

**GUIDELINES ON
TOXICOLOGICAL DATA REQUIREMENTS
FOR
PESTICIDE REGISTRATION**

**Pesticides Board
Malaysia
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PREFACE

The Pesticides Board in its efforts to further upgrade its services to the public, in particular companies applying for the registration of pesticides has prepared these guidelines to supplement the existing ones. These supplementary guidelines published in 4 booklets provide information in greater detail on the requirements for registration on the following aspects:

- i) Products chemistry;
- ii) Efficacy;
- iii) Toxicology and
- iv) Residue

In the preparation of these guidelines references were made to some international and national guidelines such as those published by FAO, OECD and the USEPA. It is hoped that with these guidelines the time taken for registration of pesticides will be reduced. Applicants who require further clarification on these guidelines or other matters related to registration may contact the Secretary of the Pesticides Board at the following address:

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Toxicological Data Requirement for Pesticide Registration

1. Introduction

1.1. Toxicological data are submitted for pesticide registration is aimed at defining possible hazards to man, non-target organisms and the environment.

1.2. Many practical recommendations can be derived from appropriate and through toxicological data such as hazard classification, restrictions on use, appropriate precautionary measures necessary to allow safe use, suggestion of appropriate diagnosis and management of a poisoned person, establishment of Acceptable Daily Intake (ADI) etc.

2. General Requirements

2.1. For the purpose of these guidelines pesticides are divided into two classes i.e. commodity and proprietary pesticides.

2.2. Commodity pesticides

2.2.1. For commodity pesticides, acute oral and acute dermal toxicity studies done on rats are required to be submitted. The detailed requirements are given in **Appendix 1**.

2.2.2. Full reports of the above studies must be made available at registration.

2.3. Proprietary pesticides

2.3.1 For proprietary pesticides, toxicological studies required to be submitted are dependent on the general use pattern and type of pesticide in question i.e. whether it is a conventional chemical or a microbial pesticide. The detailed toxicological requirements needed for **chemical** and **microbial** pesticides are given **Table A** and **Table B** of **Appendix 1** respectively.

2.3.2 Full reports of the above studies must be made available at registration.

2.4. Letter of consent authorizing the applicant to use the data for registration purposes must be provided if another company's data is submitted.

3. Acceptable protocol

- 3.1. All toxicological studies should be carried out based on internationally accepted protocols. Toxicological study protocols such as those produced by The Environmental Protection Agency of United States (USEPA), Organization for Economic Cooperation and Development (OECD), the Ministry of Agriculture, Forestry and Fisheries of Japan may be used.
- 3.2. As examples of acceptable protocols, the Pesticides Board has also come out with minimum requirements for each test protocol as outlined in **Appendix 2** and **Appendix 3** which can also be referred.
- 3.3. Protocols for acute oral and acute dermal studies are given in more detail as in **Table C** and **Table D** of **Appendix 3**. This is in order to provide applicants who wish to generate data locally with complete guidelines and reporting format.
- 3.4. In conducting the toxicological studies, the applicants are required to rigidly comply with Good Laboratory Practice standards.

4. Exemptions

- 4.1 The following are pesticides which can be registered without the submission of toxicological data.
 - i. boric acid
 - ii. borax
 - iii. copper oxychloride
 - iv. copper sulphate
 - v. cupric sulphate pentahydrate
 - vi. cuprous oxide
 - vii. cupric hydroxide
 - viii. sodium dichromate
 - ix. sulphur
 - x. sodium chlorate
 - xi. phosphorous acid

TABLE A : TOXICOLOGICAL DATA REQUIREMENTS FOR REGISTRATION OF CHEMICAL PESTICIDES

Data Required	General Use Pattern					Remarks
	Food Crop	Non Food Crop	In-door	Out Door	Fores try	
<u>ACUTE TESTING</u>						
Acute oral	R	R	R	R	R	(1)
Acute dermal	R	R	R	R	R	(1), (2)
Acute inhalation	R	R	R	R	R	(3)
Eye irritation	R	R	R	R	R	
Skin irritation	R	R	R	R	R	(1)
Dermal sensitization	R	R	R	R	R	
Acute delayed neurotoxicity	R	R	R	R	R	(4)
<u>SUBACUTE TESTING</u>						
90 days feeding study	R	R	R	R	R	
<u>CHRONIC TESTING</u>						
Chronic feeding study	R	R	CR	CR	CR	(6)
Carcinogenicity study	R	R	CR	CR	CR	(7)
<u>SPECIAL STUDIES</u>						
Teratogenicity	R	R	CR	CR	CR	(8)
Reproduction	R	R	CR	CR	CR	(9)
Mutagenicity	R	R	R	R	R	
Metabolic	R	R	CR	CR	CR	(10)
<u>Wildlife Hazards</u>						
Fish acute toxicity	R	R	CR	R	R	(13)
Other tests on fish	CR	CR	CR	CR	CR	(13)
Avian acute oral toxicity	R	R	CR	R	R	(14)
Other tests on avian	CR	CR	CR	CR	CR	(14)
Honey bee acute toxicity	R	R	CR	R	R	(15)
Effects on other wildlife	CR	CR	CR	CR	CR	(16)
<u>Human Toxicological Data</u>						
Industrial exposure data	CR	CR	CR	CR	CR	(11)
Accidental data	CR	CR	CR	CR	CR	(11)
Suicidal data	CR	CR	CR	CR	CR	(11)
Volunteers data	CR	CR	CR	CR	CR	(11)
Poisoning symptoms	R	R	R	R	R	
Antidote statements	R	R	R	R	R	
Protective clothing	CR	CR	CR	CR	CR	(12)

