is less than or equal to 5kΩ, and the test substance is not a surfactant or neutral organic, then it is corrosive.

For surfactants or neutral organics which give TER values less than or equal to 5kΩ, dye penetration can be carried out. If the mean disc dye content is greater than or equal to the mean disc dye content of the 36% HCl positive control obtained concurrently, then the test substance is a true positive and is therefore corrosive. If the mean disc dye content is less than the mean disc dye content of the 36% HCl positive control obtained concurrently, then the test substance is a false positive and is therefore non-corrosive.

2.2.2 Human skin model assay

The negative control OD value represents 100% cell viability; hence, the OD values obtained for each test sample can be used to calculate a percentage viability relative to the negative control. The cut-off percentage cell viability value distinguishing corrosive from non-corrosive test materials (or discriminating between different corrosive classes) must be clearly defined in the prediction model before the method is validated, and the subsequent validation study must show that the cut-off value is appropriate (e.g. see reference 2).

3. REPORTING

TEST REPORT

The test report must include at least the following information:

Test substance:

– identification data, physical nature and, where relevant, physicochemical properties.

Similar information should be provided for reference substances, if used.

Test conditions:

– details of test procedure used;

– description and justification of any modifications.

Results:

– tabulation of resistance values (TER assay) or percentage cell viability values (human skin model assay) for the test material, positive and negative controls and any standard reference chemicals, including data for replicates/repeat experiments and mean values;

– description of any other effects observed.

Discussion of the results.

Conclusions

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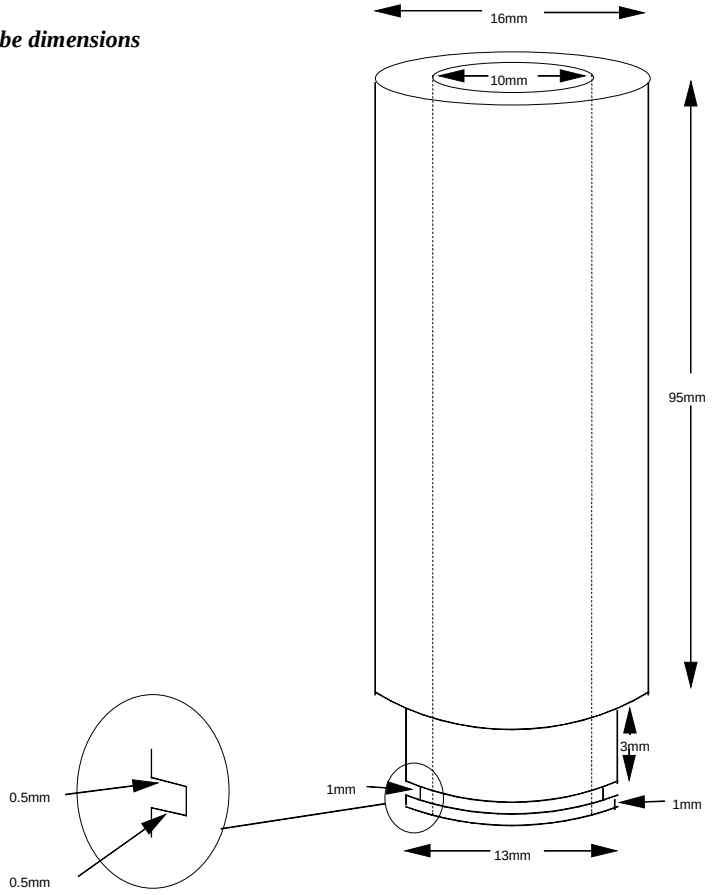
Figure 1

