

**GUIDANCE DOCUMENT ON TOXICOLOGY
FOR
REGISTRATION OF
CHEMICAL PESTICIDES
IN
INDIA**



Ministry of Agriculture & Farmers Welfare
Department of Agriculture, Co-Operation & Farmers Welfare
Central Insecticides Board & Registration Committee
Directorate of Plant Protection Quarantine & Storage
NH-IV, Faridabad – 121001 (Haryana) India

September 2017

PREFACE

Pesticides are inherently toxic in nature, and are regulated in India by a Legislation called Insecticides Act, 1968, “An Act to regulate the import, manufacture, sale, transport, distribution and use of insecticides with a view to prevent risk to human beings or animals, and for matters connected therewith and rules framed thereunder. The registration is granted by registration committee only after verifying the safety and efficacy of pesticides. To ensure the safety of pesticides, the manufacturers generate required data on toxicity as per the guidelines available on the website of CIB&RC.

“Gaintonde Committee report” was the guidance document since 1970 till March 2011, India became signatory to Mutual Acceptance of Data (MAD) with Organization of Economic Cooperation & Development (OECD) and hence, need was felt to revise the document along with guidelines & protocols. Therefore, keeping in view the requirement and to be at par globally, a guidance document along with data requirement was prepared by toxicologists of CIB&RC namely Dr. Sushil K. Khurana, Dr. Sandhya Kulshreshtha & Dr. Sarita Bhalla under the able guidance of Dr. D Kanungo in 2014. According to MAD agreement, the document was prepared with the aim to avoid repetition of toxicity studies thereby reducing the use of large number of animals. This would enable to bridge the gap and remove the potential trade barrier between India & other OECD countries. The document entitled “Guidance Document of Toxicology for the Registration of Pesticides in India” was appreciated in Registration Committee (RC) in its 351st meeting & Foreword was written by the then Chairman Dr. Gurbachan Singh and was made available on the website of CIB&RC for its implementation w.e.f. 01/01/2015.

To address the concerns raised by stakeholders including Industry, Pesticides Association & Animal Welfare Organizations during a workshop conducted in July 2015, the 360th RC held on Dec. 11, 2015 decided to form a new committee as per Agenda item No. 11.6 which reads as:

“After finalization of above guidance document several suggestions/comments are being received from stakeholders, PETA etc. in the Sectt. of CIB & RC. To address these issues a meeting may be convened under the Chairmanship of Dr. S.K. Khurana involving toxicity experts in the Sectt. of CIB & RC, Pesticide Associations Member (one member each from CLI, CCFI & PMFAI), one member from PETA & One member from Humane International Animal Welfare Society. Dr. Sarita Bhalla, Spl. Gr.-I will coordinate this work and if any fruitful recommendations are emerged, those will put up to next meeting of RC”.

The document has been revised & amended accordingly. The aim was to revise the document within the ambit of The Insecticides Act, 1968 also taking care of human safety, and animal welfare concerns and impact on the health of human beings & animals. The Foreword is written by earlier Chairman of the Registration Committee Dr. J. S. Sandhu, Deputy Director General (CS), ICAR.

The new edition is revised & updated document has been approved in the 376th RC for uploading on the website of the CIB&RC by Chairman Dr. S.K. Malhotra who has also written a Foreword.

भारत सरकार
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कृषि, सहकारिता एवं किसान कल्याण विभाग
कृषि भवन, नई दिल्ली-110001



Government of India
Ministry of Agriculture and Farmers Welfare
Department of Agriculture, Cooperation
and Farmers Welfare
Krishi Bhawan, New Delhi-110001

Dr. S.K. Malhotra
Agriculture Commissioner

Tel : 011-2338 3549
011-2338 1012
Email: ag.comm@nic.in

Dated: 30th October, 2017

FOREWORD

To be at par globally, the Guidance Document on Toxicology for Registration of Chemical Pesticides in India was prepared to harmonize the toxicological protocols in accordance to OECD, keeping the provisions of the Insecticides Act., 1968 and Insecticides Rules 1971 in view. The first issue was released in 2014, however, this document has been revised and amended in 2017.

Toxicology is a field where safety to human beings and animals arising from the use of pesticide is a major concern. The protocols and the guidance document is prepared with safety as its major goal for generation of toxicity data under Good Laboratory Practice (GLP) norms. This new set of Guidance document is scientifically justified and not only covers the detailed protocols but also provides Data Requirements for pesticides to be introduced and registered for use in the country.

The efforts of the toxicologists of CIB&RC viz. Dr. Sushil K. Khurana, Dr. Sandhya Kulshrestha and Dr. Sarita Bhalia is highly appreciated in bringing out such a voluminous document.

I convey my best wishes to all the stakeholders who would be benefitted by using this revised Guidance Document that would further enable them to keep abreast with latest scientific developments.

(S. K. Malhotra)

डॉ. जीत सिंह सन्धू
उपमहानिदेशक (फसल विज्ञान)

Dr. Jeet Singh Sandhu
Deputy Director General
(Crop Science)



भारतीय कृषि अनुसंधान परिषद्
कृषि भवन, डा. राजेन्द्र प्रसाद मार्ग, नई दिल्ली - 110001

INDIAN COUNCIL OF AGRICULTURAL RESEARCH
KRISHI BHAWAN, DR. RAJENDRA PRASAD ROAD, NEW DELHI-110001

FOREWORD

The Guidance Document on Toxicology for Registration of Chemical Pesticides in India was released in 2014 w.e.f. Jan 1, 2015. This document replaced the Gaitonde Committee Report (GCR) on Protocols for Toxicology and Data Requirements.

India had formulated the new set of Guidance document to harmonize toxicological protocols in accordance to OECD, being a signatory to 'Mutual Acceptance of Data' (MAD). This document apart from protocols, also provides Data Requirements for pesticides to be introduced & registered for use in the country for the first time. Further, making it mandatory for all toxicological data to be generated under Good Laboratory Practice (GLP) norms.

The document had received applaud from all stakeholders concerned for the attempt of the Indian Regulators to amend the GCR guidance document. However, there were some concerns on the same. To address the concerns of all stakeholders this document has been revised and amended in accordance to the provisions of the Insecticides Act., 1968 and Insecticides Rules 1971 and the OECD.

Relevant suggestions/inputs from all stakeholders including industry pesticide associations (CCFI, CL India & PMFAI) and International Animal Welfare Organizations i.e. PETA & Humane Society International have been taken into consideration. We also acknowledge the inputs received from international toxicologists and technical/ scientific contribution from Vipin Saini.

The efforts of the sub-committee under the able guidance of Dr. S.K. Khurana and Dr. Sandhya Kulshrestha and Dr. Sarita Bhalla is acknowledged & appreciated.

Support of the Secretariat of the Toxicology Division is also appreciated.

I believe that revision of this Guidance Document would be useful to all stakeholders and enable us to keep abreast with today's scientific world. Toxicology being a major concern during use of pesticides, this document would be a good guide for complying towards the safety & the environmental impact to human beings & animals. Harmonization of Indian guidelines with OECD principles also would lead to reduction in the use of animals by avoiding repetition of toxicological studies.



(JS Sandhu)

July 28, 2017
New Delhi



डा गुरबचन सिंह

अध्यक्ष, कृ.वै.च.मं. एवं रजिस्ट्रेशन कमेटी

Dr. GURBACHAN SINGH

CHAIRMAN, ASRB & Registration Committee

कृषि वैज्ञानिक चयन मंडल

(भारतीय कृषि अनुसंधान परिषद)

कृषि अनुसंधान भवन-1, पूसा, नई दिल्ली-110012



AGRICULTURAL SCIENTISTS RECRUITMENT BOARD

(INDIAN COUNCIL OF AGRICULTURAL RESEARCH)

KRISHI ANUSANDHAN BHAVAN – I, PUSA, NEW DELHI – 110012

Tel: (O) 25843295, 25841272 Fax: 91 –11–25846540

E– mail: gurbachansingh@icar.org.in

FOREWORD

Food, nutrition and environmental security for the nation is a major concern for the Policy makers in general and Ministry of Agriculture in particular. Agro chemicals including pesticides are likely to play a significant role in enhancing productivity per unit area. However, due to inherent toxic properties of pesticides, their manufacture, import and use are regulated globally under respective national regulations, and India is no exception. The Registration Committee, constituted under the comprehensive regulation in India, called the Insecticides Act, 1968, registers these pesticides only after satisfying itself regarding the efficacy and its safety to human beings, animals and environment. To ensure the safety of pesticides, the manufacturers generate required data on toxicity as per the guidelines prescribed under “Gaitonde Committee Report” which was floated in late seventies. As Chairman of the Registration Committee, I got convinced through various representations, discussions and analysis that the protocols/guidelines being currently used need updating. Further, developed countries are marching ahead towards globally harmonized protocols based on novel scientific technology and conception. This exercise would also facilitate the Mutual Acceptance of Data (MAD) by concerned regulators of different countries. Moreover, India also became a signatory of Mutual Acceptance of Data with Organisation for Economic Cooperation and Development (OECD). These changing scenarios made Registration Committee to think on similar lines for reviewing its own guidelines and come up with a set of updated, science based robust guidelines for harmonization so that these could facilitate the concept of Mutual Acceptance of Data.

For this purpose, a sub-committee was constituted under Chairmanship of Dr. D. Kanungo, the then Additional Director General, Ministry of Health and Family Welfare, Govt. of India and a renowned regulatory toxicologist. He was duly supported by toxicology experts Dr Sushil K. Khurana, Dr S. Kulshrestha, Dr Sarita Bhalla, Dr. Balakrishnanmurthy, Dr Shalini Chawla; and representatives of Industry Associations: Dr Mithyantha, Dr J. C. Majumdar and a bunch of young experts from the Industry ably led by Mr Rajesh Dhawan.

The committee evolved a set of recommendations to be named as "Kanungo Committee Report". The Registration Committee accepted the report in principle. However, to suggest study-wise protocols in detail, a group was assigned the job of finalising the toxicological guidelines in the line of OECD for the approval of Registration Committee. The group comprising of Dr. Sushil K. Khurana, Consultant (Pathology), Dr. (Mrs.) Sandhya Kulshrestha, Consultant (Pharmacology) and Dr. (Mrs.) Sarita Bhalla, Specialist Grade-I with constant guidance of Dr. D. Kanungo came out with a set of guidelines. I am told that while accomplishing this enormous task, this group has taken into cognizance the OECD guidelines, EPA guidelines, Australian guidelines, Japanese guidelines and also EU guidelines. I am also informed that these guidelines were got peer reviewed by one of the UK Regulatory Toxicologist, Dr Ian Dewhurst. While congratulating these experts who have accomplished this onerous task to facilitate pesticide regulatory authorities to compete with their counterparts else where globally, my special thanks goes to Dr. Ian Dewhurst. Secretarial and Technical Assistance by Ms Ekta Gupta, APPO (Toxicology) is also acknowledged and appreciated.

This booklet contains chapters on "Protocols on Toxicological Studies" and "Data Requirement" for new chemical molecules for submission to regulatory body under the Insecticides Act, 1968 and rules framed thereunder.

I am confident that this compilation will be helpful for all stakeholders including the pesticides manufacturers and shall bridge the gap for which there was longstanding demand. Full adherence to these guidelines shall imply that our test facilities today produce safety data of comparable rigor and quality as the test facilities throughout OECD. OECD countries will now be able to accept our data for registration of pesticides. Equally, non-members will also be able to accept Data from OECD as per the conditions for 'Mutual Acceptance of Data'. This will help removing a potential trade barrier between India and OECD countries for marketing chemicals abroad.


25/9/2014
(Gurbachan Singh)

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ACRONYMS

- μCi : Microcurie ($1\mu\text{Ci} = 37,000 \text{ Bq}$)
- 2-AAF : 2-acetylaminofluorene
- 4-NQO : 4-nitroquinoline-N-oxide
- 7, 12-DMBA : 7, 12- dimethylbenzanthracene
- AChE : Acetylcholinesterase
- ADME : Absorption, Distribution, Metabolism & Excretion
- AGD : Ano-Genital Distance
- ALP : Alkaline Phosphatase
- APTT : Activated Partial Thromboplastin Time
- AUC : Area Under Curve
- BALT : Bronchus Associated Lymphoid Tissue
- BMD : Benchmark Dose
- Bq : Becquerel
- BrdU : Bromodeoxyuridine
- CaCO_3 : Calcium Carbonate
- CAS No : CAS Registry Numbers
- CHE : Cholinestrase
- CHO : Chinese Hamster Ovary Cell Line
- CI : Confidence Interval
- C_{max} : Maximum Concentration
- COHb : Carboxyhemoglobin
- CTL : Cytotoxic T-lymphocyte
- DL : Dominant lethal
- DNA : Deoxyribonucleic acid
- DPM : Disintegrations Per Minute
- DRFS : Dose Range Finding Study
- DTH : Delayed-type hypersensitivity
- ELISA : Enzyme-Linked Immunosorbent Assay
- ET : Exposure Time
- EU : European Union
- F1 : First Generation
- F2 : Second Generation

- FCA : Freund's Complete Adjuvant
- GALT : Gut Associated Lymphoid Tissue
- GC : Guanine cytosine
- GGT : Gamma Glutamate Transferase
- GI : Gastro-Intestinal
- GLP : Good Laboratory Practice
- HPLC : High Performance Layer Chromatography
- IC : Inhibitory Concentration
- IV : Intravenous
- LC50 : Lethal Concentration 50
- LD50 : Lethal Dose 50
- LFT : Liver Function Test
- LLNA : Local Lymph Node Assay
- LNC : Lymph Node Cells
- LOAEL : Lowest Observable Adverse Effect Level
- LOEC : Lowest Observed Effect Concentration
- LOEL : Lowest Observable Effect Level
- LSC : Laser Scanning Cytometer
- MCH : Mean Corpuscular Hemoglobin
- MCHC : Mean Corpuscular Hemoglobin Concentration
- MCV : Mean Corpuscular Volume
- MetHb : Methemoglobin
- MLC : Mixed Lymphocyte Culture
- MMAD : Mass Median Aerodynamic Diameter
- MN : Micronucleus
- MTD : Maximum Tolerated Dose
- MTT : 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide
- NC : Negative Control
- NMR : Nuclear Magnetic Resonance
- NNG : Net Nuclear Grain
- NOAEL : No Observed Adverse Effect Level
- NOEC : Lowest Observed Effect Concentration
- NOEL : No Observed Effect Level
- NTE : Neuropathy Target Esterase

- OD : Optical Density
- OECD : Organization for Economic Co-operation and Development
- OP : Organophosphates
- OPPTS : Office of Prevention, Pesticides and Toxic Substances
- P : Parental
- PBS : Phosphate-Buffered Saline
- PC : Positive Control
- PFC : Plaque-Forming Cell
- PHA : Phytohaemagglutinin
- PND : Post Natal Day
- ppm : Parts Per Million
- PS : Performance Standards
- PT : Prothrombin Time
- QC : Quality control
- RFT : Renal Function Test
- RH : Relative Humidity
- RhE : Reconstructed Human Epidermis
- RICC : Relative Increase in Cell Count
- ROC : Residue of Concern
- RPD : Relative Population Doubling
- SDS : Sodium Dodecyl Sulphate
- SGOT : Serum Glutamate Oxaloacetate Transferase
- SGPT : Serum Glutamate Pyruvate Transferase
- SI : Stimulation Index
- SRBC : Sheep Red Blood Cell
- T_{1/2} : Half- life
- TCA : Trichloroacetic Acid
- TK : Toxicokinetic
- TLC : Thin Layer Chromatography
- T_{max} : Maximum Time
- TRR : Total Radioactive Residue
- UDS : Unscheduled DNA Synthesis
- US EPA : United States Environmental Protection Agency

General Guidance For Toxicity Testing

1. All toxicity studies must be conducted in accordance with the Guidelines for the Testing of Pesticides prescribed in this report.
2. The studies must be conducted in Good Laboratory Practice (GLP) certified laboratory. The study reports should be accompanied with the GLP certification from the Study Director and Quality Assurance (QA) certificate from QA unit of the laboratory. Non-GLP or non- Guideline-compliant studies will be considered on their scientific merit as additional information/study.
3. Studies should use testing regimes which cover the most likely routes/modes of human exposure.
4. Studies must be conducted using active constituents or formulations containing an impurity level comparable to that present in the commercial product. All toxicity studies must include details on the composition of the material used in the study, including solvents and vehicles. For chiral compounds the enantiomer ratio used in all toxicity studies must be clearly indicated.
5. Each study must clearly identify the name and address of the laboratory which performed the study, the names of the responsible scientists, the report number, the dates when the study was performed and the report was written.
6. Studies should be designed to assist in the establishment of No-Observed-Adverse-Effect Levels (NOAELs) in repeated exposure studies and to provide evidence of potential short- and long-term hazards, from which an estimate of the pesticide's relative safety can be deduced.
7. Details of studies must include:
 - route of administration
 - dose levels
 - the number of animals per dose level, their origin, sex, weight and maturity, all parameters studied
 - the frequency at which observations were made
 - the duration of each study
 - time of administration in relation to the observations and effects observed
 - the rationale for dose selection.
 - dose selection
8. Each report must accompany:
 - Quality Assurance Statement.
 - Statement of compliance of study as per GLP Principles/Standard.
 - Test Substance characterization: its identification, CAS Number. if available, Physico-chemical properties (color, physical state), Analytical Test Report / Certificate of Analysis.

9. The observation period, observational methods utilized and description of pre-mortem and non-lethal effects, their appearance, time course and reversibility.
10. The number of animals died or killed for humane reasons, time of death and reasons for humane killing.
11. A detailed methodology/procedure and individual animal data should be given.
12. When indicated, as in delayed deaths, the inclusion of gross pathology and possibly histopathology.
13. The LD50/ LD50 Cut Off Value/Category as per the Insecticides Rules, 1971 should be given in consonance with the methodology adopted.
14. Statistical methodology (Name with reference), if adopted, also need to be given.
15. Statement regarding the time period of retention of raw data/test article/specimen to be given.

Reports must include detailed results for the individual animals in the studies, together with statistical analyses of results. Summary tables or diagrams should be included where these will assist in reviewing data (e. g. bodyweight, haematology) or where they will permit sets of data to be compared on the same page (e.g those for control and treated animals). Studies also should include summarized reports of histopathological examinations, in tabular form, so that the incidence of observations can be studied in relation to dosage, sex and duration of treatment.

Additional toxicological studies on individual metabolites will be necessary in situations where the metabolites formed through plant metabolism or photodegradation differ from those identified in mammalian metabolism studies. These studies are required so that a judgement can be made regarding which compounds should be included in the residue definition for risk assessment purposes.

PROTOCOLS
ON
TOXICOLOGICAL STUDIES

ACUTE TOXICITY STUDIES

Acute Oral

Acute Dermal

Acute Inhalation

Primary Skin Irritation

In Vitro Skin Irritation: Reconstructed Human Epidermis Test Method

Acute Eye Irritation

Skin Sensitization

Acute toxicity studies examine the adverse effects arising from administration of a single dose of a substance or multiple doses given within 24 hours. The substance is usually administered by the oral, dermal and inhalation routes.

The degree of hazard presented by pesticides depends on many complex factors. Although no single factor is completely reliable, the acute toxicity of a chemical probably gives the most useful immediate indication of the potential acute hazards to human users and bystanders and probably also to domestic animals.

Classification of pesticides under the Indian National Regulation is primarily based on acute toxic and irritancy effects and therefore demands, amongst other things, data on acute oral, dermal and inhalation toxicities. Additional specialised studies are used to assess the skin and eye irritancy and skin sensitisation potential of the substance.

To permit assessment of the acute toxicology of a pesticide to exposed humans, studies in animals should examine the most likely routes and forms of exposure. They should be performed with both the active constituent and the products to be marketed..

Acute oral/dermal/inhalation toxicity studies should be performed in at least one mammalian species. The rat is the preferred rodent species for oral studies. LD₅₀ and LC₅₀ values are normally required for hazard classification purposes. Reports should include details of the observed toxic signs, reasons for death and other data which will enable assessment of acute toxic potential.

For skin and eye irritation studies, the rabbit is an acceptable species but properly validated alternatives to the usual *in vivo* test would be suitable. Eye irritation tests may be unnecessary in the case of substances or formulations where chemical or physical properties suggest this form of toxicity is likely, e.g pH above 11.5 or below 2.

A skin sensitization study is also required to test for possible hypersensitivity reactions to the chemical. Guinea pigs are normally used for sensitization studies. Internationally validated *in-vitro* alternative methods, such as the murine local lymph node assay (LLNA *in vivo*), are also acceptable.

ACUTE ORAL TOXICITY STUDY

I. Acute Toxic Class Method

Principle of the test

A stepwise procedure is adopted with the use of 3 animals of a single sex per step. Absence or presence of compound-related mortality of the animals dosed at one step will determine the next step, i.e.;

- no further testing is needed,
- dosing of three additional animals, with the same dose,
- dosing of three additional animals at the next higher or the next lower dose level.

Test animals: Rat is the preferred rodent species, as a regulatory requirement. Normally females (nulliparous and non-pregnant) generally being slightly more sensitive than males, are used. However, if males have been shown to be more sensitive with structurally related substances then this sex should be used and adequate justification is to be provided.

Age: Healthy young adult animals should be of age between 8 and 12 weeks at the time of dosing.

Weight: The weight should fall in an interval within ± 20 % of the mean weight of any previously dosed animals.

Housing and Feeding Conditions

Temperature - $22 \pm 3^\circ\text{C}$

Relative Humidity – 50-60% (However, humidity should not be below 30% or exceed 70% at any given point of time).

Lighting – 12 hours light and dark cycle

Diet and water – Standard laboratory diet specific to the species and filtered water, free from contamination.

Acclimatization: The animals should be acclimatized to the laboratory conditions for at least 5 days by keeping in individual cages prior to dosing.

Administration method

Administer the test substance in a single dose by gavage. However, if it is not possible to administer the compound in a single dose, it may be given in smaller fractions over a period not exceeding 24 hours and justification for the deviation should be given.

When necessary during dose preparation, the use of an aqueous solution (water based) is recommended, followed in order of preference by use of a solution/suspension/emulsion in

oil (like corn oil) and then possibly solution with other vehicles. The toxicological characteristics of the vehicle should be known if other than water is used.

Animals should be fasted prior to dosing. The feed should be withheld for overnight. However, water may be provided *ad libitum*. Following the period of fasting, the animals should be weighed and the test substance administered. The fasted body weight of each animal is determined and the dose is calculated according to the body weight. After the substance has been administered, food may be withheld for further 3–4 hours.

Where a dose is administered in fractions over a period of time, the animals may be provided with food and water depending on the length of the period.

Number of animals

Three animals are used for each step.

Dose levels and Administration of Doses

Select the starting dose from one of four fixed levels, 5, 50, 300 and 2000 mg/kg body weight and conduct tests in accordance with **Annex 1**.

The initial dose level selected should be the one which is most likely to produce mortality in some of the dosed animals. However, in case no information is available on acute toxicity of the test substance then it is recommended to start with a dose of 300 mg/kg body weight.

Determine the interval between dosing at each step according to the duration and severity of toxic symptoms. One should not proceed to the next dosing until survival or death is confirmed for the previously dosed animals.

Limit Test

If the available information indicates that the mortality is not likely at the highest dose level (2000 mg/kg body weight), then a limit test should be conducted.

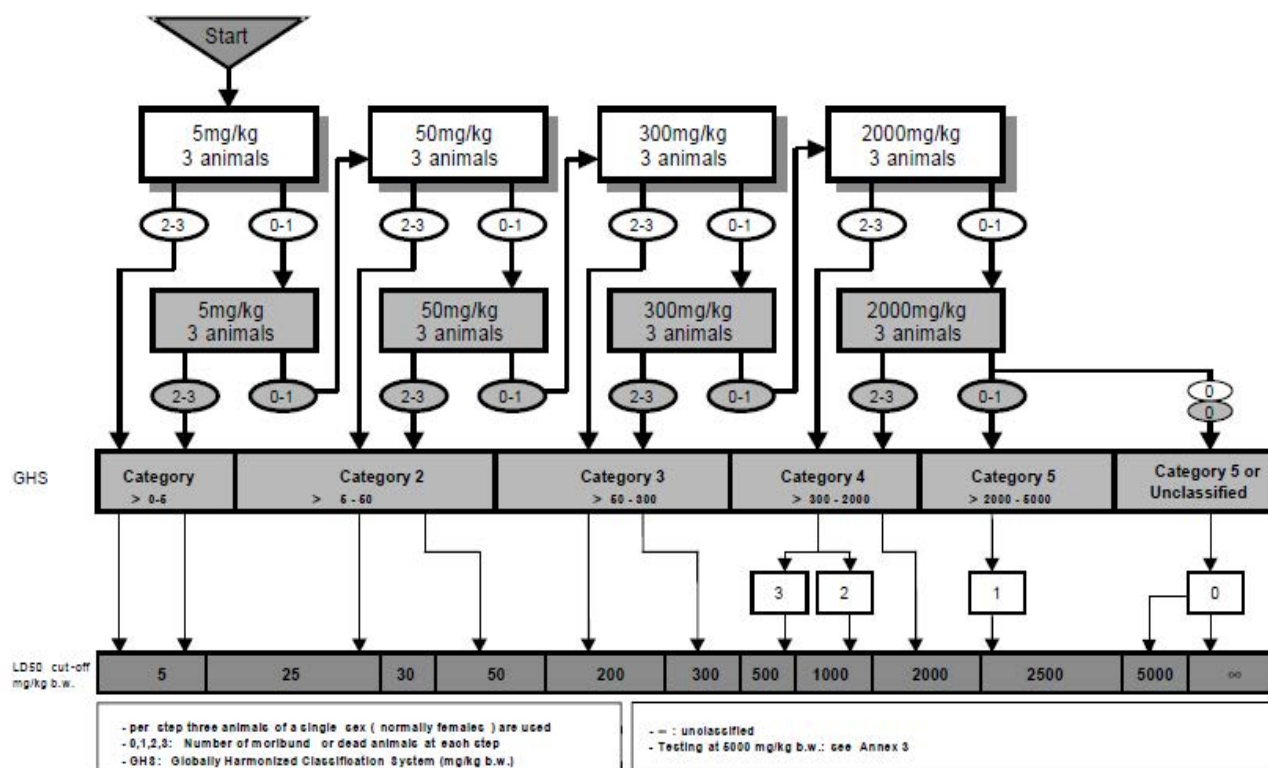
A limit test at one dose level of 2000 mg/kg body weight may be carried out with six animals (three animals per step). If there is 0-1 death at a dose level of 2000 mg/kg administered to 3 animals during first step, 2000 mg/kg should be administered to an additional 3 animals. If the test substance causes again 0-1 death after the second administration too, there is no need to administer doses exceeding 2000 mg/kg.