KEY: R = Required; CR = Conditionally Required;

TABLE B : TOXICOLOGICAL DATA REQUIREMENTS FOR REGISTRATION OF MICROBIAL PESTICIDES

Data Required	General Use Pattern					Remarks
	Food Crop	Non Food Crop	In-door	Out Door	Fores try	
<u>ACUTE TESTING</u>						
Acute oral/ pathogenicity	R	R	R	R	R	(3)
Acute dermal	R	R	R	R	R	
Acute inhalation	R	R	R	R	R	
Eye irritation/ infectivity	R	R	R	R	R	
Skin irritation	R	R	R	R	R	
Hypersensitivity study	R	R	R	R	R	
<u>SUBACUTE TESTING</u>						
Sub-acute/ pathogenicity	CR	CR	CR	CR	CR	(5)
<u>CHRONIC TESTING</u>						
Chronic feeding study	CR	-	-	-	-	(5)
Carcinogenicity study	CR	-	-	-	-	(5)
<u>SPECIAL STUDIES</u>						
Teratogenicity	CR	CR	CR	CR	CR	(5)
Mutagenicity	CR	CR	CR	CR	CR	(5)
<u>Wildlife Hazards</u>						
Fish acute toxicity	R	CR	CR	CR	CR	(5)
Other tests on fish	CR	CR	CR	CR	CR	(5)
Avian acute oral toxicity	R	CR	CR	CR	CR	(5)
Other tests on avian	CR	CR	CR	CR	CR	(5)
Honey bee acute toxicity	R	CR	CR	CR	CR	(5)
Effects on other wildlife	CR	CR	CR	CR	CR	(5)
<u>Human Toxicological Data</u>						
Industrial exposure data	CR	CR	CR	CR	CR	(11)
Accidental data	CR	CR	CR	CR	CR	(11)
Suicidal data	CR	CR	CR	CR	CR	(11)
Volunteers data	CR	CR	CR	CR	CR	(11)
Poisoning symptoms	R	R	R	R	R	
Antidote statements	R	R	R	R	R	
Protective clothing	CR	CR	CR	CR	CR	(12)

KEY: R = Required; CR = Conditionally Required; (-) = Normally Not required

Remarks:

1. Not required if test material is a gas or is highly volatile.
2. Not required if test material is corrosive to skin or has pH less than 2 or more than 11.5.
3. Required for commodity pesticides if the product consists of, or under condition of use will result in an inhalable material.
4. Required if test material is an organophosphate pesticide.
5. Required if significant signs of infectivity and adverse effects are seen in acute study or unusual persistence of the microbes.
6. Required if sub-acute study indicates significant toxic effects.
7. Required if result of mutagenicity study is positive.
8. Required if exposure to females is significant.
9. Required if the use of the product is likely to result in human exposure over a portion of the human lifespan which is significant in terms of frequency of exposure, magnitude of exposure or duration of exposure (for example; pesticides for use in treated fabric for wearing, constant release pesticides used indoor in aerosol form).
10. Required if, chronic study and carcinogenicity study are required.
11. Required if, such data are available.
12. Required if special safety protective clothing are required to be worn when handling the product.
13. Required if the product is intended to be applied directly to water or expected to be transported to water from the use site and significant exposure to aquatic organisms is anticipated.
14. Required if significant exposure to birds is expected.
15. Required if significant exposure to honey bees is expected.
16. Required if significant exposure to other wildlife is expected.

GENERAL USE PATTERNS

Food crops

1. Agricultural crops for human consumption
2. Veterinary

Nonfood crops

1. Crop for smoking and chewing
2. Medicinal crops
3. Ornamental plants
4. Lawn and turf grasses
5. General soil treatments
6. Recreational areas
7. Roads, tracks and paved areas
8. Antifouling treatments
9. Public health

Indoor

1. Houseplants pesticides
2. Household insecticides
3. Rodenticides for household use
4. Pet animals pesticides
5. Commercial and industrial uses
 - Eating establishment
 - Transportation facilities
 - Buildings and structures
6. Terminate control inside buildings

Forestry

1. Forest trees including dead trees, logs and stumps
2. Forest tree nurseries
3. Non-ornamental trees including rubber trees

Outdoor

1. Domestic ornamental plantings
2. Termite control outside buildings
3. Wood treatments

GUIDELINES FOR TOXICITY STUDIES**Test substance for tests**

Unless otherwise specified, the technical grade of the active ingredient shall be tested. For mixtures of more than one active ingredient, the technical grade of each ingredient shall be tested. Tests on the actual end-use product will also be acceptable.

1. ACUTE INHALATION TOXICITY STUDY**1.1. Purpose**

The purpose of this study is to provide information on health hazards likely to arise from short-term exposure to a pesticide via the inhalation route.

1.2. Test animals

The rat shall be used. The age, sex and numbers are as stipulated in the acute oral toxicity study.

1.3. Dose levels and selection

At least 3 dose levels shall be used and spaced appropriately to produce test group with a range of toxic effects and mortality rates. The data should be sufficient to produce a dose-response curve and permit an acceptable determination of the LC50 value.

1.4. Exposure conditions

Using inhalation equipment, animals should be exposed for at least 4 hours to the test substance in graduated concentrations, allowing sufficient time for chamber equilibrium. Description of the outline of the inhalation equipment and its operation should be included in the test report.

1.5. Observation period

This should be at least 14 days. Clinical observations should be carried out at least once daily.