PTFE tube dimensions



Electrode dimensions

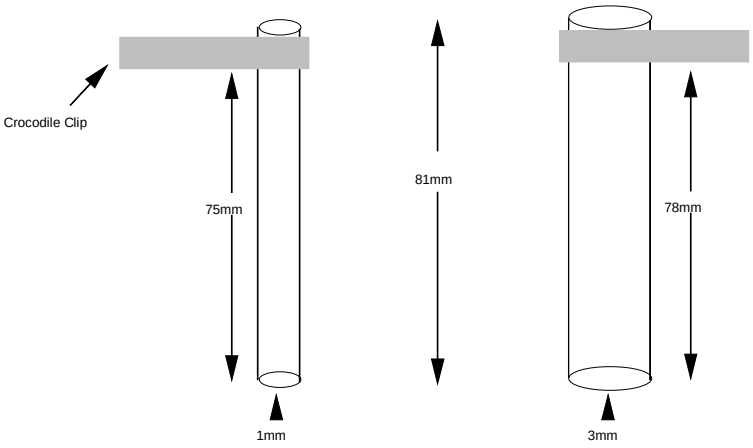
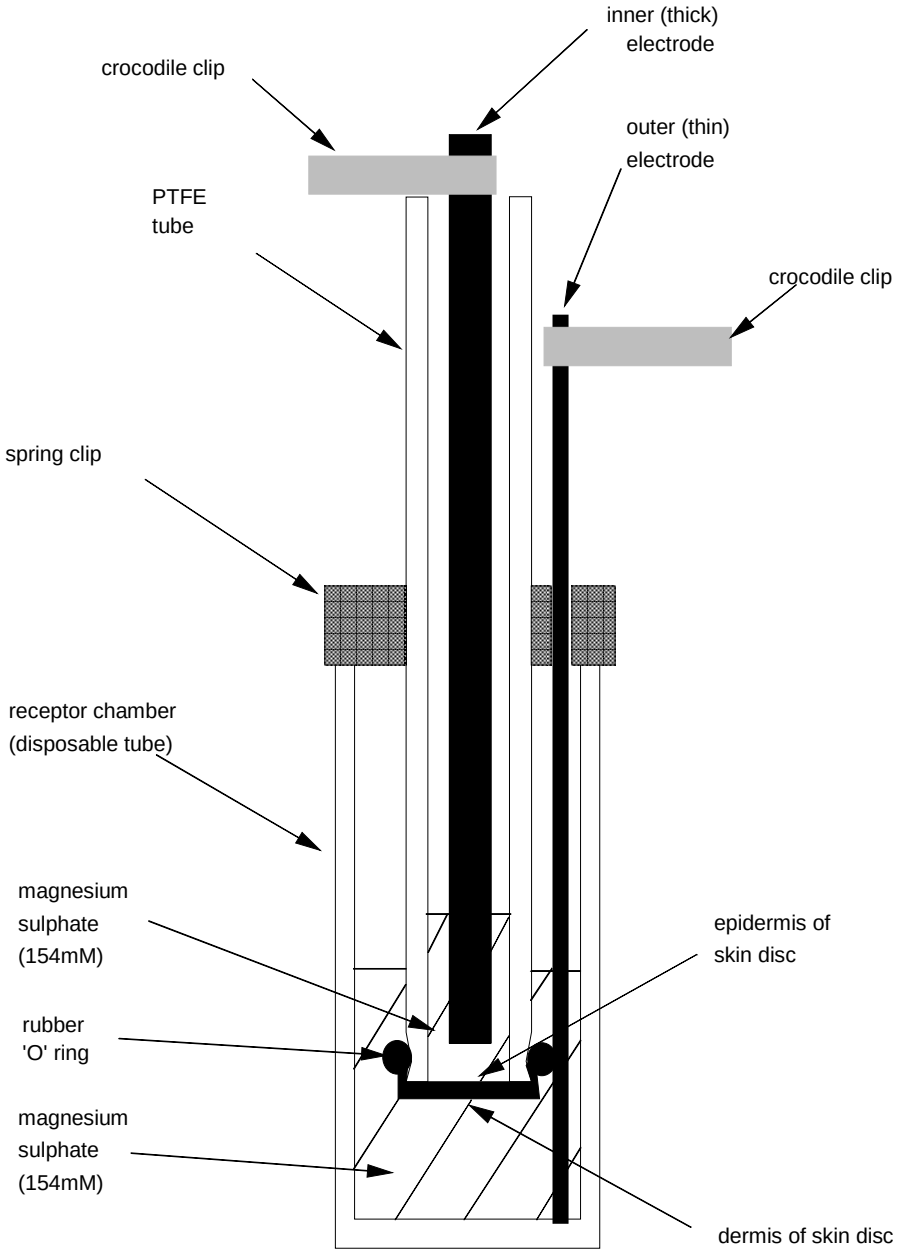


Figure 2

Apparatus for the rat skin TER assay



B. 41. PHOTOTOXICITY - *IN VITRO* 3T3 NRU PHOTOTOXICITY TEST

1. METHOD

1.1 INTRODUCTION

Phototoxicity is defined as a toxic response that is elicited after the first exposure of skin to certain chemicals and subsequent exposure to light, or that is induced similarly by skin irradiation after systemic administration of a chemical.

Information derived from the *in vitro* 3T3 NRU phototoxicity test serves to identify the phototoxic potential of a test substance, i.e. the existence or absence of possible hazards likely to arise from a test substance in association with exposure to UV and visible light.