Testing with dose level of 5000 mg/kg body weight may be considered only if results of such tests have direct relevance in relation to protection of animal or human health or environment.

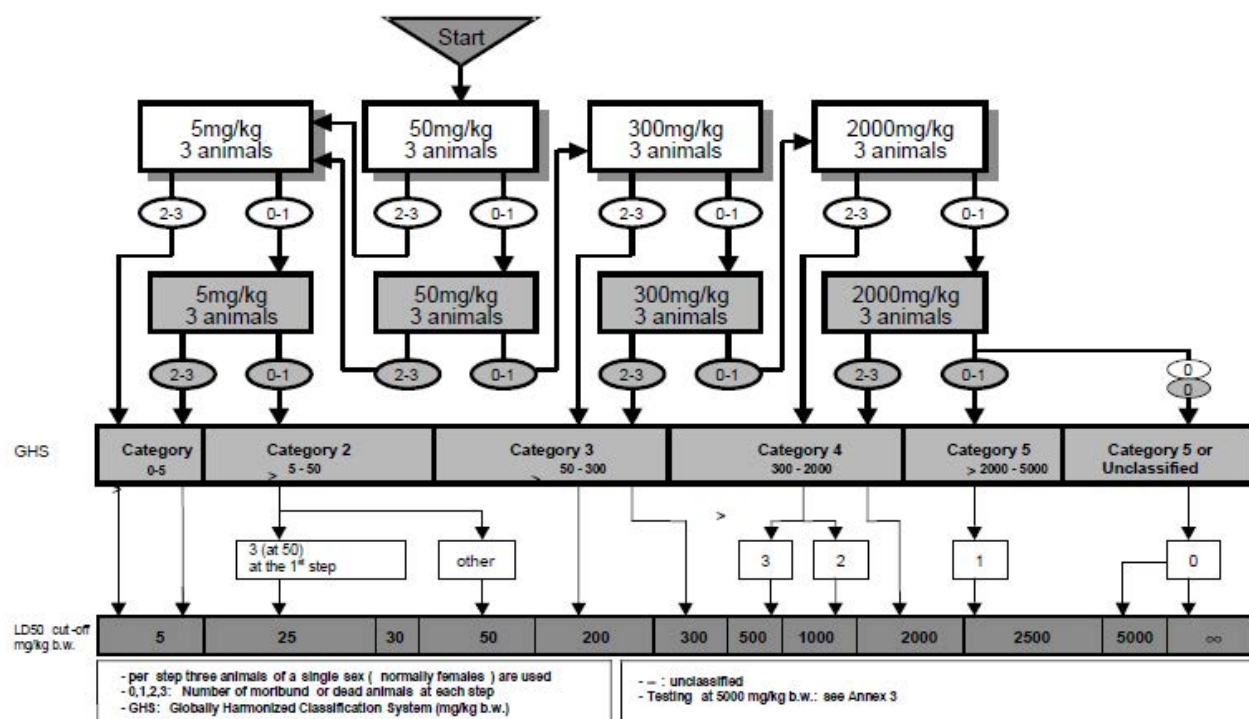
Observation period

Animals should be observed for at least 14 days. However, depending on toxic reactions and time of onset and length of recovery period, the period may be extended when considered necessary.

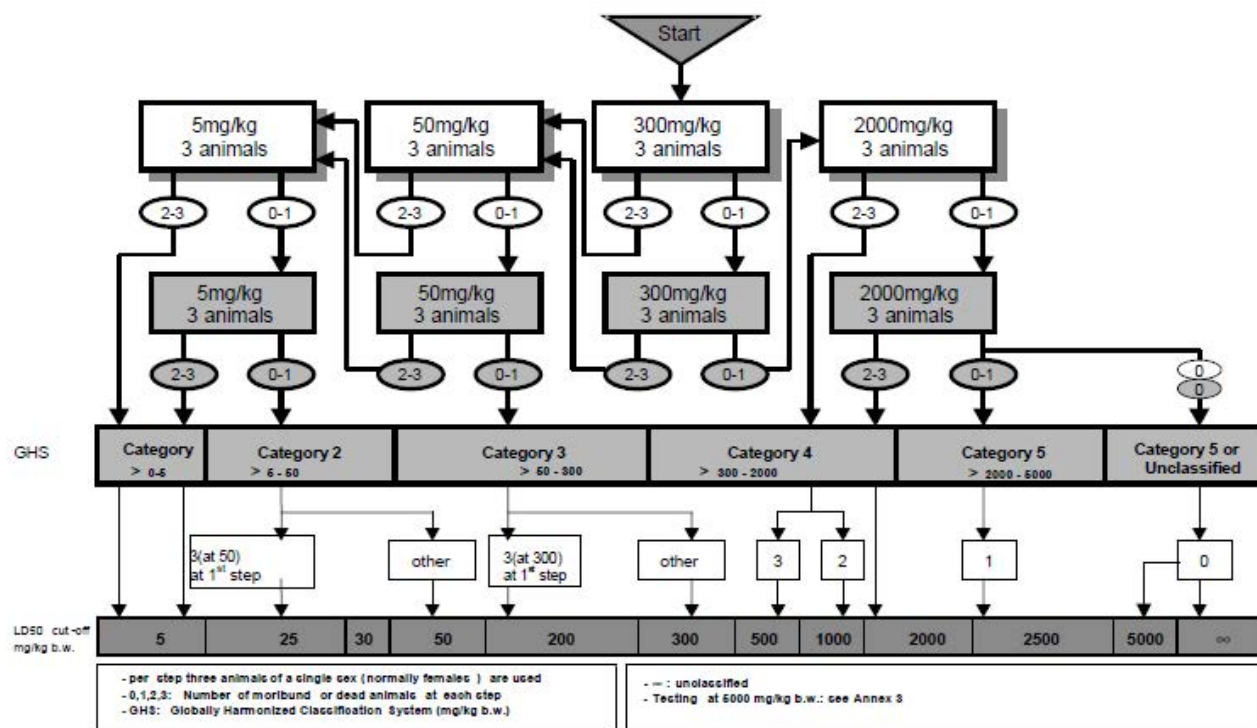
ANNEX 1a: TEST PROCEDURE WITH A STARTING DOSE OF 5 MG/KG BODY WEIGHT



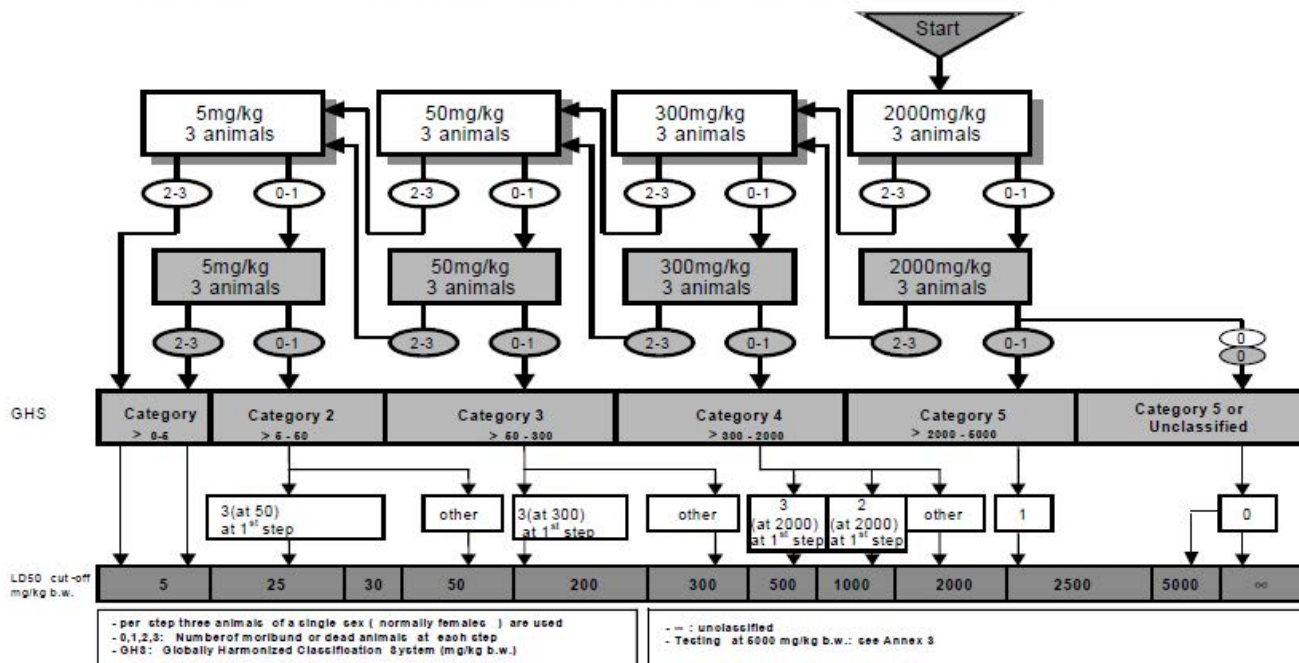
ANNEX 1b: TEST PROCEDURE WITH A STARTING DOSE OF 50 MG/KG BODY WEIGHT



ANNEX 1c: TEST PROCEDURE WITH A STARTING DOSE OF 300 MG/KG BODY WEIGHT



ANNEX 1d: TEST PROCEDURE WITH A STARTING DOSE OF 2000 MG/KG BODY WEIGHT



Observation and examination

- i) Observations should be made on individual animals at least once during the first 30 minutes after dosing, periodically during the first 24 hours (with special attention given during the first 4 hours), thereafter at least once daily during the observation period.
- ii) Observations should include condition of skin and fur, eyes and mucus membrane, respiratory, circulatory, autonomic and central nervous system, somato-motor activity and behavioral pattern. Specific observations should be made for tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma.
- iii) Test animals should be weighed individually, shortly prior to the administration of test substance and at least weekly thereafter. If a test animal dies, weigh it at the time of death. All surviving animals are to be weighed before they are humanely killed.
- iv) All test animals should be subjected to gross necropsy to record gross pathological findings. Histopathology may be considered in the organs showing significant adverse effects.

Result assessment

The method enables a judgment with respect to classifying the test substance to one of a series of toxicity classes defined by fixed LD50 cut-off values. .

Toxic Endpoint

It is basically LD50, however, the method is not with intention to calculate a precise LD50, but allow for the determination of defined exposure ranges where lethality is expected since death of a proportion of the animals is the major endpoint of this test. Animals humanely killed due to compound related distress and pain should be recorded as compound related deaths. Details of the test procedure are described in **Annex 1**.

(Reference: OECD Test Guideline 423, 2001)

Note (TESTING AT DOSES ABOVE 2000 mg/kg):-

When testing is required at the dose of 5000mg/kg, only one step (i.e. three animals) is required. If the first animal dosed dies, then dosing precedes at 2000mg/kg in accordance with the flow chart given above. If the first animal survives, two further animals are dosed. If only one of the three animals dies, the LD50 value is expected to exceed 5000mg/kg. If both animals die, then dosing proceeds at 2000mg/kg.

II. Up-and-Down Procedure

Principle of the test

Estimation of LD50 with a confidence interval (CI).

Stepwise approach is adopted with one animal per step. If the first dosed animal survives, the second animal receives a higher dose. If the first animal dies or appears moribund, the second animal receives a lower dose. Same dosing pattern follows for each subsequent animal. The LD50 and Confidence Intervals are calculated by employing appropriate statistical methodology (BMDP, 1990).

Test animals: The preferred rodent species is the rat. Normally females (nulliparous and non-pregnant) are used. However, if males have been shown to be more sensitive with structurally related substances then this sex should be used and adequate justification should to be provided.

Age: Healthy young adult animals should be of age between 8 and 12 weeks at the time of dosing.

Weight: Body weights of the animals to fall in an interval within $\pm 20\%$ of the mean weight of any previously dosed animals in the study one animal per step

Housing and Feeding Conditions

Temperature - $22 \pm 3^\circ\text{C}$

Relative Humidity – 50-60% (However, humidity should not be below 30% or exceed 70% at any given point of time).

Lighting – 12 hours light and dark cycle

Diet and water – Standard laboratory diet specific to the species and filtered water free from contamination.

Acclimatization: The animals should be acclimatized to the laboratory conditions for at least 5 days by keeping in individual cages prior to dosing.

Number of animals: One animal per step is used.

Dose Levels and Administration of Doses

The test substance is administered in a single dose by gavage. However, if it is not possible to administer the compound in a single dose, it may be given in smaller fractions over a period not exceeding 24 hours and justification for the deviation should be given.

When necessary during dose preparation, the use of an aqueous solution (water based) is recommended, followed in order of preference by use of oil (like corn oil) and then possibly solution with other vehicles. The toxicological characteristics of the vehicle should be known if other than water is used.

Animals should be fasted prior to dosing. The feed should be withheld for overnight in case of rats. However, water may be provided ad-libitum. Following the period of fasting, the animals should be weighed and the test substance administered. The fasted body weight of each animal is determined and the dose is calculated according to the body weight. After the substance has been administered, food may be withheld for further 3–4 hours in rats

Where a dose is administered in fractions over a period of time, the animals may be provided with food and water depending on the length of the period.

Limit Test

The limit test at 2000 mg/kg is primarily conducted in situations where sufficient information is available indicating that the test material is likely to be nontoxic. Information about the toxicity of the test material can be gained from knowledge about similar tested compounds or similar tested mixtures or products. In case of little or no information about its toxicity, or in which the test material is expected to be toxic, the main test should be performed.

For limit test, dose one animal at 2000mg/kg dose. If animal dies, then main test is to be conducted. If animal survives, then dose additional four animals sequentially. However, if three animals die, the limit test is terminated and main test is performed. If minimum three animals are survived then LD50 is > 2000 mg/kg.

Main Test

For the main test, single animals are dosed in sequence at minimum 48 h intervals. However, the time intervals between dosing is determined by the onset, duration, and severity of toxic signs. Treatment of an animal at the next dose should be delayed until one is confident of survival of the previously dosed animal. For selecting the starting dose, all available information, including information on structurally related substances and results of any other toxicity tests on the test material, should be used to approximate the LD50 as well as the slope of the dose-response curve.

The first animal is dosed a step below the best preliminary estimate of the LD50. If the animal survives, the second animal receives a higher dose. If the first animal dies or appears moribund, the second animal receives a lower dose. The dose progression factor should be chosen to be the antilog of $1/(\text{the estimated slope of the dose-response curve})$ and should remain constant throughout testing (a progression of 3.2 corresponds to a slope of 2). When there is no information on the slope of the substance to be tested, a dose progression factor of 3.2 is used. Using the default progression factor, doses would be selected from the sequence 1.75, 5.5, 17.5, 55, 175, 550, 2000 (or 1.75, 5.5, 17.5, 55, 175, 550, 1750, 5000 for specific cases).

If no estimate of the substance's lethality is available, dosing should be initiated at 175 mg/kg. If animal tolerance to the chemical is expected to be highly variable (i.e., slopes are expected to be less than 2.0), consideration should be given to increasing the dose progression factor beyond the default on a log dose scale (i.e., 3.2 progression factor) prior to starting the test. Similarly, for test substances known to have very steep slopes, dose progression factors smaller than the default

should be chosen (**Annex 2** includes a table of dose progressions for whole number multiples of slope ranging from 1 to 8 with starting dose 175 mg/kg).

Observation period

Animals should be observed for at least 14 days. However, depending on toxic reactions and time of onset and length of recovery period, the period may be extended when considered necessary.

Observation and examination

- i) Observations should be made on individual animals at least once during the first 30 minutes after dosing, periodically during the first 24 hours (with special attention given during the first 4 hours), thereafter at least once daily during the observation period.
- ii) Observations should include condition of skin and fur, eyes and mucus membrane, respiratory, circulatory, autonomic and central nervous system, somato-motor activity and behavioural pattern. Specific observations should be made for tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma.
- iii) Test animals should be weighed individually, shortly prior to the administration of test substance and at least weekly thereafter. If a test animal dies, weigh it at the time of death. All surviving animals are to be weighed before they are humanely killed.
- iv) All test animals should be subjected to gross necropsy to record gross pathological findings. Histopathology may be considered in the organs showing significant adverse effects.

Result assessment

The test procedure, in addition to estimation of LD50 and Confidence Intervals allows the observation of signs of toxicity.

Toxic Endpoint

The testing is ended when one of the following is observed:

- A) 3 consecutive animals survive at the upper bound.
- B) 5 reversals occur in any 6 consecutive animals tested.
- C) At least 4 animals have followed the first reversal and the specified likelihood-ratio exceed the critical value.

The LD50 and Confidence Intervals are calculated by employing appropriate statistical methodology (BMDP, 1990).

(Reference: OECD Test Guideline 425, 2008).

Annexure 2 Table on Dose Progressions

Choose a Slope and Read Down the Column All doses in mg/kg bw

Slope =	1	2	3	4	5	6	7	8
	0.175*	0.175*	0.175*	0.175*	0.175*	0.175*	0.175*	0.175*
							0.24	0.23
					0.275	0.26		
				0.31			0.34	0.31
			0.375			0.375		
								0.41
					0.44		0.47	
		0.55		0.55		0.55		0.55
					0.69		0.65	
								0.73
			0.81			0.82		
				0.99			0.91	0.97
					1.09	1.2		
							1.26	1.29
	1.75	1.75	1.75	1.75	1.75	1.75	1.75	1.75
							2.4	2.3
					2.75	2.6		
				3.1			3.4	3.1
			3.75			3.75		
					4.4			4.1
							4.7	
		5.5		5.5		5.5		5.5
					6.9		6.5	
								7.3
			8.1			8.2		
				9.9			9.1	9.7
					10.9	12		
							12.6	12.9
	17.5	17.5	17.5	17.5	17.5	17.5	17.5	17.5
							24	23
					27.5	26		
				31			34	31
			37.5			37.5		
					44			41
							47	
		55		55		55		55

Slope =	1	2	3	4	5	6	7	8
							65	
					69			73
			81			82		
				99			91	97
					109	120		
							126	129
	175	175	175	175	175	175	175	175
							240	230
					275	260		
				310			340	310
			375			375		
					440			410
							470	
		550		550		550		550
							650	
					690			730
			810			820		
				990			910	970
					1090	1200		
							1260	1290
	1750	1750	1750	1750	1750	1750	1750	1750
							2400	2300
					2750	2600		
				3100				3100
						3750	3400	
								4100
	5000	5000	5000	5000	5000	5000	5000	5000

* If lower doses are needed, continue progressions to a lower dose.

III. Fixed Dose Method

Principle of the test

Groups of animals of a single sex are dosed in a stepwise procedure using the fixed doses of 5, 50, 300 and 2000 mg/kg (and 5000 mg/kg in exceptional cases when justified). The initial dose level, as selected on the basis of a sighting study, is the dose expected to produce some signs of toxicity without causing severe toxic effects or mortality. Further groups of animals may be dosed at higher or lower fixed doses, depending on the presence or absence of signs of toxicity or mortality until

the dose causing evident toxicity or no more than one death is identified, or if no effects are seen at the highest dose or when deaths occur at the lowest dose, is determined.

Test animals: Rat is the preferred rodent species,. Normally females (nulliparous and non-pregnant) generally being slightly more sensitive than males, are used. However, if males have been shown to be more sensitive with structurally related substances then this sex should be used and adequate justification should to be provided.

Age: Healthy young adult animals should be of age between 8 and 12 weeks at the time of dosing.

Weight: The weight should fall in an interval within ± 20 % of the mean weight of any previously dosed animals.

Housing and Feeding Conditions

Temperature - $22 \pm 3^\circ\text{C}$

Relative Humidity – 50-60% (However, humidity should not be below 30% or exceed 70% at any given point of time).

Lighting – 12 hours light and dark cycle

Diet and water – Standard laboratory diet specific to the species and filtered water free from contamination.

Acclimatization: The animals should be acclimatized to the laboratory conditions for at least 5 days by keeping in their cages prior to dosing.

Number of animals

(a) Sighting study

Use 1 animal for each dose level.

(b) Main study

Use 5 animals for each dose level. However, for dose levels that have been implemented in the sighting study, 4 animals are added to the 1 animal used in the sighting study.

Administration method

Administer the test substance in a single dose by gavage. However, if it is not possible to administer the compound in a single dose, it may be given in smaller fractions over a period not exceeding 24 hours.

When necessary during dose preparation, the use of an aqueous solution (water based) is recommended, followed in order of preference by use of a solution/suspension/emulsion in oil (like corn oil) and then possibly solution with other vehicles. The toxicological characteristics of the vehicle should be known if other than water is used.

Animals should be fasted prior to dosing. The feed should be withheld for overnight in case of rats. However, water may be provided ad-libitum. Following the period of fasting, the animals should be weighed and the test substance administered. The fasted body weight of each animal is determined and the dose is calculated according to the body weight. After the substance has been administered, food may be withheld for further 3–4 hours in rats.

Where a dose is administered in fractions over a period of time, the animals may be provided with food and water depending on the length of the period.

Dose Levels and Administration of Doses

(a) Sighting study

The sighting study is conducted to determine the appropriate starting dose for the main study. For this, conduct the study in accordance with **Annex “3-a”** using the doses of 5, 50, 300, and 2000 mg/kg of body weight. As the initial dose level, select a dose at which evident toxicity is expected to be manifested (cognizance can be taken from *in vivo* and *in vitro* data from the same chemical and from structurally related chemicals). When no information is available regarding the acute toxicity of the test substance, it is desirable to start with a dose of 300 mg/kg. A period of at least 24 hours will be allowed between the dosing of each animal.

Testing with Dose level of 5000 mg/kg may be considered only if results of such tests have direct relevance in relation to protection of animal or human health or environment.

In cases where an animal tested at the lowest fixed dose level (5 mg/kg) in the sighting study dies, the normal procedure is to terminate the study and regard LD50 as 5 mg/kg of body weight and assign the substance to GHS Category 1. There is no need to conduct the main test. However, if further confirmation of LD50 is needed, an optional additional procedure may be conducted..

(b) Main study

In principle only moderately toxic doses are administered and the doses that are expected to be lethal are avoided.

The starting dose is selected based on the sighting study. Depending on the outcome of the starting dose level, one of three actions will be required:

- i) either stop testing and assign the appropriate hazard classification class;
- ii) test at a higher fixed dose; or
- iii) test at a lower fixed dose. However, to protect animals, a dose level that caused death in the sighting study will not be revisited in the main study (**see Annex 3b**).

A total of 5 animals of one sex will normally be used for each dose level tested. The five animals will be made up of one animal from the sighting study dosed at the selected dose level together with an additional four animals (except, unusually, if a dose level used on the main study was not included in the sighting study).

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

Limit test

The limit test may be conducted if the knowledge from other similar compounds tested indicates that the test substance is unlikely to be toxic.

A sighting study with 1 animal, starting dose of 2000 mg/kg (or exceptionally 5 000 mg/kg) followed by dosing of further four animals at this level in main test serves as a limit test.

Observation period

Animals should be observed for at least 14 days. However, depending on toxic reactions and time of onset and length of recovery period, the period may be extended when considered necessary.

Observation and examination

- i) Observations should be made on individual animals at least once during the first 30 minutes after dosing, periodically during the first 24 hours, (with special attention given during the first 4 hours), thereafter at least once daily during the observation period.
- ii) Observations should include condition of skin and fur, eyes and mucus membrane, respiratory, circulatory, autonomic and central nervous system, somato-motor activity and behavioural pattern. Specific observations should be made for tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma.
- iii) Test animals should be weighed individually, shortly prior to the administration of test substance and at least weekly thereafter. If a test animal dies, weigh it at the time of death. All surviving animals are to be weighed before they are humanely killed.
- iv) All test animals should be subjected to gross necropsy to record gross pathological findings. Histopathology may be considered in the organs showing significant adverse effects.

Result assessment

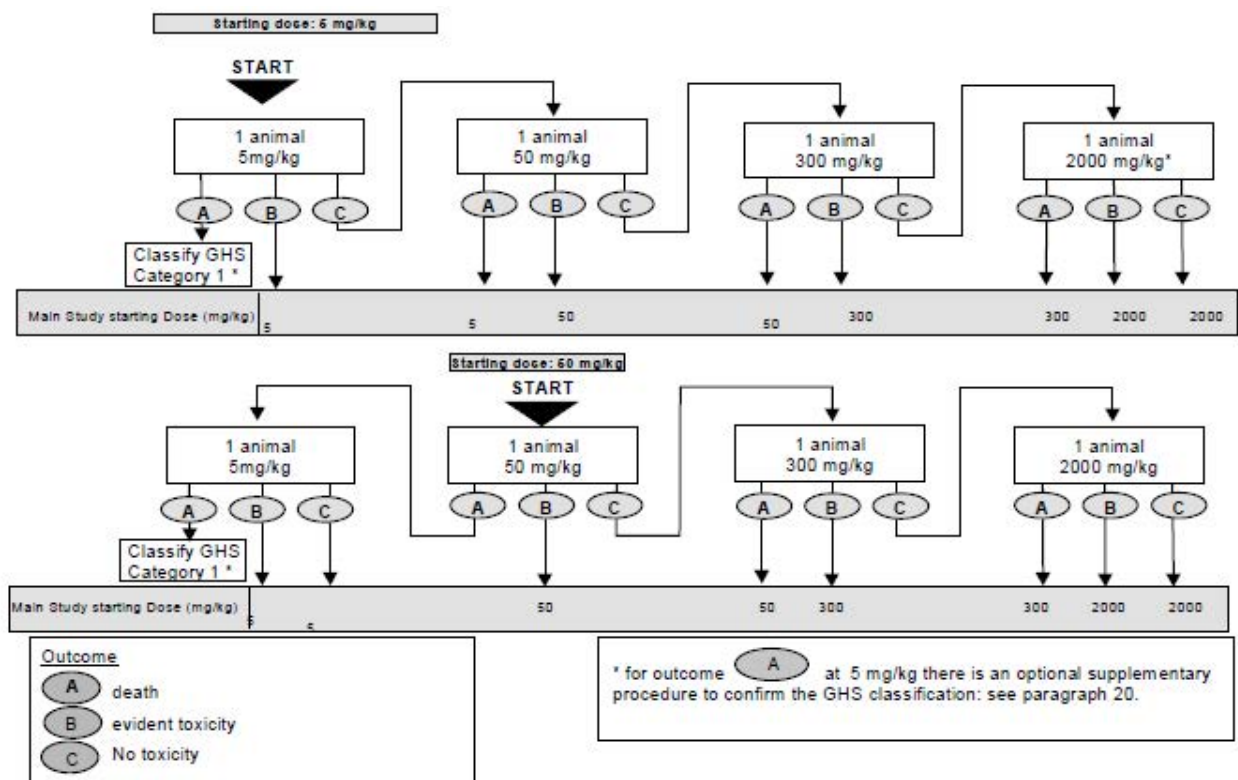
The method enables a judgement with respect to classifying the test substance to one of a series of toxicity classes.