1.6. Pathology

Same as stipulated in the acute oral toxicity study.

2. PRIMARY EYE IRRITATION STUDY**2.1. Purpose**

The purpose of this study is to obtain information on whether a hazard or hazard are likely to arise from exposure of the eyes to the test substance.

2.2. Test substance

Strongly acidic (pH 2 or less) and strongly alkaline (pH 11.5 or more) substance need not be tested.

2.3. Test animals

At least 6 young adult albino rabbits shall be used.

2.4. Test procedure

For testing liquids, a dose of 0.1 ml should be used. For solids and pastes, the amount used should have a volume of 0.1 ml or weight of 0.1 g. Solid or granular substances should be ground to a fine dust first. The test substance should be placed in the conjunctival sac of one eye and the lids then held together for about one second. The other eye remains as a control.

The eyes of the test animal should not be washed out for 24 hours following application of the dose.

2.5. Clinical examination and scoring

The eyes should be examined at 1, 24, 48 and 72 hours. The grades of ocular reaction should be recorded. If no evidence of irritation is seen, the study is terminated. Extended observation may be necessary if there is persistent corneal involvement or other ocular irritation, for up to 21 days.

3. DERMAL SENSITISATION STUDY

3.1. Purpose

The study is used to identify the possible hazard to a population repeatedly exposed to the test substance.

3.2. Test substance

The end-use product should be used. Strongly acidic or alkaline substances (pH 2 or less, or pH 11.5 or greater) might not be tested.

3.3. Test animals

The young adult guinea pig is the preferred species. The number used depends on the method employed.

3.4. Test methods

Any of the following seven test methods would be acceptable. Use of a positive control substance to test for reliability of the test system is recommended.

Draize Test
Freund's Complete Adjuvant Test
Mauer Optimisation Test
Buehler Test
Open Epicutaneous Test
Guinea Pig Maximisation Test
Split Adjuvant Technique

4. ACUTE DELAYED NEUROTOXICITY STUDY

4.1. Purpose

This screening procedure is for detecting delayed neurotoxic potential of the test substance.

4.2. Test animals

The adult domestic laying hen is recommended in sufficient numbers such that at least six survive the observation period.

4.3. Dose levels and selection

A preliminary LD₅₀ test should be performed in unprotected hens to establish the dose levels to be used in this test. The selected dose level should not be less than the unprotected dose. Doses of test substance higher than 5,000 mg/kg need not be tested.

4.4. Controls

In addition to an untreated control group, a positive control group should be used consisting of at least 4 hens treated with a known delayed neurotoxicant, such as TOCP.

4.5. Administration of dose

The test substance is preferably administered orally in a single dose by gavage or using gelatine capsules. After a short while, a protective agent e.g. atropine should be administered, to prevent death due to cholinergic effects.

4.6. Observation of animals

All hens should be observed at least once daily up to 21 days after administration. Signs of toxicity including the time onset should be recorded. The hens should be subjected to a period of forced motor activity at least twice a week to enhance the observation of minimal responses. If neurotoxic responses are not observed or if equivocal responses are seen, then the dose should be repeated and observations made for another 21 days.

4.7. Pathology

The examination should be performed on specific nerve tissues such as brain, spinal cord, terminal peripheral nerves. Sections of the proximal region of the tibial nerve and its branches should also be taken for examination. Staining of nervous tissue sections should be made with appropriate myelin or axon-specific stain as well as hematoxylin-eosin stain.

5. SUBCHRONIC ORAL TOXICITY (90-DAY) STUDY

5.1. Purpose

This study permits the determination of the no-observed effect level and toxic effects associated with continuous or repeated exposure to a substance for a period of 90 days. It also provides information on possible health hazards likely to arise from repeated exposures over a limited period of time.

5.2. Test animals

At least two mammalian species should be used. The rat and dog are preferred. Young, healthy animals (at least 10 of each sex) should be used for the rodent species while 8 (4 of each sex) should be used for the non-rodent species, at each dose level. If interim sacrifices are planned, the number should be increased appropriately.

5.3. Administration

The test substance may be administered in the diet or in capsules. For rodents, it may be administered by gavage or in the drinking water.

5.4. Dose levels and selection

At least 3 dose levels and additional control group should be used. This can be either an untreated group or a vehicle control group. If the toxic properties of the vehicle is not known, then both untreated and vehicle control groups are required. The highest dose level in rodents should result in toxic effects but not produce an incidence of fatalities which would prevent a meaningful evaluation. For non-rodents, there should be no fatalities.

A satellite group may be treated with the high dose level for 90 days and observed a further 28 days for reversibility, persistence or delayed occurrence of toxic effects.

5.5. Exposure period

The test substance should be administered to the animals, for a period of 90 days. For practical considerations, dosing on a 5-days-per-week basis is acceptable.

5.6. Observation of animals

Cageside and clinical examinations are required, the former at least once each day. Clinical examinations should include:

5.6.1. Hematology determinations i.e. hematocrit, hemoglobin concentration, erythrocyte count, total and differential leukocyte count and a measure of clotting potential (e.g. clotting time, prothrombin time etc.) These determinations should be made at the end of the test period for all survivors. For non-rodents, they should be made at the beginning and once or twice through the test period as well as at the termination.

5.6.2. Clinical biochemistry determinations on blood e.g. calcium, phosphorus, sodium, fasting glucose, SGPT, SGOT, urea nitrogen, albumen, blood creatinine etc. These determinations should be carried out on all survivors at the end of the test period. For non-rodents, these examinations should be done once or twice through the test period as well as at the termination.