Since the toxicological endpoint of the *in vitro* test is determination of *photocytotoxicity*, induced by the combined action of a chemical and light, compounds that are phototoxic *in vivo* after systemic application and distribution to the skin, as well as compounds that act as photoirritants after topical application to the skin, can be identified by the test.

The *in vitro* 3T3 NRU phototoxicity test was developed and validated in a joint EU/COLIPA project from 1992-1997 (1)(2)(3), to establish a valid *in vitro* alternative to the various *in vivo* tests in use. In 1996 an OECD workshop recommended an *in vitro* tier testing approach for phototoxicity assessment (4).

Results from the *in vitro* 3T3 NRU phototoxicity test were compared with acute phototoxicity / photoirritation effects *in vivo* in animals and humans, and the test has been shown to give excellent predictivity for these effects. The test is not designed to predict other adverse effects that may arise from the combined action of a chemical and light, e.g. *photogenotoxicity*, *photoallergy*, and *photocarcinogenicity*, although many chemicals which show these specific properties will react positive in the *in vitro* 3T3 NRU phototoxicity test. In addition, the test is not designed to permit an assessment of *phototoxic potency*.

A sequential approach to phototoxicity testing of chemicals is set out in Annex 1.

1.2 DEFINITIONS

Irradiance: the intensity of ultraviolet (UV) or visible light incident on a surface, measured in W/m^2 or mW/cm^2.

Dose of light: the quantity (= intensity × time) of ultraviolet (UV) or visible radiation incident on a surface, expressed in Joules (= W × s) per surface area, e.g. J/m^2 or J/cm^2.

UV light wavebands: The designations recommended by the CIE (Commission Internationale de L'Eclairage) are: UVA (315-400nm), UVB (280-315nm) and UVC (100-280nm). Other designations are also used: the division between UVB and UVA is often placed at 320nm, and the UVA may be divided into UV-A1 and UV-A2 with a division made at about 340nm.

Cell viability: parameter measuring total activity of a cell population (e.g. uptake of the vital dye Neutral Red into cellular lysosomes) which, depending on the endpoint measured and the test design used, correlates with the total number and / or vitality of the cells.

Relative cell viability: cell viability expressed in relation to negative (solvent) controls which have been taken through the whole test procedure (either +UV or -UV), but not treated with a test chemical.

Prediction model: an algorithm used to transform the results of a toxicity test into a prediction of toxic potential. In the present test guideline, PIF and MPE can be used for transformation of the results of the in vitro 3T3 NRU phototoxicity test into a prediction of phototoxic potential.

PIF (Photo Irritation Factor): a factor generated by comparing two equally effective cytotoxic concentrations (EC_{50}) of the test chemical obtained in the absence (-UV) and in the presence (+UV) of a non-cytotoxic irradiation with UVA/vis light.

MPE (Mean Photo Effect): a novel measure derived from mathematical analysis of the complete shape of two concentration response curves obtained in the absence (-UV) and in the presence (+UV) of a non-cytotoxic irradiation with UVA/vis light.

Phototoxicity: an acute toxic response that is elicited after the first exposure of skin to certain chemicals and subsequent exposure to light, or that is induced similarly by skin irradiation after the systemic administration of a chemical.

Photoirritation: a sub-species of the term 'phototoxicity', which is used to describe only those phototoxic reactions which are produced at the skin after exposure to chemicals (topically or orally). These photoxic reactions lead always to non-specific cell damage (sunburn like reactions).

Photoallergy: an acquired immunological reactivity, which does not occur on first treatment with chemical and light, and needs an induction period of one or two weeks before skin reactivity can be demonstrated.

Photogenotoxicity: a genotoxic response observed with a genetic endpoint, which is elicited after the exposure of cells to a non-genotoxic dose of UV/visible light and a non-genotoxic chemical.

Photocarcinogenicity: carcinogenicity induced by repeated application of light and a chemical. The term 'photo co-carcinogenesis', is used if UV induced tumorigenesis is enhanced by a chemical.

1.3 REFERENCE SUBSTANCES

Besides the positive control chemical *Chlorpromazine,* which should be concurrently tested in each assay, for newly establishing the 3T3 NRU phototoxicity test it is recommended to use as reference chemicals a subset from the chemicals used in interlaboratory trials with the present test (1)(3)(13).