Toxic Endpoint

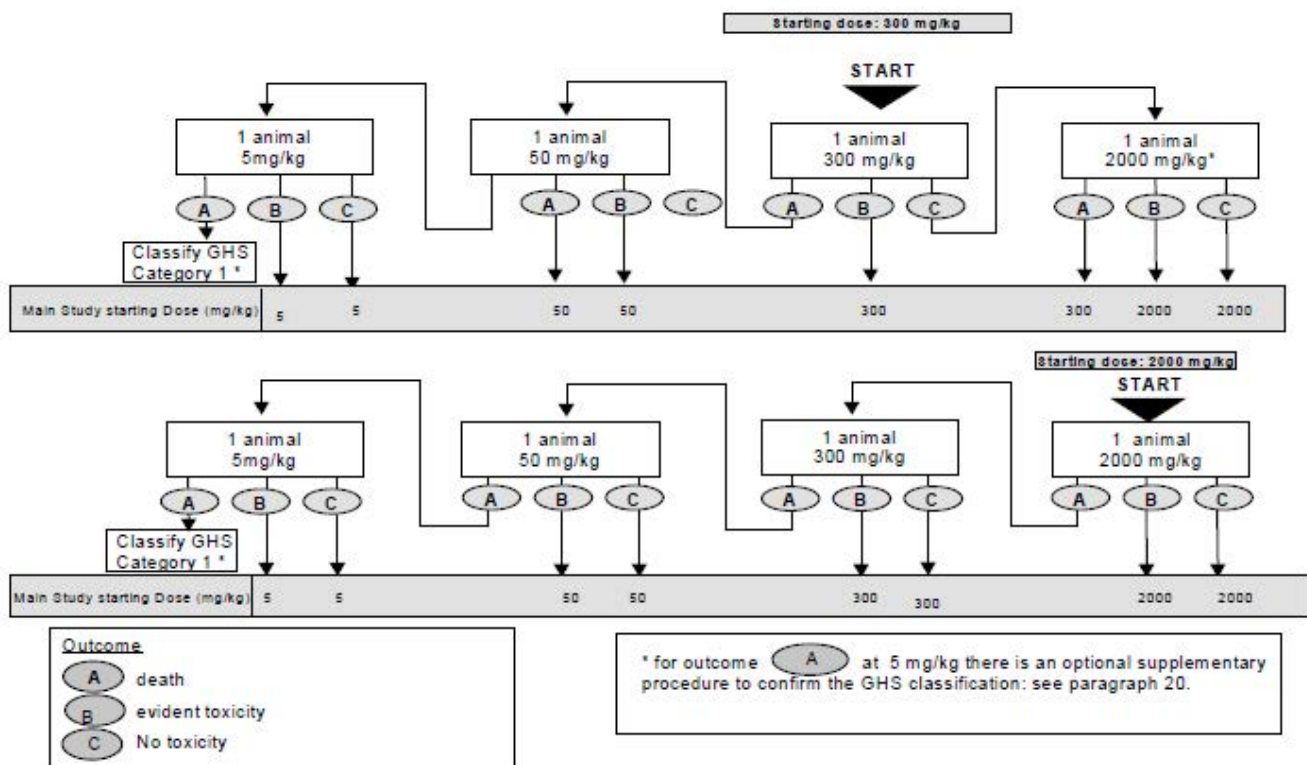
The approach avoids using death of animals as an endpoint and rely instead on the observation of clear signs of toxicity at one of the series of fixed dose levels.

(Reference: OECD Test Guideline 420, 2001).

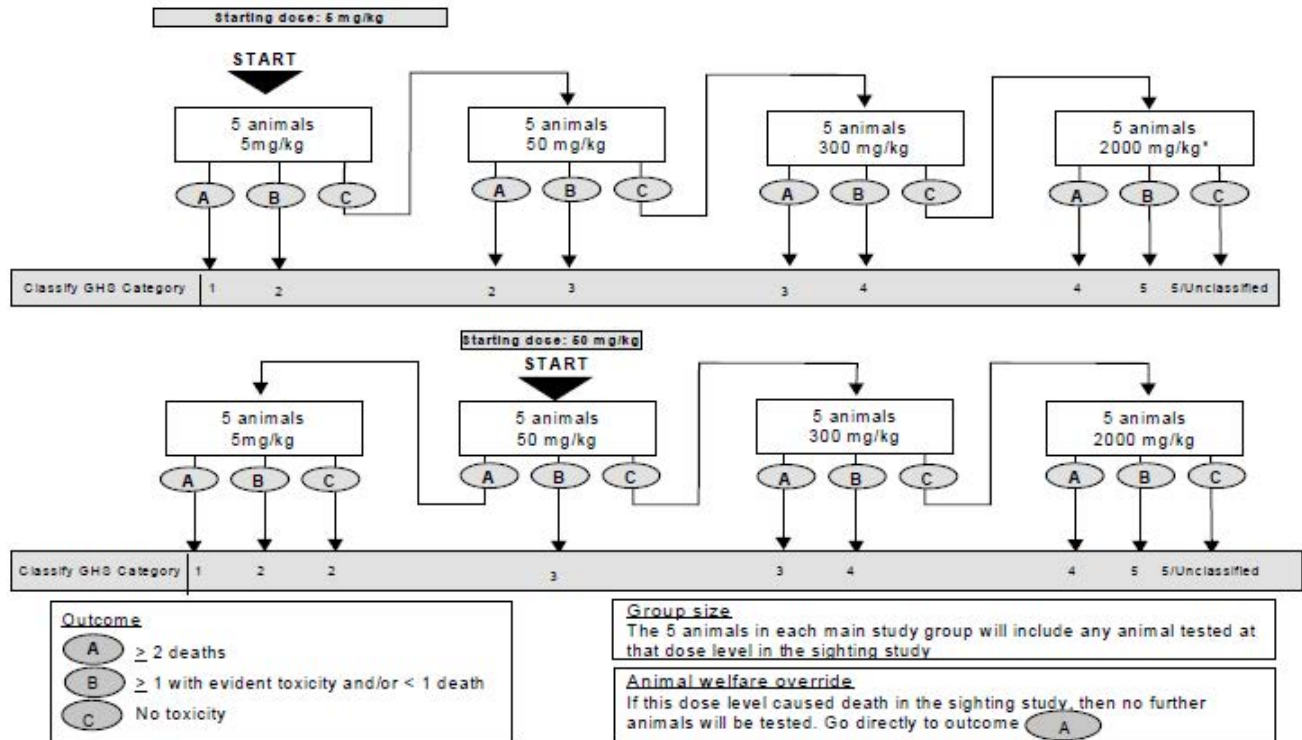
FLOW CHART FOR THE SIGHTING STUDY



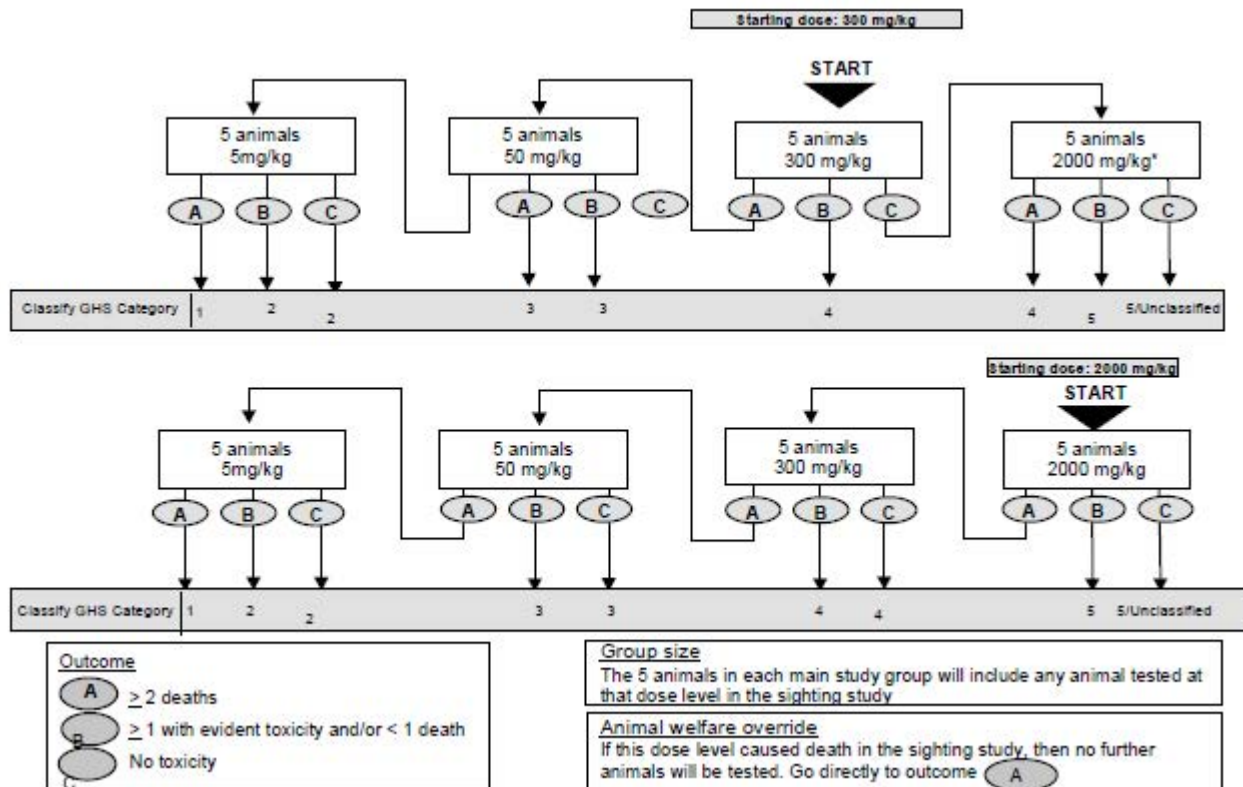
3b: FLOW CHART FOR THE SIGHTING STUDY



3c: FLOW CHART FOR THE MAIN STUDY



3d: FLOW CHART FOR THE MAIN STUDY



ACUTE DERMAL TOXICITY STUDY

Objective

The objective of this study is to obtain scientific information regarding health hazards that may result from a single dermal exposure to the agricultural chemical and calculate LD50 for providing the basis of laying down the measure of safety protection in pesticide production and application.

Principle of the test

The test substance is applied to the skin of the test animals in different doses and the animals are observed for effects and mortality. Animals showing severe signs of toxicity/distress are humanely killed. At the end of the test all surviving animals are to be sacrificed. Necropsy is performed on all dead, killed and sacrificed animals.

Test animals: Rat or Rabbit

Age: Healthy young adult rats should be of age between 6-9 weeks while rabbits not older than 2 years.

Weight: Rats: 200–300 g; **Rabbits:** 2.0–3.0 kg.

Housing and Feeding Conditions

Temperature - $22 \pm 3^\circ\text{C}$ for rodents and $20^\circ (\pm 3^\circ)$ for rabbits

Relative Humidity – 50-60% (However, humidity should not be below 30% or exceed 70% at any given point of time).

Lighting – 12 hours light and dark cycle

Diet and water – Standard laboratory diet specific to the species and filtered water free from contamination.

Acclimatization: The animals should be acclimatized to the laboratory conditions for at least 5 days by keeping in individual cages prior to dosing.

Number of animals

Five animals to be used at each dose level. They should all be of the same sex. If females are used, they should be nulliparous and non-pregnant. After completion of the study as described above, at least one group of five animals of the other sex are dosed (preferably at intermediate / higher dose) to establish that animals of this sex are not markedly more sensitive to the test substance. However, if information already exist that one particular sex is more sensitive than other, animals of only that sex should be tested.

Administration method

Animals are prepared approximately 24 hrs before the test by clipping or shaving the fur on the dorsal area of skin. Care must be taken to avoid abrading the skin, which would alter its permeability. The test substance should be applied uniformly over the shaved or clipped area which is approximately 10 percent of the body surface area. There may be cases in which test substances of high toxicity may be applied to a smaller area, but as much of the area should be covered with as thin and uniform a film as possible.

Liquid test substances are generally used undiluted. 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.

Dose levels and Administration of Doses

At least three dose levels should be taken, spaced appropriately to produce test groups 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 the LD50.

Exposure period of test material

The test substance applied is to be held in contact with skin for a period of 24 hours. For this the application site may be covered with porous gauze, and secure it with non-irritating tape so as to preserve contact with the skin and the test animals are not able to ingest the test substance.

At the end of the exposure period, residual test substance should be removed, where practicable using water or an appropriate solvent.

Limit Test

A limit test at one dose level of at least 2 000 mg/kg bodyweight may be carried out in a group of five male and five female animals. If compound related mortality does not occur in limit test, main study need not be conducted.

Observation period

Animals should be observed for at least 14 days. However, depending on toxic reactions and time of onset and length of recovery period, the period may be extended when considered necessary.

Observations and examination

- i) Carefully observe the general condition of animals, frequently during the day on which the test substance is administered, and thereafter at least once daily.
- ii) Observations should include condition of skin and fur, eyes and mucus membrane, respiratory,

circulatory, autonomic and central nervous system, somato-motor activity and behavioural pattern. Specific observations should be made for tremors, convulsions salivation, diarrhea, lethargy, sleep and coma.

- iii) Test animals should be weighed individually, shortly prior to the administration of test substance and at least weekly thereafter. If a test animal dies, weigh it at the time of death. All surviving animals are to be weighed before they are humanely killed.
- iv) All test animals should be subjected to gross necropsy to record gross pathological findings. Histopathology may be considered in the organs showing significant adverse effects.

Result assessment

LD₅₀ to be calculated by any accepted / recognized method (Litchfield and Wilcoxon, 1949; Bliss, 1938; Finney, 1971 & 1977; Weil, 1952; Thompson, 1947; Miller and Tainter, 1944)

Toxic Endpoint

Death or severe toxicity in animals. Animals humanely killed due to compound related distress and pain should be recorded as compound related deaths.

(Reference: OECD Test Guideline 402, 1987).

ACUTE INHALATION TOXICITY

Objective

To obtain scientific information regarding health hazards that may result from a single exposure to the agricultural chemical via inhalation which possibly gets into body through respiratory tract.

Principle of the Test

To provide a concentration-response relationship ranging from non-lethal to lethal outcomes to derive a median lethal concentration (LC50). Groups of animals are exposed to a limit concentration (limit test) or a series of concentrations in a stepwise procedure for a duration of 4 hrs. The animals are observed for mortality. Moribund animals or animals in distress should be humanely killed and considered in the same way as animals that died due to exposure.

Test animals: Rat is the preferred species and justification should be provided if other species are used. Females should be nulliparous and non-pregnant.

Age: On the exposure day, animals should be healthy young adults 8 to 12 weeks of age.

Weight: On the exposure day, body weights should be within $\pm 20\%$ of the mean weight for each sex of any previously exposed animals of the same age.

Housing and Feeding Conditions

Temperature - $22 \pm 3^\circ\text{C}$

Relative Humidity – 50-60% (However, humidity should not be below 30% or exceed 70% at any given point of time).

Lighting – 12 hours light and dark cycle

Diet and water – Standard laboratory diet specific to the species and filtered water, free from contamination

Acclimatization: The animals should be acclimatized to the laboratory conditions for at least 5 days by keeping in individual cages prior to dosing. Animals should also be acclimatized to the test apparatus for a short period prior to testing, as this will reduce the stress caused by introduction to the new environment.

Exposure method

Expose animals to test substance as a gas, vapour, aerosol or a mixture thereof, for 4 hours using Standard Inhalation Equipment. The preferred mode of exposure is nose- only (also include head only, snout –only). Water may be provided during whole body exposure. Do not provide food.

The flow of air through the chamber should be carefully controlled, continuously monitored, and recorded at least hourly during each exposure. Particle size should be performed for all aerosols and for vapours that may condense to form aerosols. The particle size (*Mass Median Aerodynamic Diameter i.e. MMAD*) of 1-4 µm is recommended or use the minimum particle size with which it is possible to conduct the test. Temperature of 22± 3°C and 30-70% humidity should be maintained in the chamber.

When a vehicle is used for maintaining the proper concentration and particle size of the test substance in the exposure environment, it is desirable to use water or a vehicle that is known to be non-toxic, or that will not affect the test results. When necessary, conduct studies with a vehicle control group. Particulate material may be mechanically processed to achieve the required particle size distribution.

Number of animals

Three animals per sex for each dose level in sighting study and limit test and 5 animals per sex for each dose level for main study.

Dose Levels and Administration of doses

- i) **Sighting study:** 3 animals (preferably more sensitive sex) are exposed to single or more concentrations to determine test article potency, identify test substance susceptibility to particular sex, if any. It will assist in selecting the dose for the main study, limit test.

If the mortality does not occur at maximum attainable concentration until 5 days of post exposure period in a sighting study, the study can be extended upto 14 days for considering it as limit test provided all the observations as per requirement of limit test have been recorded for sighting study.

- ii) **Limit Test:** Limit test is undertaken when test substance is known or expected to be non-toxic i.e eliciting a toxic response only above the regulatory limit concentration. Investigator should justify for avoiding the sighting study subject to authentication about non toxicity of the product by inhalation route.

Single group of 3 males and 3 females is exposed to the test article at a limit concentration of 5 mg/L (aerosol with respirable particle size) and 20 mg/L for vapour and gases. If a test article's physical or chemical properties make it impossible to attain a limit concentration, the maximum attainable concentration should be tested. It is important to maintain the respirable particle size (MMAD) of 1-4µm. For aerosols it is possible at the concentration of 2 mg/litre with most of the test substances. Therefore, aerosol testing for concentration more than 2mg/litre should only be done if respirable particle size can be achieved. If no lethality is demonstrated, no further detailed testing for acute inhalation toxicity is needed. If compound-related mortality or moribundity is produced, further study may need to be considered and the results of limit test may be used as sighting study for further testing at other concentrations.

- iii) Main Test (Number of animals, dose levels etc.):** 5 male and 5 female animals (or 5 animals of the susceptible sex, if known) are exposed per concentration level. A minimum of three concentration levels should be used. Alternatively, assign 5 animals, all of the same sex, to each group (at least 3 dosage levels). In addition, administer the substance to at least 1 group of the other sex (preferably at middle or high concentration), to confirm that the other sex does not have a notably high susceptibility.

Observation period

Animals should be observed for at least 14 days. However, depending on toxic reactions and time of onset and length of recovery period, the period may be extended when considered necessary.

Observation and Examination

- i) Animals should be observed frequently during the exposure period and then at least twice after the exposure on that day. Thereafter, at least once daily during the observation period.
- ii) Observations should include condition of skin and fur, eyes and mucus membrane, respiratory, circulatory, autonomic and central nervous system, somato-motor activity and behaviour pattern. Specific observations should be made for tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma.
- iii) Individual animal weights should be recorded once during the acclimatization period, just prior to exposure (day 0), and at least on days 1, 3 and 7 (and weekly thereafter), and at the time of death. Surviving animals are weighed and humanely killed at the end of the post exposure.
- iv) All gross pathological changes should be recorded for each animal with particular attention to any changes in the respiratory tract. Histopathology may be considered in the organs showing significant adverse effects.

Monitoring of Exposure Conditions

- Airflow should be hourly measured during each exposure.
- Monitoring of test atmosphere concentration: O₂ concentration should be at least 19% and CO₂ concentration should not exceed 1%. If there is reason to believe that these standards cannot be met, oxygen and carbon dioxide concentration should be measured.
- Monitoring and recording of nominal concentration and actual concentration of test article.
- Particle size distribution of aerosols should be recorded at least twice during four hours exposure.
- Relative humidity at least thrice during four hours duration.

Result assessment

LC₅₀ to be determined with any recognized statistical method (Chanter and Heywood, 1982; Finney, D.G, 1971; Finney, D.J, 1977).

Toxic Endpoint

Mortality or moribund animals. Animals humanely killed due to compound related distress and pain should be recorded as compound related deaths.

N.B.

- i) The protocol is not suitable for testing of specialized materials such as poorly soluble isometric or fibrous materials or manufactured nanomaterials.
- ii) Testing of concentrations that are expected to cause severe pain or distress in case of test substances having corrosive or irritant properties, should be avoided.
- iii) The granular formulation which is non-friable and specifically made to be un-inhalable need not be tested for acute inhalation toxicity. However, attrition test should be carried out with such formulation to show that respirable particles are not produced when granular material is handled. If an attrition test produces respirable particles then the inhalation toxicity test should be undertaken.

(Reference: OECD Test Guideline 403, 2009)

PRIMARY SKIN IRRITATION / ACUTE DERMAL IRRITATION / CORROSION

Objective

To assess the skin irritation/corrosion potential of pesticides in mammals and its reversibility.

Animal welfare Approach for skin irritation test:

In view of animal welfare concerns, it is recommended that all existing information be analyzed for weight-of-the-evidence prior to undertaking the detailed test for corrosion/irritation of the substance. When a substance is judged to be corrosive on the basis of a weight-of-the-evidence analysis (viz. - strong acid or strong alkali; evidence of corrosivity/irritation from structurally related substances; results of other *in-vitro* or *ex-vivo* test) no further animal testing is needed. Where insufficient data / information are available, application of sequential / simultaneous testing (three patches test) is recommended. If a determination of corrosiveness or irritation cannot be made from 3 patch testing strategy on single animal, then test containing maximum 3 animals to be considered.

Principle of in-vivo test:

The test substance is applied to a patch on the skin of test animal for certain duration. The degree of irritation /corrosion and its reversibility is assessed and scored. The untreated skin area serves as control. Severely distressed animals are to be humanely killed.

Test Animals: The albino rabbit is the preferred animal species.

Age: Healthy young adult rabbit (not older than 2 years).

Housing & Feeding Conditions

Temperature - $20\pm 3^{\circ}\text{C}$

Relative Humidity – 50-60% (However, humidity should not be below 30% or exceed 70% at any given point of time)

Lighting – 12 hours light and dark cycle

Diet and water – Standard laboratory diet specific to the species and filtered water, free from contamination

Acclimatization: The animals should be acclimatized to the laboratory conditions for at least 5 days by keeping them in individual cages prior to dosing.

Application method

The study should be conducted on animals with healthy, intact skin. Approximately 24 hours before the test, fur should be removed from the test area by clipping or shaving from the dorsal area of the trunk of the animals. Care should be taken to avoid abrading the skin.

The test substance should be applied to a small area (approximately 6 cm²) of skin and covered with a gauze patch, which is held in place with non-irritating tape. In cases in which direct application is not possible (e.g., liquids or some pastes), the test substance should first be applied to the gauze patch, which is then to be applied to the skin. Access to the patch and ingestion of the test substance by the animal should be prevented. Untreated skin areas of the test animal serve as the control.

Liquid test substances are generally used undiluted. When testing solids grind it, as appropriate, and moisten it with minimum water, if considered necessary, or, where necessary with another suitable vehicle. When vehicles other than water are used, the potential influence of the vehicle should be minimal on test substance irritation to the skin.

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

Number of Animals

Maximum of 3 animals are used. It is strongly recommended that firstly the test be performed initially using one animal. If the results indicate corrosive property of the test substance then no further testing should be done. If corrosive effect is not observed in one animal initially exposed to test substance then to confirm the test may be performed on two additional animals. If an irritant effect is observed in the initial test, the confirmatory test may be done by exposing two additional animals simultaneously or in sequential manner.

Dose level

Single dose of 0.5 ml of liquid or 0.5 g of solid or paste is to be applied on the test area.

Exposure duration of Test Material

For products which are likely to be corrosive, up to three test patches to be applied sequentially to the animal. The first patch is removed after 3 minutes. If no serious skin reaction is observed, a second patch is applied at a different site and removed after 1 hour. Based on the results of these applications if it is indicated that exposure can humanely be allowed to extend to 4 hours, a third patch is applied for 4 hrs.

In cases when test substance is not expected to produce corrosion but may be irritating, a single patch should be applied to one animal for four hours. The irritant or negative response should be confirmed using up to two additional animals, each with one patch, for an exposure period of four hours.

Observation Period

The duration of the observation period should be sufficient to evaluate fully the reversibility of the effects observed. To determine the reversibility of effects, the animals should be observed up to 14 days after removal of the patches. If reversibility is seen before 14 days, the experiment should be terminated at that time.

Observations and Examination

Animals should be examined for signs of erythema and edema as per grading of skin reactions guideline table (0-4 Score, as given below), and the responses scored at 60 minutes, and then at 24, 48 and 72 hours after patch removal.

Besides the observation regarding dermal irritation, other toxic effects such as defatting of the skin and any systemic adverse effects (e.g., effects on clinical signs of toxicity and body weight), should be fully described and recorded. Histopathological examination should be done to clarify equivocal responses.

Result Assessment

Table: Grading of Skin Reactions

Erythema and Eschar Formation

No erythema	0
Very slight erythema (barely perceptible)	1
Well defined erythema.	2
Moderate to severe erythema	3
Severe erythema (beef redness) to eschar formation preventing grading of erythema.	4

Maximum possible: 4

Oedema Formation

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

Maximum possible: 4

Toxic Endpoint

Presence of irritation/corrosion.