5.6.3. Urinalysis – only on high dose and control animals, at the end of the test period. Tests on appearance, protein, glucose, ketone, occult blood contents and also microscopy of sediments should be carried out.

5.6.4. Ophthalmological examination – only on high dose and control groups, to be made prior to administration of the test substance and at termination of study.

5.7. Pathology

Gross necropsy is required on all animals including those which died or were found in moribund condition and sacrificed. The major organs should be weighed i.e. liver, kidneys, adrenals and testes. For non-rodents the thyroid and parathyroid should also be weighed.

5.8. Tissue preservation

All gross lesions and certain tissues such as the brain, thyroid, lungs, heart, bone marrow, liver, salivary glands, kidney, spleen, intestines etc. or their representative samples should be preserved in a suitable medium.

5.9. Histopathological examination

Full histopathology should be performed on the organs and tissues mentioned above, of all rodents in the control and high dose groups, all non-rodents and all rodents that died or were killed during the study. The examinations should include all gross lesions and target organs.

In addition certain organs (liver, lungs and kidneys) should also be examined in the low and intermediate dose groups.

6. CHRONIC TOXICITY STUDY

6.1. Purpose

The study is meant to determine the effects of a substance in a mammalian species following prolonged and repeated exposure. Under the conditions of this test, effects which require a long latent period or are cumulative should become manifest.

6.2. Test animals

Equal numbers of both sexes of at least two mammalian species, a rodent and non-rodent, should be used, whose characteristics are well-known e.g. rat and dog. Dosing of rats should begin as soon as possible after weaning, and of dogs when they are between 4 and 6 months of age.

For rodents it is preferable to perform interim sacrifice, therefore the total number of animals should be increased appropriately.

6.3. Administration of dose

Generally, the animals should receive the test substance in their diet.

6.4. Dose levels and selection

At least three dose levels should be used together with a control group. When the test substance is administered mixed in a vehicle other than food, and the toxic properties of the said vehicle is unknown, both untreated and vehicle control groups are required.

6.5. Exposure period

The duration of the exposure period should be 24 months for rats, 18 months for mouse and 12 months for dogs.

6.6. Observation of animals

Careful clinical examination should be made at least each week. Records should be kept of the body weights, food week. Records should be kept of the body weights, food consumption, sign of toxicity including onset and death; also time of death.

6.7. Clinical pathology

If possible, the same animals should be used in the interim examinations. These include hematology, clinical biochemistry, urinalysis and ophthalmological examinations. At least 10 rodents/sex/dose should be used, and all animals in the non-rodent group. The examinations should be done at least every 6 months and at study termination.

6.8. Pathology

Complete gross necropsy should be done on all animals, including those which died during the experiment or were killed in moribund condition. Specific organs and tissues should be preserved for possible future histopathological examination. These include all gross lesions, brain, heart, spleen, uterus, kidneys, adrenals, muscles, spinal cord, lymph nodes, eyes etc.

6.9. Histopathological examination

This should be done for all non-rodents. For rodents, all animals in the control, highest dose group and all those which died or were killed during the study should be examined. All gross lesions, target organs, lungs, liver and kidneys should be examined. Examination of organs in the other test group depend on the organs showing effects in the highest dose group.

7. ONCOGENICITY STUDY

7.1. Purpose

In this long-term carcinogenicity study, the purpose is to observe test animals for a major portion of their life span for the development of neoplastic lesions during or after exposure to various doses of a test substance by an appropriate route of administration.

This study may be combined with the chronic toxicity study, if appropriate.

7.2. Test animals

Equal numbers of both sexes of two mammalian species, preferably rat and mouse, should be used. Strains that have sufficient historical background data on spontaneous tumours are preferred. For rodents, at spontaneous tumours are preferred. For rodents, at least 50 males and 50 females should be used at each dose level including control. If interim sacrifice is planned, the number should increase appropriately.

7.3. Administration of dose

In principle, the animals should be given the test substance in their diet.

7.4. Dose levels and selection

At least three dose levels should be used with a control. This can be either an untreated group or a vehicle control group. If the toxic properties of the vehicle is not known, then both untreated and vehicle control group are required.

7.5. Exposure period

This should comprise the majority of the normal life span of the strain of animals to be used, e.g. not less than 24 months for rats and 18 months for mice.

7.6. Observation of animals

Careful clinical observation should be made at least twice each week, where special attention should be paid to tumour development. Other observations include body weight and food consumption.

7.7. Haematology determinations

Blood smears and differential blood count should be obtained from all surviving animals of the high dose and control groups at 12 and 18 months and also terminal sacrifice. If relevant, the same samples should be taken from the other groups.

7.8. Pathology

Gross necropsy and tissue preservation (as per chronic toxicity study) should be carried out. In addition, visible tumours and lesions suspected to be tumorous should be preserved. This also holds for histopathology, where the examinations to be done are also the same as for the chronic toxicity study.

8. REPRODUCTION STUDY

8.1. Purpose

This study is meant to provide general information concerning the effects of the test substance on reproductive function including estrus cycles, mating behaviour, conception, parturition, lactation, weaning and growth and development of offspring. It may also provide information about the effects of the test substance on neonatal morbidity, mortality and may generate preliminary data on teratogenesis.

Appendix 2 (page 9)

8.2. Number of generations

Generally, two generations should be treated and observed. For F₁, and F₂ generations, testing should be performed in the first offspring (first litter) and the second offspring only where necessary.