1.4 INITIAL CONSIDERATIONS

Many types of chemicals have been reported to induce phototoxic effects (5)(6)(7)(8). The only common feature is their ability to absorb light energy within the sunlight region. According to the first law of photochemistry (Grotthaus-Draper's Law) photoreaction requires sufficient absorption of light quanta. Thus, before biological testing according to the present test guideline is considered, a UV/vis absorption spectrum of the test chemical should be determined (e.g. according to OECD Test Guideline 101). If the molar extinction / absorption coefficient is less than 10 litre $\times$ mol^{-1} $\times$ cm^{-1}, the chemical has no photoreactive potential and does not need to be tested in the *in vitro* 3T3 NRU phototoxicity test or any other biological test for adverse photochemical effects (Annex 1).

1.5 PRINCIPLE OF THE TEST METHOD

Four mechanisms have been identified by which absorption of light by a (chemical) chromophore can result in a phototoxic response (7). All of them result in cell damage. Therefore, the *in vitro* 3T3 NRU phototoxicity test is based on a comparison of the cytotoxicity of a chemical when tested in the presence and in the absence of exposure to a non-cytotoxic dose of UVA/vis light. Cytotoxicity in this test is expressed as a concentration dependent reduction of the uptake of the vital dye, Neutral Red (NR, (9)) 24 hours after treatment with the test chemical and irradiation.

Balb/c 3T3 cells are maintained in culture for 24 h for the formation of monolayers. Two 96-well plates per test chemical are then preincubated with eight different concentrations of the chemical for 1 h. Thereafter one of the two plates is exposed to a non-cytotoxic UVA/vis light dose of 5 J/cm² UVA (+UV experiment), whereas the other plate is kept in the dark (-UV experiment). In both plates, the treatment medium is then replaced by culture medium and after another 24 h of incubation, cell viability is determined by Neutral Red Uptake (NRU) for 3 h. Relative cell viability, expressed as percentage of untreated negative controls, is calculated for each of the eight test concentrations. To predict the phototoxic potential, the concentration responses obtained in the presence (+UV) and in the absence (-UV) of irradiation are compared, usually at the EC_{50} level, i.e. at the concentration inhibiting cell viability by 50 % cf. untreated controls.

1.6 QUALITY CRITERIA

UVA sensitivity of the cells, historical data: Cells should be regularly checked for sensitivity to UVA. Cells are seeded at the density used in the *in vitro* 3T3 NRU phototoxicity test, irradiated the next day with UVA doses from 1-9 J/cm², and cell viability is determined one day later using the NRU assay. Cells meet the quality criteria, if their viability after irradiation with 5 J/cm^2 UVA is not less than 80% of the viability of dark controls. At the highest UVA dose of 9 J/cm^2, viability should not be less than 50% of that of dark controls. This check should be repeated about every 10th passage of the cells.

UVA sensitivity of the negative control cells, current test: The test meets the quality criteria if negative controls (cells in Earl's Balanced Salt Solution (EBSS) with or without 1% dimethylsulfoxide (DMSO) or 1% ethanol (EtOH)) in the +UVA experiment show a viability of not less than 80% of that of non-irradiated cells in the same solvent of the concurrent dark experiment (-UVA).

Viability of negative controls: The absolute optical density ($OD_{540\ NRU}$) measured in the NR extract of the negative controls indicates whether the 1×10^4 cells seeded per well have grown with normal doubling time during the two days of the assay. A test meets the acceptance criteria if the mean $OD_{540\ NRU}$ of untreated controls is ≥ 0.2

Positive control: A known phototoxic chemical shall be tested concurrently with each *in vitro* 3T3 NRU phototoxicity test. Chlorpromazine (CPZ) was used as positive control in the EU/COLIPA validation study and is therefore recommended. For CPZ tested with the standard protocol in the *in vitro* 3T3 NRU phototoxicity test, the following test acceptance criteria were defined: *CPZ irradiated (+UVA)*: EC_{50}= 0.1 to 2.0 µg/ml, *CPZ non-irradiated (-UVA)*: EC_{50} = 7.0 to 90.0 µg/ml. The Photo Irritation Factor (PIF), i.e. the shift of EC_{50} should be at least 6.

Other known phototoxic chemicals, suitable for the chemical class or solubility characteristics of the test chemical being evaluated, may be used as the concurrent positive controls, in place of CPZ. In this case, based on historical data, the ranges of EC_{50} values and PIF or MPE (Mean Photo Effect) should be adequately defined as acceptance criteria for the test.

1.7 DESCRIPTION OF THE TEST METHOD

1.7.1 Preparations

1.7.1.1 Cells

A permanent mouse fibroblast cell line - Balb/c 3T3, clone 31 - either from ATCC or from ECACC was used in the validation study, and is therefore recommended. Other cells or cell lines may be successfully used with the same test protocol, if the culture conditions are adapted to the specific needs of the cells, but equivalency must be demonstrated.