(Reference: OECD Test Guideline 404, 2015)

IN VITRO SKIN IRRITATION: RECONSTRUCTED HUMAN EPIDERMIS TEST METHOD

Objective

To assess the *in-vitro* skin irritation component of the tiered testing.

Animal welfare Approach for skin irritation test:

In view of animal welfare concerns, it is recommended that a tiered testing strategy for the determination of skin corrosion/irritation, using validated *in vitro* and *ex vivo* test methods, thus avoiding pain and suffering of animals.

Principle of in-vivo test

The test chemical is applied topically to a 3-dimensional RhE model comprising of non-transformed human-derived epidermal keratinocytes, which have been cultured to form a multi-layered, highly differentiated model of the human epidermis. It directly covers the initial step of the inflammatory cascade/mechanism of action (cell and tissue damage resulting in localized trauma) that occurs during irritation *in-vivo*. The RhE-based test methods, in the absence of any vascularization in the *in vitro* test system, measure the initial events in the cascade, e.g. cell-tissue damage.

Procedure for skin irritation assessment RhE Test Method Components

General conditions

Non-transformed human keratinocytes should be used to reconstruct the epithelium. Multiple layers of viable epithelial cells (basal layer, stratum spinosum, stratum granulosum) should be present under a functional stratum corneum. Stratum corneum should be multilayered containing the essential lipid profile to produce a functional barrier with robustness to resist rapid penetration of cytotoxic benchmark chemicals, e.g. sodium dodecyl sulphate (SDS) or Triton X-100. The barrier function should be demonstrated and may be assessed either by determination of the concentration at which a benchmark chemical reduces the viability of the tissues by 50% (IC50) after a fixed exposure time, or by determination of the exposure time required to reduce cell viability by 50% (ET50) upon application of the benchmark chemical at a specified, fixed concentration. The containment properties of the RhE model should prevent the passage of material around the stratum corneum to the viable tissue, which would lead to poor modelling of skin exposure. The RhE model should be free of contamination by bacteria, viruses, mycoplasma, or fungi.

Functional conditions

Viability

The assay used for determining the magnitude of viability is the MTT-assay (MTT (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide), originally for measuring cell survival/proliferation)

The RhE model users should ensure that each batch of the RhE model used meets defined criteria for the negative control (NC). The optical density (OD) of the extraction solvent alone should be sufficiently small, i.e. $OD < 0.1$. The tissues treated with NC are stable in culture (provide similar viability measurements) for the duration of the test exposure period.

Barrier function

The stratum corneum and its lipid composition should be sufficient to resist the rapid penetration of cytotoxic benchmark chemicals, e.g. SDS or Triton X-100, as estimated by IC₅₀ or ET 50.

Morphology

Histological examination of the RhE model should be provided demonstrating human epidermis-like structure (including multilayered stratum corneum).

Reproducibility

The results of the positive and negative controls of the test method should demonstrate reproducibility over time.

Quality control (QC)

The RhE model should only be used if the developer/supplier demonstrates that each batch of the RhE model used meets defined production release criteria, among which those for viability, barrier function and morphology are the most relevant. An acceptability range (upper and lower limit) for the IC₅₀ or the ET₅₀ should be established by the RhE model developer/supplier. Only results produced with qualified tissues can be accepted for reliable prediction of irritation classification.

Application of the test and control

At least three replicates should be used for each test chemical and for the controls in each run. For liquid as well as solid chemicals, sufficient amount of test chemical should be applied to uniformly cover the epidermis surface while avoiding an infinite dose, i.e. ranging from 26 to 83 $\mu\text{L}/\text{cm}^2$ or mg/cm^2 should be used. For solid chemicals, the epidermis surface should be moistened with deionised or distilled water before application, to improve contact between the test chemical and the epidermis surface. Whenever possible, solids should be tested as a fine powder. A nylon mesh may be used as a spreading aid in some cases. At the end of the exposure period, the test chemical should be carefully washed from the epidermis surface with aqueous buffer, or 0.9% NaCl.

Exposure duration and temperature

Depending on the RhE test methods used, the exposure period ranges between 15 and 60 minutes, and the incubation temperature between 20 and 37°C. These exposure periods and temperatures are optimized for each individual RhE test method and represent the different intrinsic properties of the test methods (e.g. barrier function).

Positive and Negative Control

Concurrent Negative control (NC) and positive controls (PC) should be used in each run to demonstrate that viability (with the NC), barrier function and resulting tissue sensitivity (with the PC) of the tissues are within a defined historical acceptance range. The suggested PC chemical is 5% aqueous SDS. The suggested NC chemicals are water or phosphate buffered saline (PBS).

Cell viability measurements

According to the test procedure, it is essential that the viability measurement is not performed immediately after exposure to the test chemical, but after a sufficiently long post-treatment incubation period of the rinsed tissue in fresh medium. A 42 hours post-treatment incubation period was found optimal during test optimisation of two of the RhE-based test methods. This period allows both for recovery from weak cytotoxic effects and for appearance of clear cytotoxic effects.

The MTT assay is a validated quantitative method which should be used to measure cell viability under this Test Guideline. The tissue sample is placed in MTT solution of appropriate concentration (e.g. 0.3 - 1 mg/mL) for 3 hours. The MTT is converted into blue formazan by the viable cells. The precipitated blue formazan product is then extracted from the tissue using a solvent (e.g. isopropanol, acidic isopropanol), and the concentration of formazan is measured by determining the OD at 570 nm using a filter band pass of maximum ± 30 nm.

Optical properties of the test chemical or its chemical action on MTT may interfere with the assay leading to a false estimate of viability. If a test chemical acts directly on the MTT (e.g. MTT-reducer), is naturally coloured, or becomes coloured during tissue treatment, additional controls should be used to detect and correct for test chemical interference with the viability measurement technique.

When performing the standard absorbance (OD) measurement, each interfering coloured test chemical is applied on at least two viable tissue replicates, which undergo the entire testing procedure but are incubated with medium instead of MTT solution during the MTT incubation step to generate a non-specific colour (NSC_{living}) control. The NSC_{living} control needs to be performed concurrently to the testing of the coloured test chemical and in case of multiple testing, an independent NSC_{living} control needs to be conducted with each test performed (in each run) due to the inherent biological variability of living tissues. The true tissue viability is then calculated as the percent tissue viability obtained with living tissues exposed to the interfering test chemical and incubated with MTT solution minus the percent non-specific colour obtained with living tissues exposed to the interfering test chemical and incubated with medium without MTT, run concurrently to the test being corrected (%NSC_{living}).

Acceptability criteria

For each test method using valid RhE model batches, tissues treated with the NC should exhibit OD reflecting the quality of the tissues that followed shipment, receipt steps and all protocol processes. Control OD values should not be below historically established boundaries. Similarly, tissues treated with the PC, i.e. 5% aqueous SDS, should reflect their ability to respond to an irritant chemical under the conditions of the test method.

Interpretation of results

The OD values obtained with each test chemical can be used to calculate the percentage of viability normalised to NC, which is set to 100%. The cut-off value of percentage cell viability distinguishing irritant from non-classified test chemicals and the statistical procedure(s) used to evaluate the results and identify irritant chemicals should be clearly defined, documented, and proven to be appropriate. The cut-off values for the prediction of irritation are given below:

- The test chemical is considered to be irritant to skin if the tissue viability after exposure and post-treatment incubation is less than or equal ($\leq$) to 50%. And in case, the test chemical is found to be non-corrosive & shows similar viability, it may be considered to be an irritant to skin.
- Depending on the regulatory framework in member countries, the test chemical may be considered as non-irritant to skin if the tissue viability after exposure and post-treatment incubation is more than ($>$) 50%.

Toxic Endpoint

Human health skin irritation

(Reference: OECD Test Guideline 439, 2015)

ACUTE EYE IRRITATION

Objective

To assess the eye irritation / corrosion potential of pesticide.

Note: In addition, the findings of dermal irritation/corrosion test is to be considered to predict eye corrosion prior to undertaking detail eye irritation test. Materials which have demonstrated definite corrosion or severe irritation in a dermal study, need not be further tested for eye irritation and the labeling will be done accordingly.

Topical anesthetics, systemic analgesics, and humane endpoints should be routinely used during acute eye irritation and corrosion in vivo testing. Balanced preemptive pain management should include (i) routine pretreatment with a topical anesthetic (e.g., proparacaine or tetracaine) and a systemic analgesic (e.g. buprenorphine), (ii) routine post-treatment schedule of systemic analgesia (e.g., buprenorphine and meloxicam), (iii) scheduled observation, monitoring, and recording of animals for clinical signs of pain and/or distress, and (iv) scheduled observation, monitoring, and recording of the nature, severity, and progression of all eye injuries. Following test substance administration, no additional topical anesthetics or analgesics should be applied in order to avoid interference with the study. Analgesics with anti-inflammatory activity (e.g., meloxicam) should not be applied topically, and doses used systemically should not interfere with ocular effects.

Animal welfare Approach for Eye Irritation test

In view of animal welfare concerns, prior to the test for acute eye irritation/corrosion, a weight-of-the-evidence analysis should be performed on the existing relevant data. Where insufficient data are available, it is recommended that they be developed through application of sequential testing.

Substances exhibiting pH extremes such as ≤ 2.0 or ≥ 11.5 may have strong local effects. Strongly acidic or alkaline substances, for example, with a demonstrated pH of 2 or less or 11.5 or greater, need not be tested owing to their predictable corrosive properties. Materials which have demonstrated definite corrosion or severe irritation in a dermal study need not be further tested for eye irritation (USEPA).

Principle of the Test

Following pretreatment with a systemic analgesic and induction of appropriate topical anesthesia, the test article is instilled in a single dose to one eye of the test animal; untreated eye serves as a control. The eyes are examined at regular intervals for irritation/corrosion; and their reversibility. Other effects in the eye and adverse systemic effects are also observed for complete evaluation of effects. Moribund or severely distressed animals are humanely killed.

Test Animals: The albino rabbit is the preferred animal species.

Age: Healthy young adult animals (not older than 2 years and having no ocular abnormality in ophthalmological examination of eye) are to be included in the study.

Housing & Feeding Conditions

Temperature - $20\pm 3^{\circ}\text{C}$

Relative Humidity – 50-60% (However, humidity should not be below 30% or exceed 70% at any given point of time).

Lighting – 12 hours light and dark cycle

Diet and water – Standard laboratory diet specific to the species and filtered water, free from contamination

Acclimatization: The animals should be acclimatized to the laboratory conditions for at least 5 days by keeping in individual cages prior to dosing.

Application Method

Before inducing the animals in the experiment, both the eyes of all test animals are to be examined within 24 hrs. before the start of test. Use of topical anesthetics and systemic analgesics are recommended to avoid or minimize pain and distress in ocular safety testing procedures prior to test substance application. The substance to be tested should be placed in a single dose in the conjunctival sac to one of the eyes of the experimental animal; the untreated eye serves as the control.

Number of animals

Maximum 3 animals. Initial test is to be performed on one animal followed by confirmatory test in 1- 2 additional animals. If a corrosive or severe irritant effect is observed in the initial test, no further testing to be undertaken. If a corrosive effect is not observed then irritant or negative response is to be confirmed by testing in sequential manner in one animal at a time, rather than exposing the two additional animals simultaneously. If the second animal reveals corrosive or severe irritant effects, the test is not continued. In case of weak or moderate irritant responses on 2nd animal it can be confirmed on 3rd rabbit.

Dose Levels

Liquids: A dose of 0.1 ml;

Solids, Pastes: A volume of 0.1 ml or a weight of not more than 100 mg.

Aerosol: In a simple burst of about one second, from a distance of 10 cm directly in front of the eye.

Note: If the test substance is solid or granular, grind it to a fine dust. Local anesthetics may be used as mentioned above. The control eye should be similarly anaesthetized.

Exposure duration

The eyes of the test animals should not be washed for at least 24 hours following instillation of the test substance, except for solids and in case of immediate corrosive or irritating effects where it should be washed after 1 hour.

If required, for some substances shown to be irritating by this test, additional testing may be indicated using animals where eyes are washed soon after instillation of the substance.

Half a minute after instillation, the eyes of the animals are washed with water for 30 sec, using a volume and velocity of flow which should not cause injury. However, this is optional.

Observations and Examination

The eyes should be comprehensively evaluated for the presence or absence of ocular lesions one hour post test substance application, followed by at least daily evaluations. The eyes should be examined at 1, 24, 48, and 72 hours as per the prescribed scale given below w.r.t grading of ocular lesions (0-4) on Cornea, Iris, Conjunctivae. If animals do not develop ocular lesions during first 3 days after instillation then study can be terminated. Any other effect/lesion in the eye (e.g. Staining, pannus etc.) or adverse systemic effect should be recorded. Fluorescein staining should be routinely used and a slit lamp biomicroscope used when considered appropriate (e.g., assessing depth of injury when corneal ulceration is present) as an aid in the detection and measurement of ocular damage, and to evaluate if established endpoint criteria for humane euthanasia have been met.

Animals with mild to moderate lesions should be observed until the lesions clear or for 21 days in order to determine their reversibility or irreversibility. Animals with severe pain or distress due to severe eye lesions known to be irreversible, should be humanely killed without delay and the substance assessed accordingly.

Result Assessment

Grading Of Ocular Lesions

Cornea

Opacity: degree of density (readings should be taken from most dense area)*

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
Nacrous area; no details of iris visible; size of pupil barely discernible	3
Opaque cornea; iris not discernible through the opacity	4

* The area of corneal opacity should be noted

Maximum possible: 4

Iris

Normal	0
Markedly deepened rugae, congestion, swelling, moderate circumcorneal hyperaemia; or injection; iris reactive to light (a sluggish reaction is considered to be an effect	1
Hemorrhage, gross destruction, or no reaction to light	2

Maximum possible: 2

Conjunctivae

Redness (refers to palpebral and bulbar conjunctivae; excluding cornea and iris)

Normal	0
Some blood vessels hyperaemic (injected)	1
Diffuse, crimson colour; individual vessels not easily discernible	2
Diffuse beefy red	3

Maximum possible: 3

Chemosis

Swelling (refers to lids and/or nictating membranes)

Normal	0
Some swelling above norma	1
Obvious swelling, with partial eversion of lids	2
Swelling, with lids about half closed	3
Swelling, with lids more than half closed	4

Maximum possible: 4

Toxic Endpoint

Irritation or corrosion of eye

(Reference: OECD Test Guideline 405, 2012; OPPTS 870.2400, 1998)

SKIN SENSITIZATION STUDIES

Objective

The objective of these studies is to assess skin sensitization potential of pesticides.

Animal Welfare Approach

The Local Lymph Node Assay (LLNA) can be used as a first stage in the assessment of skin sensitization potential. If a positive result is seen, a test substance may be designated as a potential sensitizer, and it may not be necessary to conduct a further guinea pig test. However, if a negative result is seen in the LLNA, then GPMT (Guinea Pig Maximisa Test or Buehler Test) must be conducted. The testing laboratory should consider all available information on the test substance prior to conducting the study. This information should be considered in order to determine whether the LLNA is appropriate for the test substance (given the incompatibility of limited types of test substances with the LLNA) and to aid in dose selection.

The LLNA is an *in vivo* method and, as a consequence, will not eliminate the use of animals in the assessment of allergic contact sensitizing activity. It has, however, the potential to reduce the number of animals required for this purpose. Moreover, the LLNA offers a substantial refinement (less pain and distress) of the way in which animals are used for allergic contact sensitization testing. However, certain limitations of LLNA are false negative findings in the LLNA with certain metals, false positive findings with certain skin irritants [such as some surfactant type chemicals], or solubility of the test substance. In addition, test substance classes or substances containing functional groups shown to act as potential confounders, may necessitate the use of guinea pig tests.

I. Local Lymph Node Assay (LLNA)

Principle of the Test

The LLNA studies the induction phase of skin sensitization and provides quantitative data suitable for dose-response assessment.

The basic principle underlying the LLNA is that sensitizers induce proliferation of lymphocytes in the lymph nodes draining the site of test substance application. This proliferation is proportional to the dose and to the potency of the applied allergen. Proliferation is measured by comparing the mean proliferation in each test group to the mean proliferation in the concurrent Vehicle Treated Control (VC) group. This is termed as the Stimulation Index (SI), which should be ≥ 3 before classifying the test substance as a potential skin sensitizer.

Test animals: Young Adult nulliparous and non-pregnant Female Mice (CBA/J or CBA/Ca strains)

Age: On the exposure day, animals should be young adults, between 8 to 12 weeks of age.

Weight: On the exposure day, body weights should be within $\pm 20\%$ of the mean weight of any previously exposed animals of the same age.

Housing and Feeding Conditions

Temperature - $22 \pm 3^\circ\text{C}$;

Relative Humidity – 50-60% (However, humidity should not be below 30% or exceed 70% at any given point of time).

Lighting – 12 hours light and dark cycle

Diet and water – Standard laboratory diet specific to the species and filtered water, free from contamination

Acclimatization: The animals are randomly selected, marked to permit individual identification by any method other than ear marking, and kept in their cages for at least five days prior to the start of dosing to allow for acclimatization to the laboratory conditions. Observe all animals and ensure that none have any skin lesions, before administering the test substance.

Test Procedure

a) Dose Range Finding Study (DRFS)

In the absence of information to determine the maximum possible concentration to be tested, a Dose Range Finding Studies (DRFS)/pre-screen test should be performed in order to define the appropriate dose level to test in the LLNA. The purpose of the pre-screen test is to provide guidance for selecting the maximum dose level to use in the main LLNA study, where information on the concentration that induces systemic toxicity and/or excessive local skin irritation is not available. The maximum concentration in case of liquids tested should be 100% or in case of solids or suspensions, it should be the maximum possible achievable concentration.

The DRFS/pre-screen test is conducted similarly as the main LLNA study, except that no assessment of lymph node proliferation is made and fewer animals per dose group are used. One or two animals per dose group are suggested. All mice will be observed daily for any clinical signs of systemic toxicity or local irritation at the application site. Body weights are recorded pre- test and prior to termination (Day 6). Both ears of each mouse are observed for erythema and scored using **Table 1**. Ear thickness measurements are taken using a thickness gauge (e.g. digital micrometer or Peacock Dial thickness gauge) on Day 1 (pre-dose), Day 3 (approximately 48 hours after the first dose), and Day 6. Additionally, on Day 6, ear thickness could be determined by ear punch weight determinations, which should be performed after the animals are humanely killed. Excessive local skin irritation is indicated by an erythema score ≥ 3 and/or an increase in ear thickness of $\geq 25\%$ on

any day of measurement. The highest dose selected for the main LLNA study will be the next lower dose in the pre-screen concentration series that does not induce systemic toxicity and/or excessive local skin irritation.

Table 1: Erythema Scores

Observation	Score
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to eschar formation preventing grading of erythema	4
In addition to a 25% increase in ear thickness, a statistically significant increase in ear thickness in the treated mice compared to control mice has also been used to identify irritants in the LLNA. However, while statistically significant increases can occur when ear thickness is less than 25% they have not been associated specifically with excessive irritation.	

b) Main Test

Number of animals and dose levels

A minimum of four animals is used per dose group and a minimum 5 groups are to be tested which include a minimum of three groups of the test substance concentration, a concurrent negative control group treated only with the vehicle for the test substance, and a positive control group.

Consecutive doses are normally selected from an appropriate concentration series such as 100%, 50%, 25%, 10%, 5%, 2.5%, 1%, 0.5%, etc. Adequate scientific rationale should accompany the selection of the concentration series used. All existing toxicological information (*e.g.* acute toxicity and dermal irritation) and structural and physicochemical information on the test substance of interest (and/or structurally related test substances) should be considered where available, in selecting the three consecutive concentrations so that the highest concentration maximizes exposure while avoiding systemic toxicity and/or excessive local skin irritation. In the absence of such information, an initial pre-screen test may be necessary.

Administration of doses

Preparation of dosing solutions: Solid test substances should be dissolved or suspended in solvents/vehicles and diluted, if appropriate, prior to application to an ear of the mice. Liquid test substances may be applied neat or diluted prior to dosing. Test substances should be prepared daily unless stability data demonstrate the acceptability of storage.

Main study experimental schedule: The experimental schedule of the assay is as follows:

Day 1: Individually identify and record the weight of each animal and any clinical observation. Apply 25 µL of the appropriate dilution of the test substance, the vehicle alone, or the Positive Control(PC), to the dorsum of each ear.

Days 2 and 3: The application procedure carried out on Day 1 should be repeated.

Days 4 and 5: No treatment.

Day 6: Record the weight of each animal. Inject 250 µL of sterile phosphate-buffered saline (PBS) containing 20 µCi (7.4×10^5 Bq) of tritiated (3H)-methyl thymidine into all test and control mice via the tail vein. Alternatively, inject 250 µL sterile PBS containing 2 µCi (7.4×10^4 Bq) of 125I-iododeoxyuridine and 10-5M fluorodeoxyuridine in all mice via the tail vein. Five hours(5 h) later, humanely kill the animals. Excise the draining auricular lymph nodes from each mouse ear and process together in PBS for each animal (individual animal approach); alternatively excise and pool the lymph nodes from each ear in PBS for each treatment group (pooled treatment group approach). Additional parameters such as scoring of ear erythema or ear thickness measurements (obtained either by using a thickness gauge, or ear punch weight determinations at necropsy) may be undertaken to further monitor the local skin response.

a) Preparation of cell suspensions

A single-cell suspension of lymph node cells (LNC) excised bilaterally using the individual animal approach or alternatively, the pooled treatment group approach is prepared by gentle mechanical disaggregation through 200 micron-mesh stainless steel gauze or another acceptable technique for generating a single-cell suspension. The LNC are washed twice with an excess of PBS and the DNA is precipitated with 5% trichloroacetic acid (TCA) at 4°C for 18h. Pellets are either re-suspended in 1 mL TCA and transferred to scintillation vials containing 10 mL of scintillation fluid for 3H-counting, or transferred directly to gamma counting tubes for 125I-counting.

b) Determination of cellular proliferation (incorporated radioactivity)

Incorporation of 3H-methyl thymidine is measured by β-scintillation counting as disintegrations per minute (DPM). Incorporation of 125I-iododeoxyuridine is measured by 125I-counting and also is expressed as DPM. Depending on the approach used, the incorporation is expressed as DPM/mouse (individual animal approach) or DPM/treatment group (pooled treatment group approach).

Observations

a) Clinical Observations

Each mouse should be carefully observed at least once daily for any clinical signs, either of local irritation at the application site or of systemic toxicity. All observations are systematically recorded with records being maintained for each mouse. Animals with excessive skin irritation/corrosion or systemic toxicity are sacrificed.

The following clinical observations may indicate systemic toxicity when used as part of an integrated assessment and therefore may indicate the maximum dose level to use in the main LLNA: changes in nervous system function (e.g. pilo-erection, ataxia, tremors, and convulsions); changes in behaviour (e.g. aggressiveness, change in grooming activity, marked change in activity level); changes in respiratory patterns (i.e. changes in frequency and intensity of breathing such as dyspnea, gasping, and rales), and changes in food and water consumption. In addition, signs of lethargy and/or unresponsiveness and any clinical signs of more than slight or momentary pain and distress, or a >5% reduction in body weight from Day 1 to Day 6, and mortality should be considered in the evaluation. Moribund animals or animals obviously in pain or showing signs of severe and enduring distress should be humanely killed.

b) Body weights

Body weights of individual animals should be measured at the start of the test and at the scheduled humane kill.

Result Assessment/Calculation of Results

Results for each treatment group are expressed as the SI. When using the individual animal approach, the SI is derived by dividing the mean DPM/mouse within each test substance group, and the PC group, by the mean DPM/mouse for the solvent/VC group. The average SI for the VCs is then one. When using the pooled treatment group approach, the SI is obtained by dividing the pooled radioactive incorporation for each treatment group by the incorporation of the pooled VC group; this yields a mean SI.

The result is considered as positive when $SI \geq 3$. However, the strength of the dose-response, the statistical significance and the consistency of the solvent/vehicle and PC responses may also be used when determining whether a borderline result is declared positive.

Collecting radioactivity data at the level of the individual mouse will enable a statistical analysis for presence and degree of dose-response relationship in the data. Any statistical assessment could include an evaluation of the dose-response relationship as well as suitably adjusted comparisons of test groups (e.g. pair-wise dosed group versus concurrent VC comparisons).

Toxic Endpoint

The methods described here are based on the use of in vivo radioactive labeling to measure an increased number of proliferating cells in the draining auricular lymph nodes. However, other end points for assessment of the number of proliferating cells may be employed provided the Performance Standards (PS) requirements are fully met.

Reliability check: Inclusion of a concurrent Positive Control (PC) is recommended and allows for an assessment. The PC should produce a positive LLNA response at an exposure level expected to give an increase in the Stimulation Index (SI) > 3 over the negative control (NC) group. The PC dose should be chosen such that it does not cause excessive skin irritation or systemic toxicity

and the induction is reproducible but not excessive (*i.e.* SI > 20). The test substances which are preferred for use as PC are 25% hexyl cinnamic aldehyde (Chemical Abstracts Service [CAS] No 101-86-0) in acetone: olive oil (4:1, v/v) and 5% mercaptobenzothiazole (CAS No 149-30-4) in *N,N*-dimethylformamide .

Although the PC test substance should be tested in the vehicle that is known to elicit a consistent response (*e.g.* acetone: olive oil; 4:1, v/v), there may be certain regulatory situations in which testing in a non-standard vehicle (clinically/chemically relevant formulation) will also be necessary. If the concurrent PC test substance is tested in a different vehicle than the test substance, then a separate VC for the concurrent PC should be included.

(Reference: OECD Test Guideline 429, 2010)

II. Guinea Pig Maximisation Test (GPMT)

Principle of the Test

The test animals are initially exposed to the test substance by intradermal injection 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.

Test animal: Male and/or female healthy young adult Guinea pig is used. If females are used they should be nulliparous and non-pregnant.

Housing and feeding conditions

The temperature of the experimental animal room should be 20° C ($\pm 3^{\circ}$ C)

Relative Humidity – 50-60% (However, humidity should not be below 30% or exceed 70% at any given point of time).

Where the lighting is artificial, the sequence should be 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. It is essential that guinea pigs receive an adequate amount of ascorbic acid.