8.3. Test animals

The rat or mouse are the preferred species.

Administration of test substance to parental (P) animals should begin immediately after weaning. Each test and the control group should contain at least 20 males and sufficient number of non-pregnant females to yield at least 20 pregnant females at parturition.

8.4. Dose levels and dose selection

At least three dose levels and a control is required where the highest dose level should induce toxicity but not mortality in dams. If a vehicle is used in administering the test substance, the control group should receive the vehicle in the highest volume used.

8.5. Experimental schedule

The diagram presented below indicates the experimental schedule for dosing, mating, parturition and sacrifice.

**Experimental Schedule for Test Substance Administration and
Animal Reproduction**

Weeks on Study	P	F₁	F₂
0	Dosing begins		
8-14 11-17	F ₁ mating period	F ₁ born and litter size adjusted to 8 pups each.	
14-20	Dosing of P animals ends. P animals are sacrificed.	F ₁ weaned; dosing of F ₁ males and females for mating begins.	
22-34		F ₁ Offspring not selected for mating are sacrificed.	
25-37		F ₁ mating.	F ₂ born and litter size adjusted to 8 pups each F ₂ weaned and F ₁ (selected for mating) are sacrificed.

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- (1) *For P generation the males and females should be dosed immediately after weaning and acclimatizing for at least 1 week. The dosing should be continued for at least 8 weeks prior to the mating period.*
 - (2) *Dosing of the P generation should continue through the mating period, pregnancy, lactation and weaning of F₁ offspring. P animals should be sacrificed after weaning of the F₂ offspring.*
 - (3) *Dosing of F₁ animals saved for mating should begin at the time they are weaned and continue to the weaning of the F₂ offspring. F₁ and F₂ animals should be sacrificed after F₂ offspring are weaned. (Dosing of F₂ animals may be extended if necessary)*

8.6. Administration of dose

In principle, the test substance should be administered in the diet. During pregnancy the dosage may be based on the body weight at Day 0 and 6 of pregnancy.

8.7. Mating procedure

For mating the F₁ offspring, 1-2 males and 1-2 females are randomly selected from as many litters as possible to produce the F₂ generation. For cross mating of the F₁ offspring, males and females from the same dose group should be mated avoiding mating of siblings. F₁ offspring not selected for mating should be sacrificed upon weaning.

Each female should be placed with a single male from the same dose group until mating is confirmed or 3 weeks have passed. Each morning the female should be examined for vaginal plug, and Day 0 of pregnancy is defined as the day vaginal plugs or sperm are found. Pairs which fail to mate should be evaluated to determine the cause of the apparent infertility.

Near parturition, pregnant females should be caged separately in delivery or maternity cages and provided with nesting materials.

8.8. Standardization of litter sizes

On Day 4 after birth, the size of each litter should be adjusted by eliminating extra pups by random selection to yield 4 males and 4 females per litter. If this is not possible, partial adjustment to 8 animals in total is permitted. However, adjustments are not appropriate for litters of less than 8 pups.

8.9. Observation of animals

Each animal should be observed at least once daily and pertinent behavioural changes or signs of toxicity recorded. The duration of gestation should be calculated from day 0 of pregnancy. Each litter should be examined as soon as possible after delivery for the number of pups, stillbirth, live births and presence of gross anomalies. From the results of all observations, mating indices, parturition indices, number of males, impregnated females and viability indices of weanlings should be calculated.

The definitions of these parameters are:

$$\text{Mating index} = \frac{\text{No. of animals mated} \times 100}{\text{No. of animals used for mating}}$$

$$\text{Pregnancy index} = \frac{\text{No. of pregnant females} \times 100}{\text{No. of males mated}}$$

$$\text{Parturition index} = \frac{\text{No. of females delivering live pups} \times 100}{\text{No. of pregnant females}}$$

$$\text{Viability index at weaning} = \frac{\text{No. of viable pups at weaning} \times 100}{\text{Adjusted no. of pups at day 4 of birth}}$$

8.10. Gross necropsy

When sacrificed, each animal should be examined macroscopically with special attention to the organs of reproduction, and these organs should be preserved for histopathological examination.

8.11. Histopathology

Histopathology of the following organs and tissues of all highest dose and control P and F₁ animals selected for mating should be performed : vagina, uterus, ovaries, testes, epididymus, seminal vesicles, prostate and pituitary.

Organs demonstrating toxicologically significant abnormalities should then be examined histopathologically in animals from the other dose groups.

9. TERATOGENICITY STUDY

9.1. Purpose

The study is aimed at obtaining information on whether the test substance has the potential to induce permanent structural or functional abnormalities during the period of embryonic development.

9.2. Test animals

At least twenty pregnant animals of a suitable species (rat, mice or hamster) or twelve rabbits should be used at each dose level and control group. The strain used should be characterized for its response to teratogens.

9.3. Dose levels and selection

At least three dose levels with a control should be used. In the case of substance of low toxicity, if a dose level of at least 1000 mg/kg produces no evidence of embryotoxicity or teratogenicity, studies at other dose levels are not necessary.

If a vehicle is used, it should not be teratogenic nor have effects on reproduction. There should then be a vehicle control group.

9.4. Exposure period

Day 0 of pregnancy is the day on which vaginal plug and/ or sperm is observed. The dose period should cover the period of major organogenesis, and may be extended to approximately 1 day before the expected delivery date.

9.5. Administration

The test substance should be administered orally by gavage at approximately the same time each day. Allowance must be made for the rapid weight gain which takes place during pregnancy when deciding the amount to be administered.