Cells should be checked regularly for the absence of mycoplasma contamination and should only be used if the results of such checking was satisfactory.

Since the UVA sensitivity of cells may increase with the number of passages, Balb/c 3T3 cells of the lowest obtainable passage number should be used, preferably less than 100. It is important that UVA sensitivity of the Balb/c 3T3 cells is regularly checked according to the quality control procedure described in this Guideline.

1.7.1.2 Media and culture conditions

Appropriate culture media and incubation conditions should be used for routine cell passage and during the test procedure. For Balb/c 3T3 cells, these are DMEM supplemented with 10% new-born calf serum, 4 mM Glutamine, Penicillin and Streptomycin, and humidified incubation at 37°C / 7.5% CO_2. It is particularly important that cell culture conditions ensure a cell cycle time within the normal historical range of the cells or cell line used.

1.7.1.3 Preparation of cultures

Cells from frozen stock cultures are seeded in culture medium at an appropriate density and subcultured at least once before they are used in the *in vitro* 3T3 NRU phototoxicity test.

For the phototoxicity test cells are seeded in culture medium at a density such that cultures will not reach confluence by the end of the test, i.e. when cell viability is determined 48 h

after the seeding of the cells. For Balb/c 3T3 cells grown in 96-well plates, 1×10^4 cells per well is the recommended cell density.

For each test chemical, cells are seeded identically in two separate 96-well plates, which are then taken concurrently through the whole test procedure under identical culture conditions, except for the time period where one of the plates is irradiated (+UVA/vis) and the other one is kept in the dark (-UVA/vis).

1.7.1.4 Metabolic activation

Whereas the use of metabolising systems is a general requirement for all in vitro tests for the prediction of genotoxic and carcinogenic potential, up to now, in the case of phototoxicology, no chemical is known for which metabolic transformation is needed for the chemical to act as a phototoxin in vivo or in vitro. Thus, it is neither considered necessary nor scientifically justified for the present test to be performed with a metabolic activation system.

1.7.1.5 Test chemical / Preparation

Test chemicals must be freshly prepared immediately prior to use, unless stability data demonstrate the acceptability of storage. Preparation under red light may be required when rapid photodegradation is likely to occur.

Test chemicals should be dissolved in buffered salt solutions, e.g. Earl's Balanced Salt Solution, (EBSS) or Phosphate Buffered Saline (PBS), which, to avoid interference during irradiation, must be free from protein components and light absorbing pH indicator colours.

Test chemicals of limited solubility in water should be dissolved in appropriate solvents at 100-fold the desired final concentration and then diluted 1:100 with the buffered salt solution. If a solvent is used it must be present at a constant volume of 1% (v/v) in all cultures, i.e. in the negative controls as well as in all concentrations of the test chemical.

Dimethylsulphoxide (DMSO) and ethanol (EtOH) are the recommended solvents. Other solvents of low cytotoxicity (e.g. acetone) may be appropriate, but they should carefully be assessed for specific properties, e.g. reaction with the test chemical, quenching of the phototoxic effect, radical catching properties.

Vortex mixing and / or sonication and / or warming to 37°C may be used, if necessary, to aid solubilization.

1.7.1.6 UV irradiation / Preparation

Light source: the choice of an appropriate light source and appropriate filtering is the most crucial factor in phototoxicity testing. UVA and visible regions are usually associated with photosensitization (7)(10), whereas UVB is of less relevance and is directly highly cytotoxic, increasing its cytotoxicity through 1000 fold from 313 to 280 nm (11). Criteria for the choice of an appropriate light source should include the essential requirement that the light source emits wavelengths absorbed by the test chemical and that the dose of light (achievable in a reasonable time) should be sufficient for the detection of known photosensitizers. Furthermore, the wavelengths and doses employed should not be unduly deleterious to the test system, which includes the emission of heat (infra red region).

The simulation of sunlight with solar simulators is considered the optimal light source. Both, Xenon arcs and (doped) mercury-metal halide arcs are used in solar simulators. The latter

have the advantage of emitting less heat and of being cheaper, but the match to sunlight is not perfect. Since all solar simulators emit significant quantities of UVB, they should be suitably filtered to attenuate the highly cytotoxic UVB wavelengths.

For the *in vitro* 3T3 NRU phototoxicity test an irradiance spectrum practically devoid of UVB should be used (UVA:UVB ~ 20:1). An example of the spectral irradiance distribution of the filtered solar simulator used in the validation study of the *in vitro* 3T3 NRU phototoxicity test has been published (3).