Acclimatization: Animals are acclimatized to the laboratory conditions for at least 5 days prior to the test.

Preparation of the animals

Before the test, animals are randomized and assigned to the treatment groups. Removal of hair is by clipping, shaving or possibly by chemical depilation. Care should be taken to avoid abrading the

skin. The animals are weighed before the test commences and at the end of the test.

Number of animals

A minimum of 10 animals is used in the treatment group and at least 5 animals in the control group. In case it is not possible to conclude that the test substance is a sensitizer, testing in additional animals to give a total of at least 20 test and 10 control animals is recommended.

Dose levels

The concentration of 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 can be determined from a pilot study using two or three animals.

Experimental Schedule

a) Induction: Intradermal Injections

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: 1:1 mixture (v/v) Freund's Complete Adjuvant (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 concentration of test substance shall be equal to that used in injection 2.

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.

b) Induction: Topical Application

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 painted 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 pulverized 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.

Challenge: Topical Application

Day 20-22 -treated and control groups

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

Observations - treated and control groups

It is recommended to undertake the blind reading of test and control animals.

- approximately 21 hours after removal of the patch the challenge area is cleaned and closely clipped and/or shaved or depilated;
- 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 below; in the Table.
- approximately 24 hours thereafter another observation (72 hours from the start of the challenge application) is made.

Table: Magnusson and 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

Rechallenge

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.

Clinical observations

All skin reactions and any unusual findings, including systemic reactions, resulting from induction and challenge procedures should be observed and recorded. Other procedures, e.g. histopathological examination, the measurement of skin fold thickness, may be carried out to clarify doubtful reactions.

Reliability check

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 sensitization properties.

In a properly conducted test, a response of at least 30% in an adjuvant test and at least 15% in non- adjuvant test should be expected for mild/moderate sensitizers. Preferred substances are hexyl cinnamic aldehyde (CAS No. 101-86-0), mercaptobenzothiazole (CAS No. 149-30-4) and benzocaine (CAS No. 94-09-7). There may be circumstances where, given adequate justification, other control substances meeting the above criteria may be used.

(Reference: OECD Test Guideline 406, 1992)

III. Buehler Test Method

Principle of Test

The test animals are initially exposed to the test substance by **topical 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.

Test animals: Male and/or female healthy young adult Guinea pig is used. If females are used they should be nulliparous and non-pregnant.

Housing & Feeding conditions

The temperature of the experimental animal room should be 20° C ($\pm 3^{\circ}$ C)

Relative Humidity – 50-60% (However, humidity should not be below 30% or exceed 70% at any given point of time).

Where the lighting is artificial, the sequence should be 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. It is essential that guinea pigs receive an adequate amount of ascorbic acid.

Acclimatization: Animals are acclimatized to the laboratory conditions for at least 5 days prior to the test.

Number of Animals

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

Dose levels

- i) The concentration of test substance used for each induction exposure should be the highest to cause mild irritation. The concentration used for the challenge exposure should be the highest non-irritating dose. The appropriate concentration can be determined from a pilot study using two or three animals.
- ii) 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.

Experimental Schedule

Induction: Topical application

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 or 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 for 6 hours.

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 for 6 hours .

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

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.

Challenge

Day 27-29 -treated and control groups

The untreated flank of treated and control animals is cleared of hair (closely-clipped). An occlusive patch 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 are held in contact by a suitable dressing for 6 hours.

Observations - treated and control groups

It is recommended to undertake blind reading of test and control animals.

- 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 Guinea-Pig Maximisation Test
- approximately 24 hours after the 30 hour observation (approximately 54 hours after application of the challenge patch) skin reactions are again observed and recorded.

Rechallenge

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. The rechallenge may also be performed on the original control group.

Clinical observations

All skin reactions and any unusual findings, including systemic reactions, resulting from induction and challenge procedures should be observed and recorded. Other procedures, e.g. histopathological examination, measurement of skin fold thickness, may be carried out to clarify doubtful reactions.

Reliability check

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 sensitization properties.

In a properly conducted test, a response of at least 30% in an adjuvant test and at least 15% in non- adjuvant test should be expected for mild/moderate sensitizers. Preferred substances are hexyl cinnamic aldehyde (CAS No. 101-86-0), mercaptobenzothiazole (CAS No. 149-30-4) and benzocaine (CAS No. 94-09-7). There may be circumstances where, given adequate justification, other control substances meeting the above criteria may be used.

(Reference: OECD Test Guideline 406, 1992)

SHORT-TERM REPEATED DOSE TOXICITY STUDIES

Repeated Dose 28 Day Oral Toxicity Study- Rodent

Repeated Dose 90 Day Oral Toxicity Study- Rodent

Repeated Dose 90 Day Oral Toxicity Study- Non Rodent

Repeated Dose Dermal Toxicity Study

Repeated Dose Inhalation Toxicity Study

REPEATED DOSE 28 DAY ORAL TOXICITY STUDY- RODENT

Objective

This study provides information on the possible health hazards likely to arise from repeated exposure over a relatively limited period of time including effects on nervous, immune and endocrine systems. The method comprises the basic repeated dose toxicity study that may be used for chemicals on which a 90 day study is not warranted (e.g. when the production volume does not exceed certain limits) or as a preliminary to a long term study. The duration of exposure should normally be 28 days.

Principle of the test:

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.

Note: In the assessment and evaluation of the toxic characteristics of a chemical, the determination of oral toxicity using repeated doses may be carried out after initial information on toxicity has been obtained by acute testing.

A 28 day study provides information on the effects of repeated oral exposure and can indicate the need for further longer term studies. It can also provide information on the selection of concentrations for longer term studies.

Test Animal: Preferred rodent species - Rat

Age: Young healthy adults should be used in the study. Females should be nulliparous and non-pregnant. Dosing should begin as soon as possible after weaning and not after the animals are 9 weeks old.

Weight: The weight variation of animal used should be minimal and not exceeding $\pm 20\%$ of the mean weight of each sex.

Housing & Feeding

Temperature - $22 \pm 3^\circ\text{C}$

Relative Humidity – 50-60% (However, humidity should not be below 30% or exceed 70% at any given point of time).

Lighting – 12 hours light and dark cycle

Diet and water – Conventional laboratory diet and water *ad libitum* may be provided.

Acclimatization: Test animals to be acclimatized to laboratory conditions for at least 5 days. Animals should be randomly assigned to various groups and each animal should be identified by a unique number.

Number of animals / dose group

10 animals (five female and five male) / dose Group. Further, an additional 10 animals (five male and five females) may be included in each control and highest dose groups for determining reversibility or persistence or delayed occurrence of toxic effects, for at least 14 days post treatment.

Number of dose groups (including recovery group)

At least 3 dose levels (unless limit test is performed) and a concurrent control should be used. The control group shall be an untreated group or a vehicle-control group (as applicable). If there are no suitable data available, a range finding study may be performed to aid the determination of the doses to be used.

Selection of Doses

The dose selection should be such that highest dose level should induce toxicity but not mortality or severe distress in animals. The lower dose levels should be in descending sequence with appropriate intervals (usually 2-4 fold) to demonstrate any dosage related response and a no-observed-adverse-effect level (NOAEL) at the lowest dose level. For selecting the initial dose level the dose range finding study, toxicological and toxico-kinetic properties of test substance and its physico-chemical properties be considered.

Dose Preparation

The test substance should be dissolved or suspended in a suitable vehicle.

Dose Administration

The test substance is usually administered by gavage or via the diet or drinking water, if the purpose of study and the physico-chemical properties of the test substance require so. While administering

the test substance via diet, care should be taken to maintain either constant dietary concentration (ppm) or a constant dose level in terms of animals body weight of the test substance. The animals are dosed with the test substance daily seven days each week for a period of 28 days.

Limit Test

If a test at dose of least 1000 mg/kg body weight/day produces no observed adverse effects, a full study using three dose levels may not be considered necessary except when human exposure indicates the need for a higher dose level to be used.

Observation period

Minimum 28 days. However, it may extend if recovery group is included in the study.

Animals in recovery group should be observed further for a period of 14 days without treatment to detect delayed occurrence, or persistence of, or recovery from toxic effects.

Type of observation

Clinical observations

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. All animals are inspected at least twice daily for signs of morbidity and mortality.

At least once prior to the first exposure and once a week thereafter, detailed clinical observations should be made in all animals. Signs of toxicity 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.

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 on fourth exposure week. However, it may be omitted when the study is conducted as a preliminary study to a subsequent sub-chronic (90- day) study. Exceptionally, functional observations may also be omitted for groups that otherwise reveal such signs of toxicity that would interfere with the functional test performance.

At necropsy, the oestrus cycle of all females could be determined (optional) by taking vaginal smears. These observations will provide information regarding the stage of oestrus cycle at the time of sacrifice and assist in histological evaluation of estrogen sensitive tissues

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.

Haematology and Clinical Biochemistry

At the end of the test period, blood samples should be collected under appropriate conditions just prior to or as part of the procedure for killing the animals.

General Methods For Blood Collection

Blood samples are collected using the following techniques:

- Blood collection not requiring anesthesia
 - Saphenous vein (rat, mice, guinea pig)
 - Dorsal pedal vein (rat, mice)
- Blood collection requiring anesthesia (local/general anesthesia)
 - Tail vein (rat, mice)
 - Tail snip (mice)
 - Orbital sinus (rat, mice)
 - Jugular vein (rat, mice)
 - Temporary cannula (rat, mice)
 - Blood vessel cannulation (rat, guinea pig, ferret)
 - Tarsal vein (guinea pig)
 - Marginal ear vein/artery (rabbit)
- Terminal procedure
 - Cardiac puncture (rat, mice, guinea pig, rabbit, ferret)
 - Orbital sinus (rat, mice)
 - Posterior vena cava (rat, mice)

In addition, any better newer method will be acceptable.

The following haematological and clinical chemistry examinations should be made at the end of the test period and when any interim blood samples may have been collected. Overnight fasting of the animals prior to blood sampling is recommended.

Haematological investigations

Haematocrit, haemoglobin concentration, erythrocyte count, total and differential leukocyte count, platelet count and a measure of blood clotting time/potential e.g PT, APTT

Clinical biochemistry Investigations

Determination in serum or plasma (For a number of measurements in serum and plasma, most notably for glucose, overnight fasting would be preferable) should include:

Sodium, potassium, glucose, total cholesterol, urea, blood urea nitrogen, creatinine, total protein and albumin, Alkaline Phosphatase (ALP), Alanine aminotransferase, Aspartate aminotransferase, Gamma glutamyl transpeptidase*, Sorbitol dehydrogenase*, Bile acids

* If not analyzed, then a justification to be provided based on the toxicological results of the target organ i.e. liver.

Urinalysis

Optionally, the following urinalysis determinations should 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 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 compound. 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.

Pathology

Gross necropsy

A detailed gross necropsy should be carried out on all animals(died or killed) This should include:

Careful examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents, liver, kidneys, adrenals, testes, epididymides, uterus, ovaries, thymus, spleen, brain and heart of all animals (except those found moribund and/or intercurrently killed) should be trimmed of any adherent tissue, and their wet weight taken as soon as possible 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 glandsstomach, small and larg 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

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.

All gross lesions should be examined.

When a recovery (satellite) group is used, animals of this group should be subjected to histopathology of tissues and organs identified .

Toxic Endpoint

Neurological effects

Note: In the presence of observed general toxicity (e.g. reduced body weight, liver , heart, lung or kidney effects, etc.) or other changes that may not be toxic responses (e.g. reduced food intake, liver enlargement), observed effects on immune, neurological or endocrine sensitive endpoints should be interpreted with caution.

(Reference: OECD Test Guideline 407, 2008)

REPEATED DOSE 90 DAY ORAL TOXICITY STUDY- RODENT

Objective

The basic objective of the study is to obtain scientific information regarding toxic effects produced, target organs affected and possibility of accumulation of test substance following its repeated oral administration for at least 90 days (covering post-weaning maturation and growth well into adulthood), and to make an estimate of a dose level at which toxic changes(adverse effects) are not observed i.e. ' No Observed Adverse Effect Level'(NOAEL). This 'NOAEL' can become the basis for selecting dose levels for chronic toxicity studies and for establishing safety criteria for human exposure. The study should also reveal information about pesticides having potential to cause neurotoxic ,immunological or reproductive organ effects so as further in-depth investigation be carried out on such pesticides.

Principle of the test

The test substance is orally administered daily to rats for a period of 90 days at different dose levels and animals are observed for any signs of toxicity clinically. Their haematological and biochemical parameters are monitored. The dead, killed (due to humane reasons) and surviving animals which are killed at the end of experiment, are necropsied.

Note: 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.

Test Animal: Preferred species - Rat

Age: Young healthy adults should be used in the study. Females should be nulliparous and non-pregnant. Dosing should begin as soon as possible after weaning and not after the animals are 9 weeks old.

Weight: The weight variation of animal used should be minimal and not exceeding 20% of the mean weight of each sex.

Housing & Feeding

Temperature - $22\pm 3^{\circ}\text{C}$

Relative Humidity – 50-60% (However, humidity should not be below 30% or exceed 70% at any given point of time).

Lighting – 12 hours light and dark cycle

Diet and water – Conventional laboratory diet and water *ad libitum* may be provided.

Acclimatization: Test animals to be acclimatized to laboratory conditions for at least 5 days. Animals should be randomly assigned to various groups and each animal should be identified by a unique number.

Number of animals / dose group

20 animals (ten female and ten male) / dose Group.

Further, an additional 10 animals (five male and five females) may be included in each control and high dose groups for determining reversibility or persistence.

Number of dose groups (including recovery group)

At least 3 dose levels (unless limit test is performed) and a concurrent control should be used. The control group shall be an untreated group or a vehicle-control group (as applicable). Based on the previous knowledge and close analogue of the chemical the reversibility study may be conducted by taking additional satellite group of 10 animals (5/sex) in high dose and control.

Selection of Doses

The dose selection should be such that highest dose level should induce toxicity but not mortality or severe distress in animals. The lower dose levels should be in descending sequence with appropriate intervals (usually 2-4 folds) to demonstrate any dosage related response and a no-observed-adverse-effect level (NOAEL) at the lowest dose level. For selecting the initial dose level the dose range finding study, toxicological and toxicokinetic properties of test substance and its physico-chemical properties be considered.

Dose Preparation

The test substance should be dissolved or suspended in a suitable vehicle.

Dose Administration

The test substance is usually given by mixing in diet. It may be given via gavage or in drinking water if the purpose of study and the physico-chemical properties of the test substance requires so. While administering the test substance via diet, care should be taken to maintain either constant dietary concentration (ppm) or a constant dose level in terms of animal's body weight of the test substance. The animals are dosed with the test substance daily seven days each week for a period of 90 days.

Limit Test

If a test at dose of least 1000 mg/kg body weight/day produces no observed adverse effects, a full study using three dose levels may not be considered necessary except when human exposure indicates the need for a higher dose level to be used.

Observation period

Minimum 90 days. However, it may extend if recovery group is included in the study.

Animals in recovery group should be observed further for a period of 28 days.

Type of observations

Clinical observations

General clinical observations should be made at least once a day preferably at the same time(s), taking into consideration the peak period of anticipated effects after dosing. All animals are inspected at least twice daily for signs of morbidity and mortality.

At least once prior to the first exposure and once a week thereafter, detailed clinical observations should be made in all animals. Signs of toxicity 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.

Ophthalmological examination 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.

Reactivity to stimuli of different types (e.g., auditory, visual and proprioceptive stimuli), assessment of grip strength and motor activity assessment should be conducted anytime between 11-13 weeks. However, it may be omitted when data on functional observations are available from other studies and the daily clinical observations did not reveal any functional deficits or if animals reveal such signs of toxicity that would interfere with the functional test performance.

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.

Haematology and Clinical Biochemistry

General Methods for Blood Collection

Blood samples are collected using the following techniques:

- Blood collection not requiring anesthesia
 - Saphenous vein (rat, mice, guinea pig)
 - Dorsal pedal vein (rat, mice)
- Blood collection requiring anesthesia (local/general anesthesia)
 - Tail vein (rat, mice)
 - Tail snip (mice)
 - Orbital sinus (rat, mice)
 - Jugular vein (rat, mice)

- Temporary cannula (rat, mice)
- Blood vessel cannulation (rat, guinea pig, ferret)
- Tarsal vein (guinea pig)
- Marginal ear vein/artery (rabbit)
- Terminal procedure
 - Cardiac puncture (rat, mice, guinea pig, rabbit, ferret)
 - Orbital sinus (rat, mice)
 - Posterior vena cava (rat, mice)

In addition, any better newer method will be acceptable.

At the end of the test period, blood samples should be collected under appropriate conditions just prior to or as part of the procedure for killing the animals. The following haematological and clinical chemistry examinations should be made at the end of the test period and when any interim blood samples may have been collected. Overnight fasting of the animals prior to blood sampling is recommended.

Haematological investigations

Haematocrit, haemoglobin concentration, erythrocyte count, total and differential leukocyte count, platelet count and a measure of blood clotting time/potential e.g PT, APTT

Clinical biochemistry Investigations

Determination in serum or plasma should include:

Sodium, potassium, glucose, total cholesterol, urea, blood urea nitrogen, creatinine, total protein and albumin, Alkaline Phosphatase (ALP), Alanine aminotransferase, Aspartate aminotransferase, Gamma glutamyl transpeptidase (optional)*, Sorbitol dehydrogenase (optional)*.

* If not analyzed, then a justification to be provided based on the toxicological results of the target organ i.e. liver.

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 chloride, calcium, phosphorus, fasting triglycerides, specific hormones, acid/base balance, methaemoglobin and cholinesterase inhibition. 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 compound. 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.

Urinalysis

Optionally, the following urinalysis determinations should 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.

Pathology

Gross necropsy

A detailed gross necropsy should be carried out on all animals (died or killed) This should include:

Careful examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents, liver, kidneys, adrenals, testes, epididymides, uterus, ovaries, thymus, spleen, brain and heart of all animals (except those found moribund and/or intercurrently killed) should be trimmed of any adherent tissue, and their wet weight taken as soon as possible 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 section of bone marrow (and/or a fresh bone marrow aspirate), skin and eyes (if changes were observed during ophthalmological examinations).

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.

All gross lesions should be examined.

When a recovery (satellite) group is used, animals of this group should be subjected to histopathology of tissues and organs identified .

Toxic Endpoint

Determination of NOAEL and LOAEL.

(Reference: OECD Test Guideline 408, 1998)

REPEATED DOSE 90 DAY ORAL TOXICITY STUDY- NON RODENTS

Objectives

The basic objective of the study is to obtain scientific information regarding toxic effects produced, target organs affected and possibility of accumulation of test substance following its repeated oral administration for at least 90 days (covering post-weaning maturation and growth well into adulthood), and to make an estimate of a dose level at which toxic changes(adverse effects) are not observed i.e. ' No Observed Adverse Effect Level'(NOAEL). This 'NOAEL' can become the basis for selecting dose levels for chronic toxicity studies and for establishing safety criteria for human exposure. The study should also reveal information about pesticides having potential to cause neurotoxic, immunological or reproductive organ effects so as further in-depth investigation be carried out on such pesticides.

Principle of the test

The test substance is orally administered to dogs for a period of 90 days at different dose levels and animals are observed for any signs of toxicity clinically. Their haematological and biochemical parameters are monitored. The dead, killed (due to humane reasons) and surviving animals which are killed at the end of experiment, are necropsied.

Test Animal: Preferred species- Beagle Dog

Age: 4 - 6 months preferred (Not more than 9 months old).

Weight: The weight variation of animals used should be minimal and not exceeding 20 percent of the mean weight for each sex.

Housing & Feeding

Conventional laboratory diet and water *ad libitum* may be provided. Artificial light with 12 hrs light and dark cycle is preferred. .

Acclimatization: Test animals to be acclimatized to laboratory conditions for at least 5 days. Animals should be randomly assigned to various groups and each animal should be identified by a unique number.

Number of animals/ dose group

4 Male + 4 Female per dose level.

Further, an additional 4 animals (two male and two females) may be included in each control and high dose groups for determining reversibility or persistence.

Number of dose groups (including recovery group)

At least three dose levels (unless limit test is performed) and a concurrent control. The control group shall be an untreated group or a vehicle-control group, if a vehicle is used in administering the test substance. Based on the previous knowledge of the chemical and a close analogue, the reversibility study may be conducted by taking additional satellite group of 4 animals (2/sex) in high dose and control (for each group).

Selection of Doses

The dose selection should be such that highest dose level should induce toxicity but not mortality or severe distress in animals. The lower dose levels should be in descending sequence with appropriate intervals (usually 2-4 fold) to demonstrate any dosage related response and a no-observed-adverse-effect level (NOAEL) at the lowest dose level. For selecting the initial dose level the dose range finding study, toxicological and toxicokinetic properties of test substance and its physico-chemical properties be considered.

Limit Test

If a test at dose of least 1000 mg/kg body weight/day produces no observed adverse effects, a full study using three dose levels may not be considered necessary except when human exposure indicates the need for a higher dose level to be used.

Dose Preparation

The test substance is usually given by mixing in diet. It may be given via gavage or in drinking water if the purpose of study and the physico-chemical properties of the test substance requires so.

Dose Administration

The animals are dosed with the test substance daily seven days each week for a period of 90 days. While administering the test substance via diet or capsule, care should be taken to maintain either constant dietary concentration (ppm) or a constant dose level in terms of animals body weight.

Observation period

Minimum 90 days. However, it may extend if recovery group is included in the study. Animals in recovery group should be observed further for a period of 28 days.

Type of observations**Clinical observations**

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. All animals are inspected atleast twice daily for signs of morbidity and mortality.

At least once prior to the first exposure and once a week thereafter, detailed clinical observations should be made in all animals. Signs of toxicity 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.

Ophthalmological examination 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.

Reactivity to stimuli of different types (e.g., auditory, visual and proprioceptive stimuli), and motor activity assessment should be conducted anytime between 11-13 weeks. However, it may be omitted when data on functional observations are available from other studies and the daily clinical observations did not reveal any functional deficits or if animals reveal such signs of toxicity that would interfere with the functional test performance.

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.

Haematology and Clinical Biochemistry

At the end of the test period, blood samples should be collected under appropriate condition just prior to or as part of the procedure for killing the animals.

Blood collection may be performed adequately in awake animals of some species using the appropriate restraint. Restraint is necessary to prevent movement that may result in laceration of a blood vessel or other organ and serious complications.

Dogs, Cats, Sheep and Calves usually require only physical restraint to collect blood.

Rabbits and Swine may require only physical restraint if they have been trained to the procedure.

Rabbits, Mice and Rats - may be placed in appropriate restraining devices.

Chemical Restraint - may be used prior to blood collection to minimize distress to the animal and to the person doing the blood collection.

Anesthesia is required to perform blood collection from the orbital sinus or by cardiac puncture because of the pain involved in the procedure and the potential for complications (including cardiac tamponade and death, or injury to the eye), even if performed by experienced personnel. Cardiac puncture is only approved for terminal blood collections unless specifically approved by the IACUC.

In addition, any better newer method will be acceptable.

The following haematological and clinical chemistry examinations should be made at the end of the test period and when any interim blood samples may have been collected. Overnight fasting of the animals prior to blood sampling is recommended.

Haematological investigations

Haematocrit, haemoglobin concentration, erythrocyte count, total and differential leukocyte count, platelet count and a measure of blood clotting time/potential e.g PT, APTT

Clinical biochemistry Investigations

Determination in serum or plasma should include:

Sodium, potassium, glucose, total cholesterol, urea, blood urea nitrogen, creatinine, total protein and albumin, Alkaline Phosphatase (ALP) Alanine aminotransferase, Aspartate aminotransferase, Gamma glutamyl transpeptidase (optional)*, Sorbitol dehydrogenase (optional)*.

* If not analyzed, then a justification to be provided based on the toxicological results of the target organ i.e. liver.

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, chloride, fasting triglycerides, specific hormones, acid/base balance, methaemoglobin and cholinesterase inhibition. 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 compound. 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.

In addition, any better newer method will be acceptable.

Urinalysis

Optionally, the following 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:

Appearance, volume, osmolality or specific gravity, pH, protein, glucose and blood/blood cells.

*Mid way urine analysis is however optional.

Pathology Gross necropsy

A detailed gross necropsy should be carried out on all animals (died or killed). This should include:

Careful examination of the external surface of the body, all orifices, and -the cranial, thoracic and abdominal cavities and their contents, liver, Gall bladder, kidneys, adrenals, testes, epididymides, uterus, thyroid (with parathyroid), thymus, spleen, brain and heart of all animals (except from those found moribund and/or intercurrently killed) should be trimmed of any adherent tissue,, and their wet weight taken as soon as possible 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, gall bladder, 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 (ophthalmological examinations to be conducted to ascertain any change. The histopathological examination of the preserved samples also required to find out the retinal damage.)

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.

All gross lesions should be examined.

When a recovery (satellite) group is used, animals of this group should be subjected to histopathology of tissues and organs identified .

Toxic Endpoint

Determination of NOAEL and LOAEL.

(Reference: OECD test Guideline 409, 1998)

REPEATED DOSE DERMAL TOXICITY STUDY

Objectives

The basic objective of the study is to obtain scientific information on possible health hazards likely to arise from repeated exposures by the dermal route over a limited period of time.

Principle of the test

The test substance is applied daily to the skin in graduated doses to several groups of experimental animals. The animals are observed daily for any signs of toxicity. Animals which die during the test are necropsied, and at the conclusion of the test the surviving animals are scarified and necropsied.

Animal Species: Rat or Rabbit

Selection of Animals: Healthy, young, adult animals with healthy skin weighing Rats: 200 – 300; Rabbits : 2 – 3 Kg and weight variation not exceeding 20 percent of the mean weight for each sex.

Housing & Feeding

Rabbits should be housed at temperature 20 ($\pm$ 3) degree C

Relative Humidity – 50-60% (However, humidity should not be below 30% or exceed 70% at any given point of time).

and 12 hours light and dark cycle.

Rats should be housed at temperature 22($\pm$ 3) degree C

Relative Humidity – 50-60% (However, humidity should not be below 30% or exceed 70% at any given point of time).

And 12 hours light and dark cycle.

Feed and water may be provided *ad libitum*.

Acclimatization: Test animals to be acclimatized to laboratory conditions for at least 5 days. Animals should be randomly assigned to various groups and each animal should be identified by a unique number.