9.6. Observation of animals

The animals should be observed at least once each day and records made of all observations including signs of toxicity, time of onset, degree, duration; also food consumption and body weight. Females showing signs of abortion or premature delivery should be sacrificed and subjected to thorough macroscopic examination.

9.7. Teratological examination

At the time of sacrifice or death during the study, the dam should be examined macroscopically for any structural abnormalities or pathological changes which may have influenced the pregnancy. Immediately after sacrifice or death, the uterus should be removed and the contents examined for embryonic or fetal death in utero where this has occurred. The number of corpora lutea should be determined, the sex of the fetuses also determined and each litter should be weighed. The mean fetal weight should be derived.

Following removal each fetus 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. For rabbits, each fetus should be examined by careful dissection for visceral anomalies and then examined for skeletal anomalies.

10 **MUTAGENICITY STUDY**

10.1. **Purpose**

The study is used to determine the ability of the test substance to affect the integrity of the mammalian cell's genetic components.

10.2. **Basic approach**

The battery of studies including the following 3 categories are required:

10.2.1. Studies to detect gene mutation:

Bacterial Reverse Mutation Assay

Principle of the method: Bacteria are exposed to the test substance both in the presence and absence of metabolic activation system and plated on minimal agar medium. After a suitable period of incubation, revertant colonies are counted and compared to the number of spontaneous revertants in a solvent control culture. The metabolic activation system stated here is the method using the mixture (S-9 MIX) of supernatant fraction of the livers of the animals pre-treated with the agent to induce microsome metabolic enzyme activity.

Tester strains : *Salmonella typhimurium* TA100, TA198, TA1535, TA1537 and other strains, and *Escherichia coli* WP2 uvr A.

Dose levels : At least 5 dose levels should be used, with the highest level producing cytotoxic effects. A solvent control, positive control which require S-9 and positive controls which do not require S-9 mix should be used for each unit of assay.

Number of plates : At least two plates for each dose and control. All plates should be incubated at 37°C for 48-75 hours. At the end of the incubation period, the number of revertant colonies per plate should be counted.

10.2.2. Studies to detect chromosomal aberration:

Chromosomal aberration – in vitro Mammalian Cytogenetics Test

Principle of the method : Following exposure to the test substance at various intervals of the cell cycle, cell cultures of established cell lines or primary cell cultures are treated with colchicines or colcemide and analysed for chromosomal abnormalities in metaphase cells.

Main requirements : Cell lines which can be used include human cells, Chinese hamster cells and human lymphocytes. At least 3 dose levels should be employed, the highest level producing 50% inhibition of growth. A compound known to produce chromosomal aberration in vitro should be used as the positive control. A solvent and untreated control should also be included. A test with metabolic activation system should also be conducted where a compound known to require activation should be used as the positive control in this case.

Number of cultures : At least 2 cultures should be used for each experimental point. For established cell lines, cultures should be treated with the test substance when they are in the exponential stage of growth. Cell cultures are treated with colchicine prior to harvesting. Each culture is harvested and processed separately for the preparation of chromosomes. At least 100 well-spread metaphase cells per culture should be analysed for chromosomal abnormalities.

10.2.3. Studies to detect genotoxic effects.

Genotoxic effect – Bacterial Repair Test

Principle of the test method : A paper disc containing the test substance is placed on an agar plate in which bacterial spores are inoculated. The diameter of inhibition zone is determined 24 hours after treatment. Tests with metabolic activation are also recommended.

Tester strains : Bacillus subtilis M45 and H 17 can be used.

Other basic requirements : At least 5 dose levels should be tested, the highest level producing cytotoxic effects. Negative controls such as kanamycin, streptomycin etc. and positive controls such as AF-2, 2-aminoanthracene etc. should be employed for each unit of assay. At least one plate should be used for each dose and control level.

11. METABOLIC STUDY

11.1. Purpose

The study is aimed at characterizing the behaviour of the test substance in the animal body such that this can be used to evaluate test results from other toxicology studies and also to extrapolate data of test done on animals to man.

11.2. Test substance

Radiochemically pure grade of the active ingredient in labelled form should be used.

11.3. Test animals

At least 1 species among rat, dog etc. should be used i.e. young adults. It is preferable to use the same animal species and strain as those being used for other toxicological studies.

11.4. Dose level and selection

At least 2 dose levels should be used, the upper dose producing toxic or pharmacologic signs and the low dose corresponding to a no-effect level. Where feasible, an additional level approximate to the potential dietary exposure should also be included.

11.5. Administration

The test substance should be administered in the diet but intravenous means can also be employed if necessary. Animals should receive a single dose but if the substance is expected to accumulate in the animal, continuous dosing should be done.

11.6. Procedures

The absorption rate and the rate, ratio and route of elimination should be determined. Periodical measurement of the test substance concentration in blood, plasma or serum is required for this. Samples of expired air, urine and feces should be collected separately from each individual animal in order to determine the excretion. These measurements should be done several times and continued until approximately 90% of the administered dose is eliminated, or for 7 consecutive days.

The distribution of the test substance and other related compounds in major organs should be determined at different time points. The major metabolites should be identified, to clarify the major metabolic pathways. The recovery of the dose in the excreted parent substance and its major metabolites should be determined at a certain period of time after administration.

12. METABOLISM IN PLANTS

The purpose of this study is to characterize the absorption / translocation of the test substance via root and foliage system of the plant and the major metabolic pathways including photochemical reactions of the test substance in the plant body. Comparison of the plant metabolites with the animal metabolites can be made combined with the findings from the animal study.