Dosimetry: The intensity of light (irradiance) should be regularly checked before each phototoxicity test, by using a suitable broadband UV-meter. The UV-meter must have been calibrated to the source. The performance of the UV-meter should be checked, and for this purpose, the use of a second, reference UV-meter of the same type and identical calibration is recommended. Ideally, at greater intervals, a spectroradiometer should be used to measure the spectral irradiance of the filtered light source and to check the calibration of the broadband UV-meter, but such instruments require skilled operation by appropriately trained persons.

A dose of 5 J/cm² (UVA) was determined in the validation study to be non-cytotoxic to Balb/c 3T3 cells and sufficiently potent to excite even weak phototoxic chemicals. To achieve 5 J/cm² within a time period of 50 min, irradiance has to be adjusted to 1.666 mW/cm². If another cell line or a different light source are used, the UVA dose may have to be slightly adapted, by using the criteria of being non-deleterious to the cells and sufficient to detect standard phototoxins. The time of light exposure is calculated in the following way:

$$t(\text{min}) = \frac{\text{irradiation dose}\,(\text{J/cm}^2) \times 1000}{\text{irradiance}\,(\text{mW/cm}^2) \times 60}$$

(1 J = 1 W sec)

1.7.2 Test Conditions

The maximum concentration of a test chemical should not exceed 100 µg/ml, since all phototoxic chemicals were detected at lower concentrations, whereas at higher concentrations the incidence of false positives (overpredictions) increases (13). The pH of the highest concentration of the test chemical should be satisfactory (pH range: 6.5 - 7.8).

The ranges of concentrations of a chemical tested in the presence (+UVA) and in the absence (-UVA) of light should be adequately determined in preceding range-finder experiments. Range and intercept of a concentration series shall be adjusted in such a way that concentration-response curves are sufficiently supported by experimental data. Geometric concentration series (with a constant dilution factor) should be used.

1.7.3 Test Procedure[14]

1.7.3.1 1st day

Prepare a cell suspension of $1x10^5$ cells/ml in culture medium and dispense 100 µL culture medium only into the peripheral wells of a 96-well tissue culture microtiter plate (= blanks). In the remaining wells, dispense 100 µL of a cell suspension of 1×10^5 cells/ml (= 1×10^4

[14] Additional details can be found in reference (12)

cells/well). For each test chemical, prepare two plates: one for determination of cytotoxicity (-UVA), and the other for determination of photocytotoxicity (+UVA).

Incubate the cells for 24 h (7.5% CO_2, 37ºC) until they form a half-confluent monolayer. This incubation period allows for cell recovery and adherence, and for exponential growth.

1.7.3.2 2nd day

After incubation, decant the culture medium from the cells and wash twice with 150 µL EBSS/PBS per well. Add 100 µL of EBSS/PBS containing the appropriate concentration of test chemical or just solvent (negative control). Apply 8 different concentrations of the test chemical. Incubate cells with the test chemical in the dark for 60 minutes (7.5% CO_2, 37ºC).

To perform the (+UVA) part of the assay, irradiate the cells at room temperature for 50 minutes through the lid of the 96-well plate with 1.7 mW/cm² UVA (= 5 J/cm²). Ventilate with a fan to prevent H_2O condensation under the lid. Keep duplicate plates (-UVA) at room temperature in a dark box for 50 min (= UVA exposure time).

Decant test solution and wash twice with 150 µL EBSS/PBS. Replace EBSS/PBS with culture medium and incubate (7.5% CO_2, 37 °C) overnight (18-22 h).

1.7.3.3 3rd day

Microscopic evaluation

Examine the cells under a phase-contrast microscope. Record changes in morphology of the cells due to cytotoxic effects of the test chemical. This check is recommended, to exclude experimental errors, but these records are not used for evaluation of cytotoxicity or phototoxicity

Neutral Red Uptake test

Wash the cells with 150 µL prewarmed EBSS/PBS. Remove the washing solution by gentle tapping. Add 100 µl NR medium and incubate at 37 °C, in a humidified atmosphere of 7.5% CO_2, for 3 h.

After incubation, remove the NR medium, and wash the cells with 150 µL EBSS/PBS. Decant and blot EBSS/PBS totally. (*Optionally*: centrifuge reversed plate.)

Add exactly 150 µL NR desorb solution (freshly prepared ethanol/acetic acid)

Shake microtiter plate rapidly on a microtiter plate shaker for 10 min, until the NR has been extracted from the cells and has formed a homogeneous solution.