Number of animals/Dose Group

5 male + 5 female (nulliparous and non pregnant) per dose level.

Further, an additional 10 animals (five male and five females) may be included in each control and high dose groups for determining reversibility or persistence based on the previous knowledge of the chemical or a close analogue.

Number of dose groups (including recovery group)

At least three dose levels (unless limit test is performed) and a concurrent control. The control group shall be an untreated group or a vehicle-control group if a vehicle is used in applying the test substance. The high dose level should produce toxic effects but not fatalities. The low dose level should not produce any evidence of toxicity and intermediate dose should produce some observable toxic effects.

Preparation of animals

Animals are randomized and assigned to treatment and control groups. Fur is removed from dorsal area of trunk of animals by clipping or shaving. Care should be taken not to damage or abrade the skin while clipping or shaving. If shaving is employed to remove the fur then it should be carried out 24 hrs. before the substance is applied on the skin.

Limit Test

If a test at dose of least 1000 mg/kg body weight/day produces no observed adverse effects, and if toxicity would not be expected based upon data from structurally related compound, a full study using three dose levels may not be considered necessary.

Application method

The test substance should be applied uniformly over shaven/clipped area which is approximately 10 per cent of the total body surface area. The test substances which are liquid are usually applied undiluted unless severe skin irritation necessitate its dilution. Solid test substances are pulverised (if required) and moistened with water or vehicle before applying. Liquid test substance are generally used undiluted. The test substance is held in place on the skin with a porous gauze dressing and non irritating tape. The test site should be further covered in a suitable manner to ensure that animals do not ingest the test substance.

Exposure Duration

The test substance is to be administered by topical (dermal) application to female and male test animals for 20* days 6 hours per day on a 7-day per week basis or total 20* doses for 4 weeks (except on weekends).

*As per OECD , 20 dosing are ok i.e 5 days a week in 4 week study.

Observation period: Daily during dosing period + 14 days in case of recovery groups (If considered)

Observations

Cage-side observations should include, but not be limited to, changes in skin and fur, eyes and mucous membranes and also respiratory, circulatory, autonomic and central nervous system,

somatomotor activity and behaviour pattern. Signs of toxicity, their time of onset, duration and severity should be recorded.

Moribund animals should be sacrificed when noticed. All surviving animals (except of recovery group) are sacrificed at the end of the dosing period.

Body weight and feed consumption should be recorded weekly.

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.

Clinical Biochemistry

Determination on blood should be carried out at the end of the test period. Blood parameters of liver and kidney function are appropriate. 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), urea nitrogen, albumen, blood creatinine, total bilirubin and total serum protein measurements.

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.

Pathology Gross necropsy

Full gross necropsy should include

- Examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents.
- 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:
 - normal and treated skin,
 - liver,
 - kidney and
 - target organs or any identified organ using different routes example oral showing gross lesions or changes in size.

Histopathology

Histological examination should be performed on the preserved organs and tissues of the high dose group and the control group. These examinations may be extended to animals of other dosage groups, if considered necessary to investigate the changes observed in the high dose group. 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.

Toxic Endpoint

Determination of NOAEL and LOAEL.

(Reference: OECD Test Guideline 410, 1981)

REPEATED DOSE INHALATION 28 DAY TOXICITY STUDY

Objective

To get information about the toxicity of test substance following repeated exposure for a limited period (28 days) by inhalation route to provide the data for quantitative inhalation risk-assessment.

Principal of the test

Groups of male and female animals are exposed for 6 hours per day for 28 days to test article at three or more concentration levels along with a negative and /or vehicle control group. Generally exposure is made for 5 days a week. The animals are observed daily for any signs of toxicity. Animals which die during the test are necropsied, and at the conclusion of the test the surviving animals are sacrificed and necropsied.

Animal Species: Rat is preferred. Justification should be provided if other species are used.

Age: Young adults 7 to 9 weeks of age.

Weight: Body weights should be within $\pm 20\%$ of the mean weight for each sex.

Housing and Feeding Conditions

Temperature - $22 \pm 3^\circ\text{C}$

Relative Humidity – 50-60% (However, humidity should not be below 30% or exceed 70% at any given point of time).

Lighting – 12 hours light and dark cycle

Diet and water – Standard laboratory diet specific to the species and filtered water, free from contamination

Acclimatization: To be acclimatized to lab conditions at least for 5 days.

Number of animals/ Concentration Group

5 male + 5 female (nulliparous and non pregnant) per concentration level/Group.

Further, an additional 10 animals (five male and five females) may be included in each control and high concentration groups for determining reversibility or persistence based on the previous knowledge of the chemical or a close analogue.

Number of concentration groups (including recovery group)

At least three concentration levels (unless limit test is performed) and a concurrent control. The control group shall be an untreated group or a vehicle-control group if a vehicle is used in applying the test substance. The high concentration level should produce toxic effects but not fatalities. The low concentration should not produce any evidence of toxicity and intermediate concentration level should produce some observable toxic effects.

Equipment: Any standard inhalation chamber.

Route of Exposure: Preferably via nose only. Rationale to be given in case of whole body exposure.

Administration Method

Animals are exposed to the test article as a gas, vapour, aerosol, or a mixture thereof. The physical state to be tested depends on the physico-chemical properties of the test article, the selected concentration, and/or the physical form most likely present during the handling and use of the test article.

Exposure Duration

6 hours per day on 5 days per week basis for 4 weeks (Total study duration of 28 days).

Chamber air flow

The flow of air through the chamber should be carefully controlled, continuously monitored, and recorded at least hourly during each exposure. In case of nose only exposure care should be taken to avoid rebreathing. Oxygen concentration should be at least 19% and carbon dioxide concentration should not exceed 1%. Chamber temperature should be maintained at $22\pm 3^{\circ}\text{C}$. The relative humidity should ideally be maintained in the range of 30 to 70%.

Particle size distribution

Particle sizing should be performed for all aerosols and for vapours that may condense to form aerosols. To allow for exposure of all relevant regions of the respiratory tract, aerosols with mass median aerodynamic diameters (MMAD) ranging from 1 to 4 μm are recommended. (Except for cases where it is not possible viz. metal fume particles which will be smaller or fibers or charged particles which may be larger).

Limit Test

Up to a maximum concentration of 5 mg/L for aerosols, 20 mg/L for vapors and 20,000 ppm for gases. Where above concentrations are not possible then at the maximum attainable concentration. In case of no lethality, full study using 3 concentrations is not necessary.

Main Test

The main study consists of three concentration levels, and also a negative control and/or vehicle control as needed .

Based on the previous knowledge of the chemical or a close analogue, the reversibility study may be conducted by taking additional satellite group of 10 animals (5 male & 5 Female) in high concentration and control (air or vehicle control as applicable). The animals are exposed to test substance for 6hrs. per day on a 5 day per week basis for 4 weeks. Feed should be withheld during the exposure period of 6 hrs. Water may be given during whole body exposure. The concurrent control animals should be handled in a similar manner as test Groups except that they are exposed to filtered air or air with same relative humidity (if water is used as vehicle) or with same concentration of vehicle as used in the study (in case of vehicle control).

Observation period

Daily during 28 days of dosing period + at least 14 days in recovery group (if considered).

Type of Observations

Clinical observations on daily basis before, during and after exposure for each animal, Cage-side observations should include, but not be limited to, changes in skin and fur, eyes and mucous membranes and also respiratory, circulatory, autonomic and central nervous system, somato motor activity and behavior patterns. Signs of toxicity, their time of onset, duration and severity should be recorded. Additional assessment like kinetics, biomonitoring, lung functions and behavioral changes may be included in the study.

Moribund animals should be sacrificed when noticed. All surviving animals (except of recovery group) are sacrificed at the end of the dosing period.

Body weight should be recorded before the first exposure, and then twice weekly and then at death or euthenesia. Feed consumption should be recorded weekly.

Clinical Pathology

Clinical pathology assessments should be made for all animals, including control and satellite (reversibility) animals if considered, when they are sacrificed. The time interval between the end of exposure and blood collection should be recorded, particularly when the reconstitution of the addressed endpoint is rapid. Sampling following the end of exposure is indicated for those parameters with a short plasma half-time (e.g., COHb, CHE, and MetHb).

Urinalysis is not required on a routine basis, but may be performed when deemed useful based on expected or observed toxicity.

The following haematology and clinical chemistry assessment are recommended to be carried out on all animals:

Haematology	
Erythrocyte count Haematocrit Haemoglobin concentration Mean corpuscular haemoglobin	Total leukocyte count Differential leukocyte count Platelet count Clotting potential (select one):
Mean corpuscular volume Mean corpuscular haemoglobin concentration Reticulocytes	Prothrombin time Clotting time Partial thromboplastin time
Clinical Chemistry	
Glucose* Total cholesterol Triglycerides Blood urea nitrogen Total bilirubin Creatinine Total protein Albumin Globulin	Alanine aminotransferase Aspartate aminotransferase Alkaline phosphatase Potassium Sodium Calcium Phosphorus Chloride
Urinalysis (optional)	
Appearance (colour and turbidity) Volume Specific gravity or osmolality pH	Total protein Glucose Blood/blood cells

(*) : Determination of fasting glucose may be carried out after overnight fasting during the last exposure week, or after overnight fasting prior to necropsy (in the latter case together with all other clinical pathology parameters).

In order to better characterize a test article's toxicity, additional parameters e.g., cholinesterase, lipids, hormones, acid/base balance, methaemoglobin or Heinz bodies, creatine kinase, myeloid/erythroid ratio, troponins, arterial blood gases, lactate dehydrogenase, sorbitol dehydrogenase, glutamate dehydrogenase, and gamma glutamyl transpeptidase may be carried out.

Gross Pathology and Organ Weights

All test animals, including those which die during the test or are removed from the study for animal welfare reasons, should be subjected to complete exsanguination (if feasible) and gross necropsy. The time between the end of each animal's last exposure and their sacrifice should be recorded. If a necropsy cannot be performed immediately after a dead animal is discovered, the animal should be refrigerated (not frozen) at a temperature low enough to minimize autolysis. Necropsies should be performed as soon as possible, normally within a day or two. All gross pathological changes should be recorded for each animal with particular attention to any changes in the respiratory tract.

The following organs and tissues that should be preserved in a suitable medium during gross necropsy for histopathological examination. Tissues and organs should be fixed in 10% buffered formalin or another suitable fixative as soon as necropsy is performed, and no less than 24-48 hours prior to trimming depending on the fixative to be used.

*Adrenals Bone marrow and/or fresh aspirate) *Brain (including sections of cerebrum, cerebellum, and medulla/pons) [Eyes (retina, optic nerve) and eyelids] *Heart *Kidneys Larynx (3 levels, 1 level to include the base of the epiglottis) *Liver *Lung (all lobes at one level, including main bronchi) Lymph nodes from the hilar region of the lung, especially for poorly soluble particulate test materials. For more in depth examinations and/or studies with immunological focus, additional lymph nodes may be considered, e.g. those from the mediastinal, cervical/submandibular and/or auricular regions. Nasopharyngeal tissues (at least 4 levels; 1 level to include the nasopharyngeal duct and the Bronchus Associated Lymphoid Tissue(BALT)) Oesophagus [Olfactory bulb] Ovaries	Seminal vesicles Spinal cord (cervical, mid- thoracic, and lumbar) *Spleen Stomach *Testes *Thymus Thyroid Trachea (at least 2 levels including 1 longitudinal section through the carina and 1 transverse section) [Urinary bladder] Uterus All gross lesions
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*. The organs should be trimmed and weighed as soon as possible after dissection to avoid drying.
 () The matter in paranthesis are Optional.

The lungs should be removed intact, weighed, and instilled with a suitable fixative at a pressure of 20-30 cm of water to ensure that lung structure is maintained Sections should be collected for all lobes at one level, including main bronchi, but if lung lavage is performed, the unlavaged lobe should be sectioned at three levels (not serial sections).

At least 4 levels of the nasopharyngeal tissues should be examined, one of which should include the nasopharyngeal duct, to allow adequate examination of the squamous, transitional (non-ciliated respiratory), respiratory (ciliated respiratory) and olfactory epithelium, and the draining lymphatic tissue Three levels of the larynx should be examined, and one of these levels should include the base of the epiglottis . At least two levels of the trachea should be examined including one longitudinal section through the carina of the bifurcation of the extrapulmonary bronchi and one transverse section.

Histopathology

A histopathological evaluation of all the organs and tissues should be performed for the control and high concentration groups, and for all animals which die or are sacrificed during the study.

Particular attention should be paid to the respiratory tract, target organs, and gross lesions. The organs and tissues that have lesions in the high concentration group should be examined in all groups. The study director may choose to perform histopathological evaluations for additional groups to demonstrate a clear concentration response. When a satellite (reversibility) group is used, histopathological evaluation should be performed for all tissues and organs identified as showing effects in the treated groups. If there are excessive early deaths or other problems in the high exposure group that compromise the significance of the data, the next lower concentration should be examined histopathologically. An attempt should be made to correlate gross observations with microscopic findings.

Toxic Endpoint

Determination of NOAEL and LOAEL.

(Reference: OECD Test Guideline 412, 2009)

NEUROTOXICITY STUDIES

Acute Neurotoxicity - Rodent

Repeated Dose Neurotoxicity – Rodent

Delayed Neurotoxicity – OP Compound – Acute Exposure

Delayed Neurotoxicity – OP Compound – Repeated Administration

Developmental Neuro Toxicity (DNT) Study

ACUTE NEUROTOXICITY – RODENT

Objective

In the assessment and evaluation of the potential human health effects of substances, it may be necessary to test for neurotoxic effects. Substances that have been observed to cause neurotoxic signs (e.g., convulsions, tremors, ataxia) in other toxicity tests, as well as those having a structural similarity to known neurotoxicants, should be evaluated for neurotoxicity.

Principle of the test

The material is administered by an appropriate route to laboratory rodents. The animals are observed under carefully standardized conditions with sufficient frequency to ensure the detection of behavioral and/or neurologic abnormalities, if present. Various functions that could be affected by neurotoxicants are assessed during each observation period.

Concurrent or historic data from the laboratory performing the testing shall provide evidence of the ability of the procedures used to detect major neurotoxic endpoints such as limb weakness or paralysis (e.g., acrylamide), CNS stimulation (e.g., β , β' -iminodipropionitrile) autonomic signs (e.g., physostigmine).

Any significant effects on behavior (delayed effects), histopathology, or inhibition of NTE in the acute study are sufficient cause to conduct the 28-day study.

Test animal: Rat is preferred rodent species. Young adult healthy animal should be used in the study. The females should be nulliparous and non- pregnant.

Age & weight of animal: Young adult between 6 to 9 weeks. The weight variation should not exceed $\pm 20\%$ of mean weight of each sex.

Housing and feeding

The temperature in animal room should be $22\pm 3^{\circ}\text{C}$

Relative Humidity – 50-60% (However, humidity should not be below 30% or exceed 70% at any given point of time).

Lighting should be artificial with 12 hrs light and 12 hrs dark cycle. Animal should be provided with conventional laboratory diet and water ad- libitum.

Acclimatization: Test animals to be acclimatized to the laboratory conditions for at least 5 days prior to the test

Route of administration: Usually oral, mixed in the diet or through gavage or in capsule.

Administration method

Administer the test substance in a single dose by gavage. However, if it is not possible to administer the compound in a single dose, it may be given in smaller fractions over a period not exceeding 24 hours and justification for the deviation should be given.

Number of animal per dose level

At least 8 animals of each sex should be used for each dose level and a concurrent control group (“sham” exposure or vehicle) is required. Subjects shall be treated in the same way as for an exposure group except that administration of the test substance is omitted.

Dose selection & dose levels

For the acute study, a single exposure group is required. The acute dose level should be chosen to maximize the amount of material, particularly in cases where some activity is expected. Selection of the dose level for the acute study may be based on a limit dose or lethal doses and other available data, e.g. on NTE inhibition.

Observation period

All animals should be observed prior to initiation of exposure. Subsequent observations should be made with sufficient frequency to ensure the detection of behavioral and/or neurologic abnormalities, if present. At minimum, observations at 1 hour, 6 hours, 24 hours, 7 days, and 14 days and monthly thereafter are recommended.

Observations and examination

The following is a minimal list of observations that shall be noted:

(A) Any unusual responses with respect to body position, activity level, coordination of movement, and gait.

- (B) Any unusual or bizarre behavior including, but not limited to, headflicking, head searching, compulsive biting or licking, self-mutilation, circling, and walking backwards. Among the variables that can affect behavior are sound level, temperature, humidity, lighting, odors, time of day, and environmental distractions.
- (C) The presence of Convulsions, Tremors, Increased levels of lacrimation and/or red-colored tears, Increased levels of salivation, Piloerection, Pupillary dilation or constriction, Unusual respiration (shallow, labored, dyspneic, gasping, and retching and/or mouth breathing), Diarrhea, Excessive or diminished urination and Vocalization.
- (D) Forelimb/hindlimb grip strength
- (E) Sensory function.

All test animals should be subjected to gross necropsy to record gross pathological findings. Histopathology may be considered in the organs showing significant adverse effects.

Result assessment

The findings of the study should be evaluated in terms of the presence or absence, incidence and severity, of any neurotoxic effects. The numerical results should be evaluated by an appropriate and generally acceptable statistical method.

(Reference: OPPTS, 870.6100, 1998)

REPEATED DOSE NEUROTOXICITY – RODENT

Objective

The objective of this study is to obtain information whether the nervous system is permanently or reversibly affected by the chemical tested and to characterize the nervous system alterations associated with exposure to the chemical, and to understand the underlying mechanism. This study will provide information on dose-and time-response relationship in order to estimate a no-observed adverse effect level (which can be used to establish safety criteria for the chemical).

Principle of the test

The test chemical is administered by oral route across a range of doses to several groups of rats for a period of 90 days. The animals are tested for determination of behavioral and/or neurological abnormalities. At the end of the test, subsets of animal of each sex from each group are perfused in situ and section of brain, spinal cord, and peripheral nerves are prepared and examined.

Test animal: Rat is preferred rodent species. Young adult healthy animal should be used in the study. The females should be nulliparous and non- pregnant.

Age & weight of animal: Young adult between 6 to 9 weeks. The weight variation should not exceed $\pm 20\%$ of mean weight of each sex.

Housing and feeding

The temperature in animal room should be $22\pm 3^{\circ}\text{C}$

Relative Humidity – 50-60% (However, humidity should not be below 30% or exceed 70% at any given point of time).

Lighting should be artificial with 12 hrs light and 12 hrs dark cycle. Animal should be provided with conventional laboratory diet and water *ad- libitum*.

Acclimatization: Test animals to be acclimatized to the laboratory conditions for at least 5 days prior to the test

Route of administration: Usually oral, mixed in the diet or through gavage or in capsule.

Number of animal per dose level

At least 20 animals (10 males and 10 females) should be used for each dose level and control group. (Please see note at the end).

Dose selection & dose levels

Dose levels should be selected by taking into account any previously observed toxicity and kinetic data available for the test compound or related materials. The highest dose level should be chosen

with the aim of inducing neurotoxic effects or clear systemic toxic effects. Thereafter, a descending sequence of dose levels should be selected with a view to demonstrating any dose-related response and a no-observed-adverse effect (NOAEL) at the lowest dose level. At least three dose groups and a control group should be used. While deciding the dose selection, if no suitable data exist then a range- finding study may be performed.

Limit Test

If a study at a dose level of 1000mg/kg body weight per day using the procedures described, produces no observable neurotoxic effects and if toxicity would not be expected based upon data from structurally related compounds, then a full study using three doses level may not be considered necessary.

Main Test (Number of animals, dose levels etc)

The test substance is administered daily for a period of 90 days. When the study is conducted as a separate study, at least 20 animals (10 females and 10 males) should be used in each dose and control group for the evaluation of detailed clinical and functional observations. At least five males and five females, selected from these 10 males and 10 females, should be perfused *in situ* and used for detailed neuro-histopathology at the end of the study. When the study is conducted in combination with a repeated dose toxicity study, adequate numbers of animals (at least 15 males and 15 females) should be used to meet the objectives of both studies.

At least three dose groups and a control group should generally be used.

Observation period

In repeated dose studies, the observation period should cover the dosage period. For animals in satellite groups which are kept without exposure during a post-treatment period, observations should cover this period as well. Table indicates the frequency of observations for various parameters.

Observations and examination

Type of observations		Study duration (90 days)
In all animals	General health condition	Daily
	Mortality/ morbidity	Twice daily
In animal selected for functional observations	Detailed clinical observations	- Prior to first exposure - Once during the first or second week of exposure - Monthly, thereafter - Detailed observation in satellite recovery group at the end of the recovery period.

Type of observations		Study duration (90 days)
	Functional tests	- Prior to first exposure - Once during the first or second week of exposure - Monthly, thereafter

Clinical observations: Clinical observations should include but not limited to changes in skin, fur, eyes, mucus membranes, occurrence of secretions and excretions and autonomic activity. Further, any unusual responses to body positions, activity level, co-ordination of movement, changes in gait, posture, reactivity to handling, placing or other environmental stimuli, clonic or tonic movement, tremors, convulsions, stereotypes or bizarre behavior or aggression should be recorded.

Functional tests

Functional tests should include sensory reactivity to stimuli of different modalities [e.g., auditory, visual and proprioceptive stimuli], assessment of limb grip strength and assessment of motor activity. (strength, coordination) for e.g landing foot splay, rotarod, righting reflex, auditory startle, learning component with repeated testing viz. Passive avoidance, olfactory, conditioning, Morris water maze, Biel or Cincinnati maze, radial arm maze and T-maze.

Body weight and food/water consumption

For studies up to 90 days duration, all animals should be weighed at least once a week and measurements should be made of food consumption (water consumption, when the test substance is administered by that medium) at least weekly.

Ophthalmology

Ophthalmological examination, using an ophthalmoscope or an equivalent suitable instrument, should be made prior to the administration of the test substance and at 13 weeks of the study, preferably on all animals but at least in animals of high dose and control groups.

Hematology and Clinical Biochemistry

When the neurotoxicity study is carried out in combination with a repeated dose systemic toxicity study, hematological examinations and clinical biochemistry determinations should be carried out as set out in the respective guideline of the systemic toxicity study.

Histopathology

Tissues from at least 5 animals/sex/group should be fixed in situ, using generally recognized perfusion and fixation techniques. The neuropathological examination should be performed in high dose group and the control group. If neuropathological alterations are observed in the high dose group,

samples from each of the potentially affected tissues from the intermediate and low dose groups should then be coded and examined sequentially. Representative sections of central and peripheral nervous system should be examined. Normally it includes the forebrain, the centre of the cerebrum, a section through hippocampus, the midbrain, the cerebellum, the pons, the medulla oblongata, the eye with optic nerve and retina, the spinal cord at cervical and lumbar swellings, dorsal root ganglia, dorsal and ventral root fibres, the proximal sciatic nerve, the proximal tibial nerve and tibial nerve calf muscle branches. The spinal cord and peripheral nerve sections should include both cross or transverse and longitudinal sections.

Result assessment

The findings of the study should be evaluated in terms of the incidence, severity and correlation of neurobehavioral and neuropathological effects and any other adverse effects observed. The numerical results should be evaluated by an appropriate and generally acceptable statistical method.

Note: This test protocol can be combined with existing protocol/ test guidelines for repeated dose toxicity studies or be carried out as a separate study. When it is combined with test guidelines for repeated dose toxicity study, attention should be paid to include sufficient number of animals in the study so as to satisfy the requirements of the observations of both studies.

(Reference: OECD Test Guideline 424, 1997)

DELAYED NEUROTOXICITY – OP COMPOUND

ACUTE EXPOSURE

Objective

The objective of this study is to obtain information on possible delayed neurotoxicity likely to arise from acute exposure. This study will provide information on certain classes of substances to cause specific types of neurotoxicity that might not be detected in other toxicity studies.

Principle of the test

The test substance is administered orally in a single dose to domestic hens. The animals are observed for behavioral abnormalities, ataxia and paralysis for 21 days. Neuropathy target esterase (NTE) estimation (24 and 48 hrs after dosing) and histopathology of neural tissue (after 21 days) is undertaken.

Test animal: Young Adult domestic laying hen (*Gallus gallus domesticus*) free from any viral infection and without abnormality of gait.

Weight/Age of animal: 8 – 12 months/standard size

Housing and feeding

Cages should be large enough for free mobility of hens so that gait can be observed clearly. Appropriate diet and free supply of drinking water should be given.

Acclimatization: Test animals to be acclimatized to the laboratory conditions for at least 5 days prior to the test.

Route of administration: Normally oral route using gavage or gelatine capsules is used.

Number of dose: Single oral administration is given to all test animals.

Dose selection/dose level

The dose selected should be based on preliminary dose selection study and should be as high as possible with upper limit of 2000 mg/kg body weight. Care should be taken to prevent mortality due to acute cholinergic effects by administering atropine or any other protective drug which does not interfere with delayed neurotoxic response.

Preliminary Dose Selection Study

With the objective to maximize the dose to be used in the main study, a preliminary study using an appropriate number of hens and dose levels should be conducted. Some lethality is necessary in the study to define an adequate main study dose.

Limit Test

If no observable toxic effects are encountered at a dose level of 2000mg/kg body weight per day and the toxicity is not expected based upon data from structurally related compounds then the higher doses need not to be tested except when human exposure indicates the need for a higher dose level to be used.

Main Test (Number of animals, dose levels etc)

One treatment, one vehicle control group and a positive control group should be used. The treatment group and the vehicle control group should contain a sufficient number of hens so that six can be killed for biochemical determinations (three at each of two time points) and six survive the 21 day observation period and can be used for pathology.

Observation period

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 they are killed according to schedule. Observations should start immediately after treatment begins.

Observations and examination

Clinical: All signs of toxicity should be recorded, including the time of onset, type, severity and duration of behavioral 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. Any moribund hens should be removed and killed and subjected to gross necropsy.

Body weight: All hens should be weighed just prior to administration of the test substance and at least once a week thereafter.

Biochemistry

Three hens from each treatment and vehicle control group are killed after 24 hours and three at 48 hours after the last dose. Three hens of positive control group should also be killed after 48 hrs. The brain and lumbar spinal cord and sciatic nerve tissue should be prepared and assayed for NTE as well as acetyl cholinesterase (AChE) on these samples.