Test plants should preferably be of the crops to be treated with the chemical or those which are close species to the crops.

Test plants should be treated by a method similar to that of the test chemical. There are two preferred method-direct application and methods which allow the plant to absorb the test substance via the root. Absorption, distribution (to edible part) and metabolism should be determined. Recovery of parent substance and metabolites in treated and untreated parts should be periodically determined.

Additional studies include metabolism in the soil and degradation in natural water, especially if the test substance demonstrates long-term residues in the soil.

13. ACUTE FISH TOXICITY STUDY

13.1. Test fish

One or more species may be used, preferably those which are readily available throughout the year, easily maintained and whose relevant economic, biological and ecological characteristics are known. They should be acclimatized at least 12 days before the test at temperatures appropriate to the species. Feeding must be stopped 24 hours before test. All fish must be exposed to water of the quality to be used in the test for at least 7 days before they are used.

13.2. Preparation of test substance

Stock solutions of the required strength are prepared by dissolving the appropriate amount of the test substance in the required volume of dilution water. The chosen test concentrations are prepared by dilution of the stock solution. The test should be carried out without adjustment of pH. Generally, no reference substances are required.

13.3. Test procedure

The usual procedure is that of the static test where there is no flow of test solution occurring – solutions may remain unchanged throughout the duration of the test. Measurements of pH, dissolved oxygen and temperature must be carried out at least daily.

The fish are exposed to the test substance at a range of concentrations, preferably for a period of 96 hours. Mortalities are recorded at 24, 48, 72, and 96 hours and the concentrations which kill 50% of the fish (LC_{50}) are determined where possible. Visible abnormalities e.g. loss of equilibrium, swimming behaviour etc. should be recorded. The maximum concentration tested producing no mortality and the minimum concentration tested producing total mortality should be recorded.

Mortality in the controls should not exceed 10% at the end of the test.

Median lethal concentrations can be calculated using internationally-accepted standard procedures.

TABLE C: PROTOCOL FOR ACUTE ORAL TOXICITY STUDIES

TEST CONDITIONS	ACUTE ORAL
1. Test substance.....	i) If the study using the formulation as the test substance involves any difficulty, technical material may be used in lieu of the formulation. ii) If technical grade of active ingredient is used as the test substance, it should be of the same composition as that used for manufacturing the formulation.
2. Test animals..... 2.1. Species..... 2.2. Age..... 2.3. Sex..... 2.4. Number..... 2.5. Animal status	At least one species of rat Young adult animals should be used Both sexes and the female should be non-pregnant For rodents, 10 animals (5 females + 5 males) for each dose level Animals should be fasted prior to the substance administration: Rat – overnight; other animals with higher metabolic rates a shorter period fasting is sufficient
3. Dose levels.....	i) At least 3 dose levels spaced appropriately to produce in test group a range of toxic effects and mortality rates. The data should be sufficient to produce a dose response curve and where possible permit an acceptable determination of LD50. ii) If a test at one dose level of at least 5000mg/kg body weight on not less than 10 rats of equal numbers of sexes using the procedure described for the study, produces no compound related mortality, then the full study using the minimum three dose levels might not be necessary.
4. Preparation of test substance.....	When necessary the test substance should be dissolved or suspended in water or a suitable vehicle. The toxic characteristics of the vehicle should be known.

TEST CONDITIONS	ACUTE ORAL
5. Administration of test substance.....	<p>i) The test substance should be administered in a single dose by gavage using a tube or suitable intubation cannula. If a single dose is not possible, the dose may be given in smaller fractions over a period not exceeding 24 hours.</p> <p>ii) The maximum volume of liquid that can be administered at one time depends on the size of the test animal. In rodents, the volume must not exceed 1 ml/100 g body weight.</p>
6. Frequency of observation.....	<p>Observations should be made frequently on the first day of the administration and at least once each subsequent day.</p>
7. Observation period...	<p>Should be at least 14 days</p>
8. Observation of the animals.....	<p>i) Cageside observations should include, but not limited to changes in:</p> <ul style="list-style-type: none"> a. The skin & fur b. Eyes & mucous membranes c. Respiratory system d. Circulatory system e. Autonomic and central nervous system f. Behavioral pattern g. Somatomotor activity h. Particular attention should be directed to observation of tremors, convulsions salivation, diarrhea, lethargy, sleep and coma. <p>ii) Individual weights of animals should be recorded shortly before the test substance is administered, weekly thereafter and at death.</p> <p>iii) The time of death should be recorded as precisely as possible.</p> <p>iv) At the end of the test surviving animals should be weighed and sacrificed.</p>

TEST CONDITIONS	ACUTE ORAL
9. Gross pathological	Necropsy of animals should be carried out and all gross pathological changes should be recorded.
10. Data and reporting 10.1. Treatment of results.....	<p>Data shall be summarised in tabular form, showing for each test group:</p> <ul style="list-style-type: none"> i) The number of animals and their body weights at the start of the test, ii) Time of death of animals a different dose levels, iii) Number of animals displaying other signs of toxicity, iv) Description of toxic effects, and v) Necropsy findings
10.2. Evaluation of results.....	<p>An evaluation of results should include the relationship, if any, between the dose of the test substance and the incidence, severity and reversibility of all abnormalities, including behavioral and clinical effects, gross lesions, body weight changes, effects on mortality, other toxicological effects.</p>
10.3. Test report...	<p>The test report should include the following information:</p> <ul style="list-style-type: none"> i) Species/strain/source used; diet, environmental conditions, ii) Tabulation of response data by sex and dose level (i.e. number of animals exposed, number of animals showing signs of toxicity; number of animals which died or were killed during the test), iii) Description of toxic effects iv) Dose response curves, for mortality and other toxic effects (when permitted by the method determination), v) LD50 values for each sex, determined at 14 days (with method of determination specified) vi) 95 percent confidence interval for the LD50 vii) Time of death after dosing viii) Body weight data ix) Gross pathological findings.