Measure the optical density of NR extract at 540 nm in a spectrophotometer, using blanks as a reference. Save the data in appropriate file format (e.g. ASCII) for subsequent analysis.

2 DATA

2.1 QUALITY AND QUANTITY OF DATA

The data should permit a meaningful analysis of the concentration-response obtained in the presence and in the absence of UVA/vis irradiation. If cytotoxicity is found, both the concentration range and the intercept of individual concentrations should be set in such a way as to allow the fit of a curve to experimental data. Due to the fact that a test chemical might not be cytotoxic up to the defined limit concentration of 100 µg/ml in the dark experiment (-UVA), but highly cytotoxic when irradiated (+UVA), the concentration ranges to be tested in both parts of the experiment may need to differ by orders of magnitude to fulfil the requirement of adequate data quality. If no cytotoxicity is found in both parts of the experiment (-UVA and +UVA), testing with a great intercept between single doses up to the highest concentration is sufficient.

There is no requirement for verification of a clear positive result by performing a repeat experiment. In addition, clear negative results need not to be verified, provided the test chemical was tested at sufficiently high concentrations. In such cases, one main experiment, supported by one or more range-finding preliminary experiments, is sufficient.

Tests with borderline results near to the cut-off line of the prediction model should be repeated for verification.

If repeat testing is considered necessary, then variation of the experimental conditions may be important to achieve a clear result. A key variable in this test is preparation of solutions of the test chemical. Hence, variation of these conditions (co-solvent, trituration, sonication) may be most relevant in the repetition of a test. Alternatively, variation of the pre-irradiation incubation time may be considered. A shorter time can be relevant for water-unstable chemicals.

2.2 TREATMENT OF RESULTS

Where possible, the concentration of a test chemical reflecting a 50% inhibition of the cellular NRU (EC_{50}) is determined. This can be done by applying any appropriate non-linear regression procedure (preferably a Hill function or logistic regression) to the concentration-response data, or by using other fitting procedures (14). Before using an EC_{50} for further calculations, the quality of the fit should be appropriately checked. Alternatively, graphical fitting methods can be used to calculate the EC_{50}. In this case, the use of probability paper is recommended (x-scale: log, y-scale: probit), as in many cases the concentration response function will become almost linear after this transformation.

2.3 EVALUATION OF RESULTS (PREDICTION MODELS)

2.3.1 Prediction model version 1: Photo-Irritation-Factor (PIF)

If both, in the presence (+UVA) and in the absence (-UVA) of light, complete concentration response curves are obtained, a Photo-Irritation-Factor (PIF) is calculated by means of the following formula:

(a) $$PIF = \frac{EC_{50}(-UV)}{EC_{50}(+UV)}$$

A PIF < 5, predicts no phototoxic potential, whereas a PIF ≥ 5 predicts phototoxic potential.

If a chemical is only cytotoxic +UVA and is not cytotoxic when tested -UVA, the PIF cannot be calculated, although this is a result that indicates phototoxic potential. In such cases, a "> PIF" can be calculated if the (-UV) cytotoxicity test is performed up to the highest test concentration (C_{max}) and this value is used for calculation of the "> PIF":

(b) $$> PIF = \frac{C_{max}(-UV)}{EC_{50}(+UV)}$$

If only a "> PIF" can be obtained, then any value >1 predicts phototoxic potential.

If both EC_{50} (-UV) and EC_{50} (+UV) cannot be calculated due to the fact that a chemical does not show any cytotoxicity up to the highest test concentration, this indicates no phototoxic potential. In such cases, a formal "PIF = *1" is used to characterise the result

(c) $$PIF = *1 = \frac{C_{max}(-UV)}{C_{max}(+UV)}$$

If only a "PIF = *1" can be obtained, this predicts no phototoxic potential.

In cases (b) and (c), concentrations achieved in the *in vitro* 3T3 NRU phototoxicity test should be carefully taken into consideration when predicting phototoxic potential

2.3.2 Prediction model version 2: Mean-Photo-Effect (MPE)

Alternatively, a novel version of the model for predicting phototoxic potential can be applied, which has been developed by using data of the EU/COLIPA validation study (15) and tested under blind conditions in a subsequent study on the *in vitro* phototoxicity of UV filter chemicals (13). This model overcomes the limitation of the PIF model in cases where an EC_{50} cannot be obtained. The model uses the "Mean Photo Effect" (MPE), a measure which is based on comparison of the complete concentration response curves. For application of the MPE model, a special computer software was developed at the Humboldt University (Berlin, D), which can be obtained free of charge.