Pathology 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.

Histopathology

Neural tissue of all survived animal which are not used for biochemical studies should be subjected for histopathology.

Sections from the following tissues should be taken for staining with appropriate myelin and axon-specific stains after fixing the tissues in situ, using perfusion techniques

- Cerebellum (mid- longitudinal level)
- Medulla oblongata
- Spinal cord (upper cervical segment, mid- thoracic and lumbo-sacral region)
- Tibial nerve (distal region) and its branches to gastronemial muscle
- Sciatic nerve

Result assessment

The findings of this study should be evaluated in terms of the incidence, severity, and correlation of behavioral, 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.

Toxic Endpoint:

Behavioral : Sign/symptoms of neurobehavioral toxicity

Biochemical : Decreased AChE and decreased NTE activity

Histopathological : Lesion in nervous system

(Reference: OECD Test Guideline 418, 1995)

DELAYED NEUROTOXICITY – OP COMPOUND

REPEATED ADMINISTRATION

Objective

The objective of this study is to obtain information on possible delayed neurotoxicity likely to arise from repeated exposures over a limited period of time. This study 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. Usually the delayed neuropathic potential of repeated exposure is determined after acute tests.

Principle of the test

The daily dose of the test substance is administered orally to domestic hens for 28 days. The animals are observed daily for behavioral abnormalities, ataxia and paralysis for 14 days after last dose. Biochemical measurements specially (NTE) are done on randomly selected hens from each group (24 and 48 hrs after last dose). After 14 days observation, remainders of the hens are killed and histopathological examination of selected neural tissue is undertaken.

Test animal: Adult domestic laying hen (*Gallus gallus domesticus*). The animals should be healthy, free from any viral disease and any abnormalities of gait.

Age of animal: 8 – 12 months

Number of animals

Sufficient number of animals should be used for each dose level and for control to ensure that 6 birds be killed for biochemical estimation and six other survive the 14 day post- treatment observation period for undertaking pathology.

Housing and feeding

Hens should be kept in cages large enough to permit free mobility and easy observation of their gait. If light is artificial then 12 hours light and 12 hrs dark cycle should be maintained. Appropriate diet and free supply of drinking water should be given.

Acclimatization: Test animals to be acclimatized to the laboratory conditions for at least 5 days prior to the test.

Route & duration of administration of test substance

The test substance should be administered orally preferably by gavage or in gelatin capsules Daily, 7 days per week for 28 days,

Dose levels

At least three dose levels and a vehicle control group should be used.

Dose selection

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 no observable toxic effects are encountered at a dose level of 1000mg/kg body weight per day and the toxicity is not expected based upon data from structurally related compounds then the higher doses need not to be tested except when human exposure indicates the need for a higher dose level to be used.

Observation period

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. Observations should start immediately after treatment begins.

Observations and examination

Observations should include, but not be limited to behavioral 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. Any moribund hens should be removed and killed and subjected to gross necropsy. All signs of toxicity - their onset, type, severity and duration should be recorded.

Body weight: All hens should be weighed just prior to administration of the test substance and at least once a week thereafter.

Biochemistry

Three birds of the control and each treatment group are killed after 24 hours and three at 48 hours after the last dose.

The brain and lumbar spinal cord and sciatic nerve tissue should be prepared and assayed for NTE as well as acetyl cholinesterase (AChE) on these samples.

Pathology 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.

Histopathology

Neural tissue of all surviving animals not used for biochemical study be fixed in situ using perfusion technique and sections be taken for histopathology examination for Brain (cerebellum, medulla oblongata), spinal cords (upper cervical, mid- thoracic and lumbo-sacral regions) and peripheral nerves (distal region of tibial nerve and its branches to gastro-nemial muscle, sciatic nerve) of high dose and control group animals. If high dose group shows some evidence of toxicity then hen of intermediate and low dose group should also be examined.

Result assessment

The findings of this study should be evaluated in terms of the incidence, severity, and correlation of behavioral, 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.

Toxic Endpoint

Determination of NOAEL

(Reference: OECD Test Guideline 419, 1995)

DEVELOPMENTAL NEUROTOXICITY – RODENT

Objective

The study is to provide data, including dose response characterization, on the potential functional and morphological effects on developing nervous system of the offspring that may arise from exposure in utero and during early life.

Principle of the test

The test substance is administered to animal during gestation and lactation. Dams and randomly selected offspring are evaluated for neurotoxicity which consist of physical development, behavioral ontogeny, motor activity, motor and sensory function, learning and memory, and the evaluation of brain weight and neuropathology during postnatal development and adulthood.

Test animal: The preferred test species is Rat.

Age of animal: Young adult nulliparous pregnant females

Housing & feeding

The animal rooms should have temperature $22\pm3^{\circ}\text{C}$ and relative humidity 50-60% (not exceeding below 30% or above 70%), artificial light sequence of 12 hours light & 12 hours dark. Animals should be housed individually or in small groups of same sex. Mating procedure should be carried out in the cages suitable for the purpose. Conventional laboratory diet and free supply of drinking water should be given.

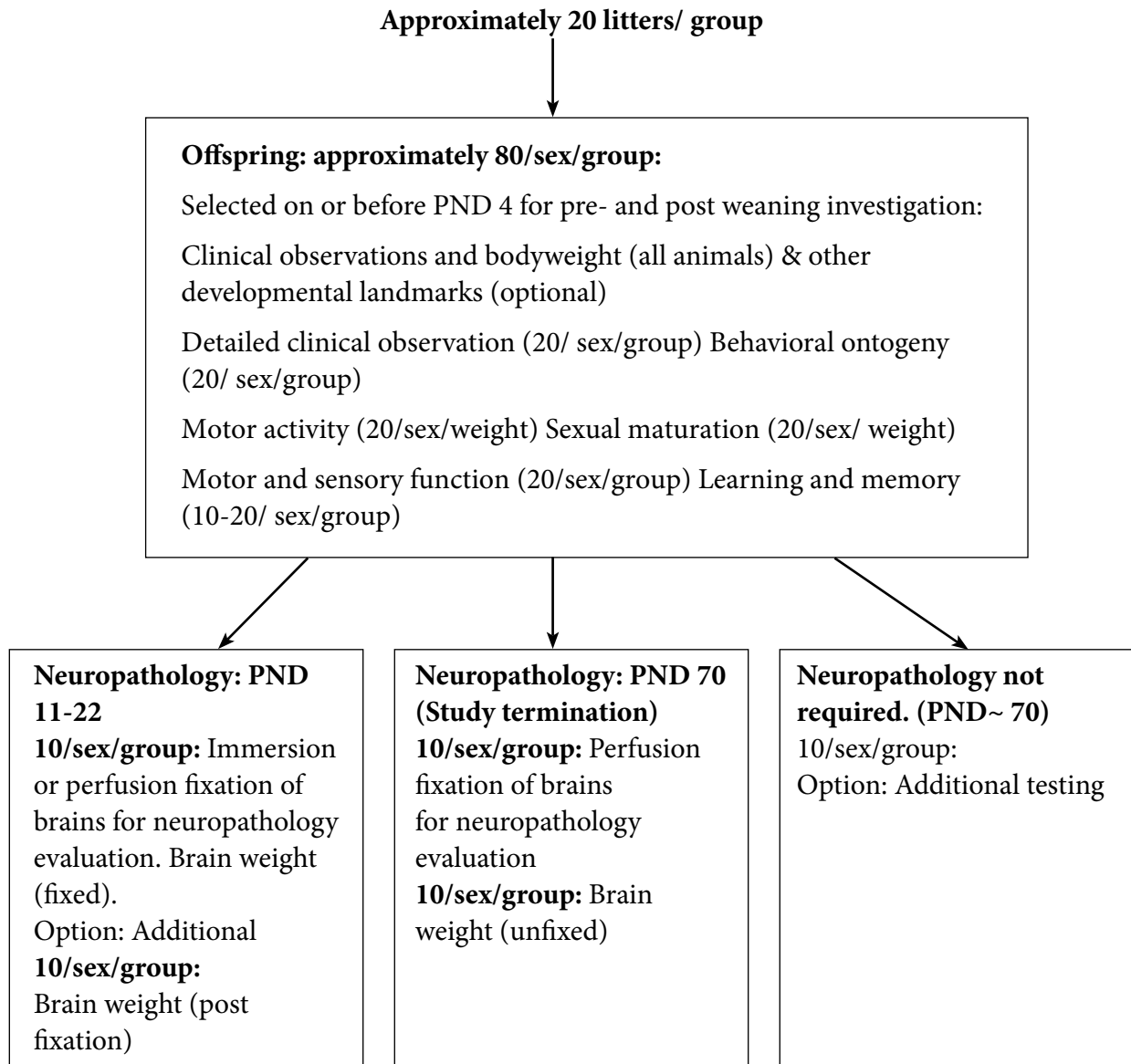
Acclimatization: Test animals to be acclimatized to the laboratory conditions for at least 5 days prior to the test

Procedure

Number and sex of animals

Each test and control group should contain a sufficient number of pregnant females to be exposed to the test substance to ensure that an adequate number of offsprings are produced for neurotoxicity evaluation. A total of 20 litters are recommended at each dose level. The pups are randomly selected from each dose group and assigned for endpoint assessment on or after post natal day (PND) 4 (day of delivery is PND 0). Selection of pups should be performed so that equal numbers of males and female from each dose group are obtained for all tests. For motor activity testing the same pair of male and female pups should be tested at all pre- weaning ages. For all other tests the same or separate pairs of male and female animals may be assigned to different behavioral tests. Different pups may need to be assigned to weanling versus adult tests of cognitive function in order to avoid confounding the effects of age and prior training on these measurements. At weaning (PND 21), pups not selected for testing can be disposed of humanely.

There are different ways to assign pups to the pre-weaning and post-weaning examination, cognitive tests, pathological examination etc. The general testing scheme for functional/ behavioral tests, neuropathology evaluation and brain weights is as shown below:



Recommended minimum numbers of animals in each dose group for pre weaning and post weaning examination are:

Clinical observations and body weight

All animals

Detailed clinical observations	20/sex (1/sex/litter)
Brain weight (post fixation) PND 11-22	10/Sex (1/ Litter)
Brain weight (unfixed) PND-70	10/Sex (1/ Litter)
Neuropathology (immersion or perfusion fixation) PND 11-22	10/Sex (1/ Litter)
Neuropathology (perfusion fixation) PND 70	10/Sex (1/ Litter)
Sexual maturation	20/sex (1/sex/litter)

Other developmental landmarks (optional)

all animals

Behavioral ontogeny	20/sex (1/sex/litter)
Motor activity	20/sex (1/sex/litter)
Motor and sensory function	20/sex (1/sex/litter)
Learning and memory	10/sex (1/litter)

Dosage

At least three dose levels and a concurrent control should be used. The highest dose level should be chosen with the aim to induce some maternal toxicity. The high dose level may be limited to 1000 mg/kg/day /body weight unless expected human exposure may indicate the need for a higher dose level may be used. A descending sequence of dose levels should be selected with a view to demonstrate any dose related response and a No- Observed- Adverse Affect Level (NOAEL).

Administration of doses

The route of administration will generally be oral (e. g. gavage, dietary, via drinking water) , but other routes (e.g. dermal, inhalation) may be used depending on the characteristics and anticipated or known human exposure routes. Justification should be provided for the route of administration chosen. The test substance should be administered at approximately the same time every day.

The test substance be administered daily to mated females from the time of implantation (GD 6) throughout lactation (PND 21) , so that the pups are exposed to the test substance during pre- and postnatal neurological development. In general, it is assumed that exposure of the pups will occur through the maternal milk; however, direct dosing of pups should be considered in those cases where there is a lack of evidence of continued exposure to offspring. Evidence of continuous exposure can be retrieved from e.g. pharmacokinetic information, offspring toxicity or changes in bio- makers.

Observations

Observations on dams

1. All dams should be carefully observed at least once daily with respect to their health condition, including morbidity and mortality.
2. A detailed clinical observation should be conducted periodically at least twice during gestational dosing period and twice during the lactation dosing period by trained technician who are unaware of animal treatment during treatment and observation period.
3. Clinically Observed signs of toxicity with magnitude (whenever feasible) should be recorded which includes:
 - Changes in skin, fur, eyes, mucus membranes.
 - Occurrence of secretions
 - Autonomic activity like lacrimation, piloerection, pupil size, unusual respiratory pattern and/or mouth breathing, any unusual sign of urination or defecation etc.
 - Unusual response with respect to body position, activity level, co-ordination of movement.
 - Changes in gait and posture
 - Reactivity to handling, placing or other environmental stimuli.
 - Presence of clonic or tonic movements, convulsions, tremors, stereotypes, bizarre behavior or aggression .
4. Body weight should be taken at the time of dosing, weekly throughout the study, on day of delivery, PND 21 (weaning)
5. Food and water consumption should be measured weekly during gestation and lactation.

Observation of offspring

1. All offspring should be examined at least daily for signs of toxicity, mortality or morbidity.
2. A detailed clinical observation of the offspring (at least one pup/sex/litter) should be conducted by trained technicians who are unaware of animal treatment, during treatment and observation period.
3. Clinical observation includes same as those described for dam and should be monitored as appropriate for the developmental stage.
4. Any gross signs of toxicity in the offspring should be recorded as they are observed, including the time of onset, degree, and duration.

Physical and developmental landmarks

Body weight may be the best indicator of physical development as many developmental landmarks (e.g. pinna unfolding, eye opening, incisor eruption) are highly correlated with it. Measurement of developmental landmarks is therefore, recommended only when there is prior evidence that these endpoints will provide additional information. Table below presents the minimum number of times when measurement should be performed. If required, additional time points at the other developmental stages may be added.

Age Periods Endpoints	Pre- weaning (b)	Adolescence (b)	Young adult (b)
Physical and developmental landmarks			
Body weight and clinical observations	Weekly (c)	At least every two weeks	At least every two weeks
Brain weight	PND 22 (d)		At termination
Neuropathology	PND 22 (d)		At termination
Sexual maturation	--	As appropriate	--
Other developmental landmarks (e)	As appropriate	--	--
Functional/ behavioural end points			
Behavioural ontogeny	At least two measures		
Motor activity (including habituation)	1-3 times	--	once
Motor and sensory function	--	Once	Once
Learning and memory	--	Once	Once

- (b) It is recommended that pups not be tested during the two days after weaning. Recommended ages for adolescent testing are: learning and memory= PND 25 $\pm$ 2; motor and sensory function=PND 25 $\pm$ 2. Recommended ages for testing young adult is PND 60-70.
- (c) Body weight should be measured at least twice weekly when directly dosing pups for adjustment of doses at a time of rapid body weight gain.
- (d) Brain weight and neuropathology may be assessed at some earlier time (e.g PND 11), if appropriate

- (e) Other developmental landmarks in addition to the body weight (e.g. eye opening) should be recorded when appropriate.

Behavioral ontogeny

Ontogeny of selected behavior should be measured in at least one pup/sex/litter during appropriate age period. Some of the behaviors for which their ontogeny could be assessed are righting reflex, negative geotaxis and motor activity

Motor activity

Motor activity should be monitored during the pre-weaning and adult age periods. Use of motor activity to measure behavioral ontogeny is strongly recommended and if so, then testing should utilize the same animals for all pre-weaning test sessions, day of weaning and at an adult age close to study termination (PND- 70). Motor activity should be monitored by an automated activity recording apparatus.

Motor and sensory function

It should be examined in detail at least once for the adolescent period and once during the young adult period (e.g. PND 60-70). The testing should ensure an adequate quantitative sampling of sensory modalities (e.g. somato-sensory, vestibular) and motor functions (e.g. strength, coordination). Example of motor and sensory functions are extensor thrust response, righting reflex, auditory startle habituation and evoked potentials.

Learning and memory tests

A test of associative learning and memory should be conducted post-weaning (e.g. 25±2 days) and for young adult (PND 60 and older).

Learning should be assessed either as a change across several repeated learning trials or sessions or in tests involving a single trial, with reference to a condition that controls for non-associative effects of the training experience. Learning component with repeated testing viz. Passive avoidance, olfactory, conditioning, Morris water maze, Biel or Cincinnati maze, radial arm maze and T-maze.

1. The test should include some measure of memory (short-term or long-term) in addition to original learning (acquisition)

Post-mortem examination

Maternal animal can be euthanized and discarded after weaning of the offspring.

Neuropathological evaluation of the offspring will be conducted using tissues from animal humanely killed at PND 22 or at an earlier time point between PND 11 and PND 22 as well as at study termination. For offspring humanely killed through PND 22, brain tissue should be evaluated; for animal humanely killed at termination, both brain and peripheral nervous system (PNS) tissue

should be evaluated. All gross abnormalities should be noted. Tissue samples from all major regions of the nervous system be taken and retained in appropriate fixative and processed for histological examination.

Neuropathological examination

The purpose of neuropathological (qualitative) examination includes:

- (i) Identification of regions within the nervous system exhibiting evidence of neuropathological alterations;
- (ii) Identification of types of neuropathological alterations resulting from exposure to the test substance;
- (iii) Determination the range of severity of the neuropathological alteration.

Representative histological sections from the tissue samples should be examined microscopically for evidence of the neuropathological alterations.

Morphometric (quantitative) evaluation should be performed as its data are valuable in the interpretation of treatment- related differences in brain weight or morphology. It may include linear or areal measurements of specific brain regions.

Brain should be examined using adequate samples taken from all major brain regions (e.g. olfactory bulbs, cerebral cortex, hippocampus, basal ganglia, thalamus, midbrain, pons, medulla oblongata and the cerebellum) taken in same plane to ensure a thorough examination.

Significant alteration indicative of developmental insult include:

- Alterations in the gross size or shape of the olfactory bulbs, cerebrum or cerebellum.
- Alterations in the relative size of various brain regions, including decreases or increases in the size of regions resulting from the loss or persistence of normally transient population of cells or axonal projections (e. g external germinal layer of cerebellum, corpus callosum)
- Alterations in proliferation, migration, and differentiation, as indicated by areas of excessive apoptosis or necrosis, clusters or dispersed population of ectopic, disoriented or malformed neurons or alterations in the relative size of various layers of cortical structures
- Alterations in pattern of myelination, including an overall size reduction or altered staining of myelinated structures
- Evidence of hydrocephalus, in particular enlargement of the ventricles, stenosis of the cerebral aqueduct and thinning of the cerebral hemispheres.
- For dose-response relationship of neuropathological alterations, first sections from high dose group are compared with those of control group. If no evidence of neuropathological alterations is found then no further analysis is required. If evidence of alterations found in high dose group, then animals from intermediate and low dose groups are examined.

Result assessment

The evaluation should include

1. The relationship between the doses of the test substance and the presence or absence, incidence, and extent of any developmental neurotoxic effect and NOAEL.
2. The relationship, if any, between observed neuropathological and behavioral alterations.
3. Appropriate statistical analyses

(Reference: OECD Test Guideline 426, 2007)

LONG TERM TOXICITY STUDIES

Carcinogenicity

Chronic Toxicity

Combined Chronic Toxicity and Carcinogenicity

CARCINOGENICITY

Objective

The objective of the test is to identify carcinogenic properties of test substance; identification of target organ(s) of carcinogenicity; characterization of tumor dose:response relationship and establishment of No Observed Adverse Effect Level (NOAEL) or Benchmark Dose (BMD) and extrapolate carcinogenic effect/ data regarding mode of action.

A combined chronic toxicity and carcinogenicity study, in lieu of separate chronic toxicity and carcinogenicity study are acceptable..

Principle of the test

The test substance is administered orally daily in graduated doses to several groups of test animals for majority of their life span. The animals are observed closely for signs of toxicity and for development of neoplastic lesions. 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.

Test animals: Rat/Mice (preferred species Rat)

Age/Sex: Young healthy adult (<8 weeks old), both male & female (Nulliparous & non pregnant)

Weight of the test animal: At the commencement of the study, the weight variation of the animal should be minimal and not exceed $\pm 20\%$ of the mean weight of all the animal within the study, separately for each sex.

Housing and feeding

Rats should be housed individually or in small groups of same sex at temperature $22(\pm 3)^{\circ}\text{C}$ and Relative Humidity – 50-60% (However, humidity should not be below 30% or exceed 70% at any given point of time) with 12 hours light and dark cycle. They should be supplied with conventional laboratory diet and unlimited supply of drinking water.

Acclimatization: Test animals to be acclimatized to the laboratory conditions for at least 7 days prior to the test.

Number of test animal

A sufficient number of animals should be used so that a thorough biological and statistical evaluation is possible. Each dose group and concurrent control group should therefore contain at least 50 animals of each sex. Each animal should be assigned a unique identification number which should be permanently marked with this number by a suitable method.

Dose group

At least three dose levels and a concurrent control (non-treated or vehicle treated) based on short term repeated toxicity studies or Toxicokinetics data of the product should be used. In case study design provides for interim kill, 10 animals per sex may be included in each dose group.

Route of administration: The test substance is normally administered orally, by gavage or via the diet or drinking water.

Duration of study

The animals are dosed with test substance daily for 24 months (rat) or 18 months (mice). Termination of the study should be considered when the number of survivors in the lower dose groups or the control group falls below 25%. Survival of each sex would normally be expected to be 50% or greater in the top dose groups to permit a robust evaluation of carcinogenic potential.

Observations

All animals should be checked for morbidity or mortality and for specific signs of toxicological relevance usually at the beginning and the end of each working day and at least once in weekend/holiday. Particular attention should be paid to tumour development; the time of onset, location, dimensions, appearance, and progression of each grossly visible or palpable tumour should be recorded.

Body weight: All animals should be weighed at the start of treatment, at least once a week for the first 13 weeks and at least monthly thereafter.

Hematology & Clinical Biochemistry Analysis

To obtain maximum information from the study, blood samples can also be collected for hematological and clinical biochemical investigations. Blood smear may also be prepared for examination, particularly if bone marrow appears to be the target organ. Though blood examination is not obligatory, at the end of study, prior to sacrificing of animals blood samples may be taken for examination.

Pathology Gross necropsy

All animals in the study should be subjected to a full, detailed gross necropsy which include careful examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal

cavities and their contents. Organ weight data can also found to be useful when there is development of tumors.

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, Adrenal gland, Aorta , Brain (including section of cerebrum, cerebellum & medulla/pons), Caecum, Cervix, Coagulating gland, Colon, Duodenum, Epididymis, Eye (including retina), Harderian gland, Heart, Ileum, Jejunum, Kidney, Lacrimal gland, Liver, Lung, Lymph nodes(both superficial & deep), Female's mammary gland, Oesophagus, Ovary , Pancreas, Parathyroid gland, Peripheral nerve, Pituitary, Prostate, Salivary gland, Seminal vesicle, Skeletal muscle, Skin, Spinal cord (at three levels: cervical, mid- thoracic & lumbar), Spleen, Stomach, Testis, Thymus, Thyroid gland, Trachea, Urinary bladder, Uterus, Vagina, Section of bone marrow and/or a fresh 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.

Histopathology

Tissues to be examined for histopathology must include:

- All tissues from the high dose and control groups;
- All tissues of animals dying or killed during the study;
- All tissues showing macroscopic abnormalities including tumours;
- When treatment- related histopathological changes are observed in high dose group, those same tissues are to be examined from all animals in all other dose groups;
- In the case of paired organ, e.g. kidney, adrenal, both organs should be examined.

Result assessment

Identification of separate NOAEL and LOAEL for toxicity parameters as well as for carcinogenicity.

(Reference: OECD Test Guideline: 451, 2009)

NOTE : ORGAN WEIGHT MAY BE EXEMPTED IN CARCINOGENICITY, HOWEVER, INFORMATION ON ORGAN WEIGHT MAY BE PROVIDED IN CHRONIC & COMBINED (CHRONIC & CARCINOGENICITY) STUDY.

CHRONIC TOXICITY

Objective

The objective of the study is to identify the -toxic properties of the test chemical along with its target organ(s), characterization of dose response relationship with the establishment of NOAEL or Bench mark Dose (BMD) and provision of data regarding probable mode of action. The study provides information on the possible health hazards likely to arise from repeated exposure over a considerable part of the entire lifespan in rodents.

A combined chronic toxicity and carcinogenicity study, in lieu of separate chronic toxicity and carcinogenicity study are acceptable.

Principle of the test

The test substance is administered preferably orally (dermal or inhalation, if relevant for human exposure) daily in graduated doses to several groups of test animals preferably for a period of 12 months. 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.

Test animals: The preferred species is Rat. Mice may be used. The animals of same strain and source as used in preliminary toxicity studies of shorter duration be preferably used.

Age/Sex: Young healthy adult (<8 weeks old), both male & female (Nulliparous & non pregnant)

Weight of the test animals: At the commencement of the study, the weight variation of the animal should be minimal and not exceed $\pm 20\%$ of the mean weight of all the animal within the study, separately for each sex.

Housing and feeding

Rats should be housed individually or in small groups of the same sex in cages at temperature 22 (± 3)° C

Relative Humidity – 50-60% (However, humidity should not be below 30% or exceed 70% at any given point of time).

Lighting with 12 hours light and dark cycle. The animals may be provided with conventional laboratory diets with an unlimited supply of drinking water.

Acclimatization: Test animals to be acclimatized to the laboratory conditions for at least 7 days prior to the test. Animals should be randomly assigned to the control and treatment groups. Each animal should be assigned a unique identification number and this number be marked on animal permanently by any suitable method.

Number of test animals

A sufficient number of animals should be used so that a thorough biological and statistical evaluation is possible. Each dose group and concurrent control group should therefore contain at least 20 animals of each sex. The study may make provision for interim kills (at 6 months) to obtain information on progression of toxicological changes, if required or inclusion of satellite group (usually at highest dose level) to monitor the reversibility of any toxicological changes or inclusion of group of sentinel animals (5 animals/ sex) to monitor the disease status, if required.

Dose group

At least three dose levels and a concurrent control (untreated or vehicle treated) should be used. The highest dose level should produce evidence of toxicity but not severe suffering or morbidity or death. Dose level spacing should be such that it demonstrates dose : response to establish NOAEL.

Route of administration: The test substance is normally administered orally, by gavages or via the diet or drinking water.