TEST CONDITIONS	ACUTE ORAL
10.4. Statement of compliance..	The report should also include statement of compliance signed by the study director that the test had been carried out in compliance with Good Laboratory Practice standards.

TABLE D: PROTOCOL FOR ACUTE DERMAL TOXICITY STUDIES

TEST CONDITIONS	ACUTE ORAL
1. Test substance.....	i) If the study using the formulation as the test substance involves any difficulty, technical material may be used in lieu of the formulation ii) If technical grade of active ingredient is used as the test substance, it should be of the same composition as that used for manufacturing the formulation.
2. Test animals..... 2.1. Species..... 2.2. Age..... 2.3. Sex..... 2.4. Number..... 2.5. Animals status...	At least one species of rat Young adult animals should be used Both sexes and the female should be non-pregnant For rodents, 10 animals (5 females + 5 males) for each dose level. Fasting not required
3. Dose levels.....	i) At least 3 dose levels spaced appropriately to produce test group with a range of toxic effects and mortality rates. The data should be sufficient to produce a dose response curve and where possible permit an acceptable determination of LD50. ii) If a test at one dose level of at least 2000mg/kg body weight on not less than 10 rats of equal numbers of sexes using the procedures described for the study. Produces no compound related mortality, then the full study using the minimum three dose levels might not be necessary.
4. Preparation of test substance.....	When necessary the test substance should be dissolved or suspended in water or a suitable vehicle. The toxic characteristics of the vehicle should be known.

TEST CONDITIONS	ACUTE ORAL
5. Administration of test substance.....	<ul style="list-style-type: none"> i) Approximately 24 hours before the test, fur should be removed from the dorsal area of the trunk of the test animals by clipping or shaving. Care should be taken to avoid abrading the skin which could alter its permeability. ii) Not less than 10 percent of the body surface are should be clear for the application of test substance. iii) When testing solids, which may be pulverised if appropriate, the test substance should be moistened with water or where necessary, a suitable vehicle to ensure good contact with the skin. When vehicle is used, the influence of the vehicle on penetration of the skin by the test substance should be taken into account. iv) The test substance should be applied uniformly over an area approximately 10 percent of the total body surface. v) The test substance should be held in contact with the skin with a porous gauze dressing and non-irritating tape for 24 hours. The test site should be further covered in a suitable manner to retain the gauze dressing and the test substance and to ensure that the animals cannot ingest the test substance. vi) At the end of the exposure period, residual test substance should be removed, where practicable using water or an appropriate solvent.
6. Frequency of observation.....	Observations should be made frequently on the first day of the administration and at least once each subsequent day.
7. Observation period...	Should be at least 14 days
8. Observation the animals.....	<ul style="list-style-type: none"> i) Cageside observations should include, but not limited to changes in: <ul style="list-style-type: none"> a. The skin & fur b. Eyes & mucous membranes c. Respiratory system e. Circulatory system

TEST CONDITIONS	ACUTE ORAL
<p>9. Gross pathological.</p> <p>10. Data and reporting.</p> <p>10.1. Treatment of results.....</p> <p>10.2. Evaluation of results.....</p> <p>10.3. Test report.....</p>	<p>e. Autonomic and central nervous system</p> <p>f. Behavioral pattern</p> <p>g. Somatomotor activity</p> <p>h. Particular attention should be directed to observation of tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma.</p> <p>ii) Individual weights of animals should be recorded shortly before the test substance is administered, weekly there after and at death.</p> <p>iii) The time of death should be recorded as precisely as possible.</p> <p>iv) At the end of the test surviving animals should be weighed and sacrificed.</p> <p>Necropsy of animals should be carried out and all gross pathological changes should be recorded.</p> <p>Data shall be summarised in tabular form, showing for each test group:</p> <p>i) The number of animals and their body weights at the start of the test,</p> <p>ii) Time of death of animals at different dose levels,</p> <p>iii) Number of animals displaying other signs of toxicity,</p> <p>iv) Description of toxic effects, and</p> <p>v) Necropsy findings.</p> <p>An evaluation of results should include the relationship, if any, between the dose of the test substance and the incidence, severity and reversibility of all abnormalities, including behavioral and clinical effects, gross lesions, body weight changes, effects on mortality, other toxicological effects.</p> <p>The test report should include the following information:</p> <p>i) Species/strain/source used; diet, environmental conditions,</p>

TEST CONDITIONS	ACUTE ORAL
<p>10.4. Statement of compliance...</p>	<ul style="list-style-type: none"> ii) Tabulation of response data by sex and dose level (i.e. number of animals exposed, number of animals showing signs of toxicity; number of animals which died or were killed during the test), iii) Description of toxic effects iv) Dose response curves, for mortality and other toxic effects (when permitted by the method of determination) v) LD50 values for each sex, determined at 14 days (with method of determination specified) vi) 95 percent confidence interval for the LD50 vii) Time of death after dosing viii) Body weight data ix) Gross pathological findings. <p>The report should also include statement of compliance signed by the study director that the test had been carried out in compliance with Good Laboratory Practice standards.</p>