2.4 INTERPRETATION OF RESULTS

A positive result in the *in vitro* 3T3 NRU phototoxicity test (PIF ≥ 5 or MPE ≥ 0.1) indicates that the test substance has phototoxic potential. If this result is obtained at concentrations below 10 µg/ml, the test chemical is also likely to act as phototoxin also under various exposure conditions *in vivo*. If a positive result is obtained only at the highest test concentration of 100 µg/mL, further considerations may be necessary for the assessment of hazard or phototoxic potency. These may include data on penetration, absorption and possible accumulation of the chemical in the skin, or testing of the chemical in a confirmatory alternative test, e.g. using a human *in vitro* skin model.

A negative result from the *in vitro* 3T3 NRU phototoxicity test (PIF < 5 or MPE < 0.1) indicates that the test substance was not phototoxic to the cultured mammalian cells under the

conditions used. In cases where the chemical could be tested up to the highest concentration of 100 µg/ml, a negative result indicates that the chemical has no phototoxic potential, and phototoxicity *in vivo* may be considered unlikely. In cases where identical concentration-toxicity responses (EC_{50}+UV and EC_{50}-UV) were obtained at lower concentrations, the interpretation of data would be the same. In contrast, if no toxicity was demonstrated (+UV and -UV) and if aqueous solubility limited concentrations to values less than 100 µg/ml, then compatibility of the test substance with the assay may be questioned and confirmatory testing should be considered (e.g. using an *in vitro* skin model, or an *ex vivo* skin model or an *in vivo* test).

3 REPORTING

TEST REPORT

The test report must include the following information:

3.1. Test chemical:

– identification data and CAS no., if known

– physical nature and purity

– physicochemical properties relevant to conduct of the study

– stability and photostability, if known

3.2. Solvent:

– justification for choice of solvent

– solubility of the test chemical in this solvent

– percentage of solvent present in treatment medium (EBSS or PBS)

3.3. Cells:

– type and source of cells

– absence of mycoplasma

– number of cell passages, if known

UVA sensitivity of cells, determined with the irradiation equipment used in the *in vitro* 3T3 NRU phototoxicity test

3.4. Test conditions:

(a) *incubation before and after treatment:*

– type and composition of culture medium

– incubation conditions (CO2 concentration, temperature, humidity)

– duration of incubation (pre-treatment, post-treatment)

(b) *treatment with the chemical:*

– rationale for selection of concentrations of the test chemical used both in the presence and in the absence of UV/vis irradiation

– in case of limited solubility of the test chemical and absence of cytotoxicity, rationale for the highest concentration tested

– type and composition of treatment medium (buffered salt solution)

– duration of the chemical treatment

(c) *irradiation:*

– rationale for selection of the light source used

– spectral irradiance characteristics of the light source

– transmission / absorption characteristics of the filter(s) used

– characteristics of the radiometer and details on its calibration

– distance of the light source from the test system

– UVA irradiance at this distance, expressed in mW/cm^2

– duration of the UV/vis light exposure

– UVA dose (irradiance × time), expressed in J/cm^2

– temperature employed to cell cultures during irradiation and for cell cultures concurrently kept in the dark

(d) *NRU test*

– composition of NR medium

– duration of NR incubation

– incubation conditions (CO2 concentration, temperature, humidity)

– NR extraction conditions (extractant, duration)

– wavelength used for spectrophotometric reading of NR optical density

– second wavelength (reference), if used

– content of spectrophotometer blank, if used

3.5. Results:

– cell viability obtained at each concentration of the test chemical, expressed in percent mean viability of controls

– concentration - response curves (test chemical concentration vs. relative cell viability), obtained in concurrent +UVA and -UVA experiments

– data analysis of the concentration response curves: if possible, computation / calculation of EC50 (+UVA) and EC50 (-UVA)

– comparison of the two concentration-response curves obtained in the presence and in the absence of UVA/vis irradiation, either by calculation of the Photo Irritation Factor (PIF), or by calculation of the Mean Photo Effect (MPE)

– classification of phototoxic potential

3.6. Test acceptance criteria:

(a) *concurrent negative control:*

– absolute viability (optical density of NR extract) of irradiated and non irradiated cells

– historical data of negative control, mean and standard deviation

(b) *concurrent positive control:*

– EC50(+UVA) and EC50(-UVA) and PIF of positive control chemical

– historical data of positive control chemical: EC50(+UVA) and EC50(-UVA) and PIF, mean and standard deviation

Discussion of the results

Conclusions

4 REFERENCES

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ANNEX 1
Role of the 3T3 NRU PT in a Sequential Approach to Phototoxicity Testing of Chemicals