Duration of study

The duration of the study will normally be 12 months for rats. The study design can also be allowed and can be applied for either shorter or longer duration with proper justification. The satellite group if included in the study for reversibility monitoring, then animals should be kept without dosing for a period of 4 weeks.

Observations

All animals should be checked for morbidity or mortality and for specific signs of toxicological relevance, usually at the beginning and the end of each day.

General clinical observations should be made at least once a day. Detailed clinical observations should be made on all animals at least once prior to the first exposure, at the end of the first week of the study and monthly thereafter. 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, stereotypies (e.g. excessive grooming, repetitive circling) or bizarre behaviour (e.g. self mutilation, walking backwards) should also be recorded.

Ophthalmological examination should also be conducted in all animals prior to the administration of the test substance & at least in the high dose and control group at termination of the study. In case any changes / toxicity observed in high dose group then other groups should also be examined.

If the test chemical indicated the potential to cause neurotoxic effects and immunotoxic effect in previous repeated dose 28 day and/or 90 day toxicity test, then further investigations should be conducted at 12 months.

Body weight: All animals should be weighed at the start of treatment, at least once a week for the first 13 weeks, and at least monthly thereafter.

Note:

Animal Welfare Approach: *“In studies involving repeated dosing, when an animal shows clinical signs that are progressive, leading to further deterioration in condition, an informed decision as to whether or not to humanely kill the animal should be made. The decision should include consideration as to the value of the information to be gained from the continued maintenance of that animal on study relative to its overall condition. If a decision is made to leave the animal on test, the frequency of observations should be increased, as needed. It may also be possible, without adversely affecting the purpose of the test, to temporarily stop dosing if it will relieve the pain or distress, or reduce the test dose.”*

Hematological Investigations

Hematological examinations should be carried out in at least 10 male and 10 females per group, at 3, 6 and 12 months, using the same animal throughout. The parameters to be investigated are: total & differential leukocyte count, erythrocyte count, platelet count, hemoglobin concentration, hematocrit, red cell indices (MCV, MCH & MCHC), prothrombin time and activated partial thromboplastin time. If the chemical has an effect on haematopoietic system, reticulocyte count and bone marrow cytology may also be undertaken.

Clinical Biochemistry Investigations

Clinical biochemical examinations should be conducted to investigate major toxic effects in tissues and, specifically, effects on kidney and liver, on overnight fasting blood samples obtained from at least 10 males and 10 females per group at the same time intervals as specified for hematological investigations, using the same animals throughout. The parameters to be investigated are: glucose, urea, creatinine, total protein, albumin, calcium, sodium, potassium, total cholesterol, SGOT, SGPT, ALP, GGT and total bilirubin. Other parameters like fasting triglycerides, specific hormones may also be appropriate depending on the toxicity of the substance.

Urinalysis

Examinations should be carried out in at least 10 male and 10 females per group, at the same interval as for hematological and biochemical investigations using the same animal throughout. These investigations need not be conducted if no effects were seen on these parameters in a previous 90 day study carried out at comparable dose levels. The parameters to be included are: appearance, volume, specific gravity, pH, total protein and glucose. Further parameters may be employed where necessary to extend the investigations of observed effects.

Pathology

Gross necropsy: All animals in the study should be subjected to a full, detailed gross necropsy which include careful examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents. Organ weights should be collected from all animals for adrenals, brain, epididymis, heart, kidneys, liver, ovaries, spleen, testes, thyroid and uterus.

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, Adrenal gland, Aorta, Brain (including section of cerebrum, cerebellum & medulla/pons), Caecum, Cervix, Coagulating gland, Colon, Duodenum, Epididymis, Eye (including retina), Harderian gland, Heart, Ileum, Jejunum, Kidney, Lacrimal gland, Liver, Lung, Lymph nodes(both superficial & deep), Female's mammary gland, Oesophagus, Ovary, Pancreas, Parathyroid gland, Peripheral nerve, Pituitary, Prostate, Salivary gland, Seminal vesicle, Skeletal muscle, Skin, Spinal cord (at three levels: cervical, mid- thoracic & lumbar), Spleen, Stomach, Testis, Thymus, Thyroid gland, Trachea, Urinary bladder, Uterus, Vagina, Section of bone marrow and/or a fresh 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.

Histopathology

Tissues to be examined for histopathology must include:

- All tissues from the high dose and control groups;
- All tissues of animals dying or killed during the study;
- All tissues showing macroscopic abnormalities;
- When treatment- related changes are observed in high dose group in target tissues, those same tissues are to be examined from all animals in all other dose groups;
- In the case of paired organ, e.g. kidney, adrenal, both organs should be examined.

Result assessment

Identification of NOAEL and LOAEL

(Reference: OECD Test Guideline: 452, 2009)

COMBINED CHRONIC TOXICITY AND CARCINOGENICTY

Objective

The objective of the study is to identify the carcinogenic properties of the test chemical, namely its potential to induce neoplastic lesions, time of appearance of neoplastic lesions and its chronic toxicity along with its target organ(s), characterization of dose response relationship with the establishment of NOAEL or BMD of a test substance by an appropriate route of administration. Also to provide data to test hypothesis regarding mode of action of substance.

Principle of the test

The test substance is administered orally daily in graduated doses to several groups of test animals for majority of their life span, normally of two year duration for carcinogenicity phase & one year for chronic toxicity phase. The animals in both phase are observed closely for signs of toxicity and for development of neoplastic lesions. 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.

Test animals: Rat/Mice (preferred species Rat)

Age/Sex: Young healthy adult (< 8 weeks old), both male & female (Nulliparous & non pregnant)

Weight of the test animal: At the commencement of the study, the weight variation of the animal should be minimal and not exceed $\pm 20\%$ of the mean weight of all the animal within the study, separately for each sex.

Housing and feeding

Rats should be housed at temperature $22(\pm 3)^{\circ}\text{C}$

Relative Humidity – 50-60% (However, humidity should not be below 30% or exceed 70% at any given point of time).

Lighting with 12 hours light and dark cycle. Conventional laboratory diet may be used with unlimited supply of drinking water.

Acclimatization: Test animals to be acclimatized to the laboratory conditions for at least 7 days prior to the test.

Number of test animal

A sufficient number of animals should be used so that a thorough biological and statistical evaluation is possible. Each dose group and concurrent control group should therefore contain at least 50 animals of each sex. An additional 2 satellite groups of 20 animals of each sex, one high dose group and one control group, should be included for evaluation of chronic toxicity and non- neoplastic pathology at 12 month.

Dose group

At least three dose levels and a concurrent control should be used. A limit of 1000mg/kg body weight/day may apply except when human exposure indicates the need for higher dose level to be used. The highest dose level should elicit evidence of toxicity while avoid suffering, severe toxicity, morbidity or death. Dose level spacing may be selected to establish a dose-response which in turn depends on objective of the study and characteristics of test substance.

Route of administration: The test substance is normally administered orally usually via the diet or drinking water. If any other route/mode is chosen then justification should be given.

Duration of the study

The duration of chronic phase of the study is normally 12 months. The high dose and control satellite groups will be terminated at this stage for evaluation of chronic toxicity and non-neoplastic pathology. The duration of the carcinogenicity phase of the study will be 24 months for rodents, representing the majority of the normal life span of the animals to be used. Survival of each sex would normally be expected to be 50% or greater in the top dose groups to permit a robust evaluation of carcinogenic potential.

Satellite groups included for reversibility monitoring should be maintained without dosing for a period of 4 weeks and not more than 1/3rd of total study duration after cessation of exposure. If the number of survivors in the lower dose groups or the control group falls below 25% then the study should be terminated. Survival for each sex should be considered separately.

Observations

Chronic toxicity phase

All animals should be checked for morbidity or mortality and for specific signs of toxicological relevance, usually at the beginning and the end of each day.

General clinical observations should be made at least once a day.

Detailed clinical observations should be made on all animals at least once prior to the first exposure, at the end of the first week of the study and monthly thereafter. 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, stereotypies (e.g. excessive grooming, repetitive circling) or bizarre behaviour (e.g. self mutilation, walking backwards) should also be recorded.

Ophthalmological examination should also be conducted in all animals prior to the administration of the test substance and at least in the high dose and control group at termination of the study.

If the test chemical indicated the potential to cause neurotoxic effects and immunotoxic effect in previous repeated dose 28 day and/or 90 day toxicity test, then further investigations should be conducted at 12 months.

Body weight

All animals should be weighed at the start of treatment, at least once a week for the first 13 weeks, and at least monthly thereafter.

Food /Water consumption and food efficiency

Measurements of food consumption and food efficiency should be made at least weekly for the first 13 weeks and at least monthly thereafter. Water consumption should be measured at least weekly for the first 13 weeks and at least monthly thereafter when the substance is administered in drinking water or if substances . Water consumption measurements should also be considered for studies in which drinking activity is altered.

Hematological Investigations

Hematological examinations should be carried out in at least 10 male and 10 females per group, at 3, 6 and 12 months, using the same animal throughout. Blood should be collected from a named site viz. cardiac puncture or from retro-orbital sinus, under anaesthesia. The investigations at three months, need not be conducted if no effects were seen on hematological parameters in a previous 90 day study carried out at comparable dose levels. The parameters to be investigated are: total & differential leukocyte count, erythrocyte count, platelet count, hemoglobin concentration, hematocrit, red cell indices (MCV, MCH & MCHC), prothrombin time and activated partial thromboplastin time. If the chemical has an effect on haematopoietic system, reticulocyte count and bone marrow cytology may also be indicated or other parameters like Heinz bodies, typical enythrocytes or methaemoglobin may be measured, if appropriate, depending on toxicity of the test substance.

Clinical Biochemistry Investigations

Clinical biochemical examinations should be conducted to investigate major toxic effects in tissues and, specifically, effects on kidney and liver, on overnight fasting blood samples obtained from at least 10 males and 10 females per group at the same time intervals as specified for hematological investigations, using the same animals throughout. These investigations at 3 months need not be conducted if no effects was seen on biochemical parameters in a previous 90 day study carried out at comparable dose levels. The parameters to be investigated are: glucose, urea, creatinine, total protein, albumin, calcium, sodium, potassium, total cholesterol, SGOT, SGPT, ALP, GGT and total bilirubin. Other parameters like fasting triglycerides, specific hormones may also be appropriate, depending on the toxicity of the substance.

Urinalysis

Examinations should be carried out in at least 10 male and 10 females per group, at the same interval as for hematological and biochemical investigations using the same animal throughout. These investigations need not be conducted at three months if no effects was seen on these parameters in a previous 90 day study carried out at comparable dose levels. The parameters to be included are: appearance, volume, specific gravity, pH , total protien and glucose. Further parameters may be employed where necessary to extend the investigations of observed effects.

Pathology

Gross necropsy

All animals in the study should be subjected to a full, detailed gross necropsy which include careful examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents.

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, Adrenal gland, Aorta, Brain (including section of cerebrum, cerebellum & medulla/pons), Caecum, Cervix, Coagulating gland, Colon, Duodenum, Epididymis, Eye (including retina), Harderian gland, Heart, Ileum, Jejunum, Kidney, Lacrimal gland, Liver, Lung, Lymph nodes(both superficial & deep), Female's mammary gland, Oesophagus, Ovary, Pancreas, Parathyroid gland, Peripheral nerve, Pituitary, Prostate, Salivary gland, Seminal vesicle, Skeletal muscle, Skin, Spinal cord (at three levels: cervical, mid- thoracic & lumbar), Spleen, Stomach, Testis, Thymus, Thyroid gland, Trachea, Urinary bladder, Uterus, Vagina, Section of bone marrow and/or a fresh 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.

Histopathology

Tissues to be examined for histopathology must include:

- All tissues from the high dose and control groups;
- All tissues of animals dying or killed during the study;
- All tissues showing macroscopic abnormalities;
- When treatment- related changes are observed in high dose group in target tissues, those same tissues are to be examined from all animals in all other dose groups;
- In the case of paired organ, e.g. kidney, adrenal, both organs should be examined.

Carcinogenicity phase

All animals should be checked for morbidity or mortality and for specific signs of toxicological relevance usually at the beginning and the end of each working day. Particular attention should be paid to tumor development; the time of onset, location, dimensions, appearance, and progression of each grossly visible or palpable tumor should be recorded.

Body weight

All animals should be weighed at the start of treatment, at least once a week for the first 13 weeks and at least monthly thereafter.

Food/Water consumption and food efficiency

Measurements of food consumption and food efficiency should be made at least weekly for the first 13 weeks and at least monthly thereafter. Water consumption should be measured at least weekly for the first 13 weeks and at least monthly thereafter when the substance is administered in drinking water or if substances . Water consumption measurements should also be considered for studies in which drinking activity is altered.

Hematology & Clinical Biochemistry and other measurements

To obtain maximum information from the study, blood samples can also be collected for hematological and clinical biochemical investigations. Data on the animals used in chronic toxicity phase of the study, normally of 12 months duration will provide information on these parameters. If blood samples are taken, these should be collected at the end of the test period, just prior to or as part of the procedure for killing the animals.

Pathology

Gross necropsy

All animals in the study should be subjected to a full, detailed gross necropsy which include careful examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents. Organ weight data can also found to be useful when there is development of tumours. Sentinel animals and other satellite animals may require necropsy on a case-by-case basis. The development of tumours confounds the usefulness of organ weight data, though it is not mandatory.

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, Adrenal gland-ileum, Aorta-peripheral nerve testis, Brain (including sections of cerebrum, cerebellum & medulla/pons), Caecum, Cervix, Coagulating gland (exorbital), rectum, Colon, Duodenum, Epididymis, Eye (including retina), Harderian gland, Heart, Ileum, Jejunum, Kidney, Lacrimal gland, Liver, Lung, Lymph nodes(both superficial & deep), Female's mammary gland, coagulating gland, Oesophagus, Ovary , Pancreas, Parathyroid gland, Peripheral nerve, Pituitary, Prostate, Salivary gland, trachea, Seminal vesicle, Skeletal muscle, Skin, Spinal cord (at three levels: cervical, mid- thoracic & lumbar), Spleen, Stomach, Testis, Thymus, Thyroid gland, Trachea, Urinary bladder, Uterus, Vagina, skin, Section of bone marrow and/or a fresh bone marrow aspirate. Gall bladder (for species other than rat). 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 know properties of the test substance should be preserved.

Histopathology

Tissues to be examined for histopathology must include:

- All tissues from the high dose and control groups;
- All tissues of animals dying or killed during the study;
- All tissues showing macroscopic abnormalities including tumours;
- When treatment- related histopathological changes are observed in high dose group, those same tissues are to be examined from all animals in all other dose groups;
- In the case of paired organ, e.g. kidney, adrenal, both organs should be examined.

Result assessment

Identification of separate NOAEL and LOAEL for toxicity parameters as well as for carcinogenicity.

(Reference: OECD Test Guideline: 453, 2009)

DEVELOPMENTAL & REPRODUCTION TOXICITY STUDIES (DART)

Developmental Toxicity Study - Rat

Developmental Toxicity Study - Rabbit

Two Generation Reproduction Toxicity Study

Extended One Generation Reproduction Toxicity Study

DEVELOPMENTAL TOXICITY STUDY - RAT

Objective

The objective of the study is to provide general information concerning the effects of prenatal exposure of test substance on the pregnant test animal and on the developing organism; this may include assessment of maternal effects as well as death, structural abnormalities, or altered growth in the foetus.

Principle of the test

The test substance is administered daily to pregnant animals at least from implantation to one day prior to the day of scheduled kill, which should be as close as possible to the normal day of delivery without risking loss of data resulting from early delivery. Shortly before caesarean section, the females are killed, the uterine contents are examined, and the fetuses are evaluated for soft tissue and skeletal changes.

Test animal: The preferred rodent species is the rat. Justification should be provided if another species is used. Healthy animals should be used that have not been subjected to previous experimental procedures. Animals should be characterized as to strain, source, sex, weight and/or age. The animals of all test groups should be of uniform weight and age. Young adult nulliparous female animals should be used at each dose level. The females should be mated with males of the same species and strain, and the mating of the siblings should be avoided.

Housing and feeding

Rats should be housed at temperature $22\pm 3^{\circ}\text{C}$

Relative Humidity – 50-60% (However, humidity should not be below 30% or exceed 70% at any given point of time).

Lighting with 12 hours light and dark cycle. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. Mating procedure should be carried out in cages suitable for the purpose.

Acclimatization: Test animals to be acclimatized to the laboratory conditions for at least 5 days prior to the test.

Number of test animals

Each test and control group should contain a sufficient number of females to result in approximately 20 female animals with implantation sites at necropsy. Groups with fewer than 16 animals with implantation sites may be inappropriate. Maternal toxicity does not necessarily invalidate the study providing it does not exceed approximately 10 percent.

Dose group

At least three dose levels and a concurrent control (sham treated or vehicle control) should be used. The highest dose should induce some developmental and/or maternal toxicity but not death or severe suffering. Intermediate dose should produce minimum observable toxic effects, while the lowest dose level should not produce any evidence of maternal or developmental toxicity. In case vehicle is used to dissolve the test substance then concurrent control should be treated with vehicle. It should be ensured that the vehicle used does not have any effect on developmental toxicity or on reproduction.

Duration of Dosing of Animals

Daily from implantation (i.e. day 5 post mating or Administration of dose should be given from the day of implant i.e. 6th day) till the day prior to scheduled caesarean section. (6th-20th day) and sacrifice on 21st day.

Route of administration: The test substance is normally administered orally by intubation.

Care should be taken to avoid unnecessary handling of animals as well as stress from outside factors like noise etc. to guard against foetal loss due to these factors.

Limit Test

If no observable toxic effects are encountered at a dose level of 1000mg/kg body weight per day and the toxicity is not expected based upon data from structurally related compounds then the higher doses need not to be tested except when human exposure indicates the need for a higher dose level to be used.

Observations

Dams: Clinical observations should be made and recorded at least once a day, preferably at the same time. The condition of the animals should be recorded including mortality, morbidity, pertinent behavioural changes, and all signs of overt toxicity.

Weight: should be recorded on day 0 (or no later than day 3 if time mated animals are supplied by breeder); on 1st day of dosing and every 3 days during dosing and on day of scheduled kill.

Food: at every three day interval and should coincide with body weight recording.

Post-mortem examination: Females should be killed one day prior to the expected day of delivery. Females showing signs of abortion or premature delivery prior to scheduled kill should be killed and subjected to a thorough macroscopic examination.

At the time of termination or death during the study, the dam should be examined macroscopically for any structural abnormalities or pathological changes. Evaluation of the dams during caesarean section and subsequent foetal analysis should be conducted preferably without knowledge of treatment group in order to minimize bias.

Examination of uterine contents: Immediately after termination or as soon as possible after death, the uteri should be removed and the pregnancy status of the animals ascertained. Uteri that appear non- gravid should be further examined to confirm the non-pregnant status.

Gravid uteri including the cervix should be weighed. Gravid uterine weights should not be obtained from animals found dead during the study.

The number of corpora lutea should be determined for pregnant animals.

The uterine contents should be examined for numbers of embryonic or foetal deaths and viable fetuses. The degree of resorption i.e. Early resorption (evidence of implantation without recognisable embryo/foetus) & Late resorption (dead embryo or foetus with external degenerative changes) should be described in order to estimate the relative time of death of the conceptus and thus the important factor to estimate the degree of developmental toxicity.

Examination of foetuses

1. The sex and body weight of each foetus should be examined.
2. Each foetus should be examined for external alterations.
3. Foetus should be examined for skeletal and soft tissue alterations (e. g. variations and malformations or anomalies). Approximately one-half of each litter should be prepared and examined for skeletal alterations (stain bone with alizarin or cartilage as well with Alcian Blue) and remainders should be prepared and examined for soft-tissue alterations. (Wilson sectioning) Categorization of foetal alterations is preferable but not required. When categorization is done, the criteria for defining each category should be clearly stated. Particular attention should be paid to the reproductive tract which should be examined for signs of altered development.

Result assessment

Identification of NOAEL and LOAEL values for - maternal, fetal and developmental effects.

(Reference: OECD Test guideline 414, 2001)

DEVELOPMENTAL TOXICITY STUDY – RABBIT

Objective

The objective of the study is to provide general information concerning the effects of prenatal exposure of test substance on the pregnant test animal and on the developing organism; this may include assessment of maternal effects as well as death, structural abnormalities, or altered growth in the foetus.

Principle of the test

The test substance is administered daily to pregnant animals at least from implantation to one day prior to the day of scheduled kill, which should be as close as possible to the normal day of delivery without risking loss of data resulting from early delivery. Shortly before caesarean section, the females are killed, the uterine contents are examined, and the fetuses are evaluated for soft tissue and skeletal changes.

Test animal: The preferred non rodent species is the Rabbit. Justification should be provided if another species is used. Healthy animals should be used that have not been subjected to previous experimental procedures. Animals should be characterized as to strain, source, sex, weight and/or age. The animals of all test groups should be of uniform weight and age. Young adult nulliparous female animals should be used at each dose level. The females should be mated with males of the same species and strain, and the mating of the siblings should be avoided.

Housing and feeding

Rabbits should be housed at temperature $18 \pm 3^{\circ}\text{C}$

Relative Humidity – 50-60% (However, humidity should not be below 30% or exceed 70% at any given point of time).

Lighting with 12 hours light and dark cycle. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. Mating procedure should be carried out in cages suitable for the purpose.

Acclimatization: Test animals to be acclimatized to the laboratory conditions for at least 5 days prior to the test.

Number of test animals

Each test and control group should contain a sufficient number of females to result in approximately 20 female animals with implantation sites at necropsy. Group having less than 16 animals with implantation sites may not be appropriate for evaluation. Maternal toxicity does not necessarily invalidate the study providing it does not exceed approximately 10 percent.

Dose group

At least three dose levels and a concurrent control (sham treat or vehicle treated) should be used. The highest dose should induce some developmental and/or maternal toxicity but not death or severe suffering. Intermediate dose should produce minimum observable toxic effects, while the lowest dose level should not produce any evidence of maternal or developmental toxicity. In case vehicle is used to dissolve the test substance then concurrent control should be treated with vehicle. It should be ensured that the vehicle used does not have any effect on developmental toxicity or on reproduction.

Duration of Dosing of Animals

Daily from implantation (i.e. day 5 post mating or Administration of dose should be given from the day of implant i.e. 6th day) till the day prior to scheduled caesarean section and sacrifice on 28th day.

Route of administration: The test substance is normally administered orally by intubation.

Care should be taken to avoid unnecessary handling of animals as well as stress from outside factors like noise etc. to guard against fatal loss due to these factors.

Limit Test

If no observable toxic effects are encountered at a dose level of 1000mg/kg body weight per day and the toxicity is not expected based upon data from structurally related compounds then the higher doses need not to be tested except when human exposure indicates the need for a higher dose level to be used.

Observations

Dams: Clinical observations should be made and recorded at least once a day, preferably at the same time. The condition of the animals should be recorded including mortality, morbidity, pertinent behavioural changes, and all signs of overt toxicity.

Weight : should be recorded on day 0 (or no later than day 3 if time mated animals are supplied by breeder); on 1st day of dosing and every 3 days during dosing and on day of scheduled kill.

Food : at every three day interval and should coincide with body weight accordingly.

Post-mortem examination: Females should be killed one day prior to the expected day of delivery. Females showing signs of abortion or premature delivery prior to scheduled kill should be killed and subjected to a thorough macroscopic examination.

At the time of termination or death during the study, the dam should be examined macroscopically for any structural abnormalities or pathological changes. Evaluation of the dams during caesarean section and subsequent foetal analysis should be conducted preferably without knowledge of treatment group in order to minimize bias.

Examination of uterine contents: Immediately after termination or as soon as possible after death, the uteri should be removed and the pregnancy status of the animals ascertained. Uteri that appear non-gravid should be further examined to confirm the non-pregnant status.

Gravid uteri including the cervix should be weighed. Gravid uterine weights should not be obtained from animals found dead during the study.

The number of corpora lutea should be determined for pregnant animals.

The uterine contents should be examined for numbers of embryonic or foetal deaths and viable fetuses. The degree of resorption i.e. Early resorption (evidence of implantation without recognisable embryo/foetus) and Late resorption (dead embryo or foetus with external degenerative changes) should be described in order to estimate the relative time of death of the conceptus and thus the important factor to estimate the degree of developmental toxicity.

Examination of foetuses

1. The sex and body weight of each foetus should be examined.
2. Each foetus should be examined for external alterations.
3. All Foetus should be examined for skeletal and soft tissue alterations (e. g. variations and malformations or anomalies). The bodies of these foetuses are evaluated by careful dissection for soft tissue alterations (Wilson sectioning), which may include procedures to further evaluate internal cardiac structure. The heads of one-half of the foetuses examined in this manner should be removed and processed for evaluation of soft tissue alterations (including eyes, brain, nasal passages and tongue), using standard serial sectioning methods or an equally sensitive method. The bodies of these foetuses and the remaining intact foetuses should be processed and examined for skeletal alterations (stain bone with alizarin or cartilage as well with Alcian Blue). Categorization of foetal alterations is preferable but not required. When categorization is done, the criteria for defining each category should be clearly stated. Particular attention should be paid to the reproductive tract which should be examined for signs of altered development.

Result assessment

Identification of NOAEL and LOAEL values for maternal, fetal and developmental effects.

(Reference: OECD Test guideline 414, 2001)

TWO GENERATION REPRODUCTION TOXICITY STUDY

Objective

The objective of the study is to provide general information concerning the effects of a test substance on the integrity and performance of the male and female reproductive systems which includes gonadal function, the oestrous cycle, mating behaviour, conception, gestation, parturition, lactation and weaning, and on the growth & development as well as integrity and performance of the offspring.

Principle of the test

The test substance is administered in graduated doses to several groups of males and females during growth, at least one complete spermatogenic cycle for males and several complete oestrous cycles for females, during their mating, during the resulting pregnancies, and through the weaning of their offspring. At weaning, the administration of the test substance is continued their offspring during their growth into adulthood, mating and production of new generation, until this new generation is weaned. Clinical observation and pathological examinations are performed on all animals for the signs of toxicity with special emphasis on effects on the integrity and performance of their reproductive system and on growth and development of the offspring.

Test animals: The rat is the preferred species.

Housing and feeding

Rats should be housed at temperature $22(\pm 3)^{\circ}\text{C}$

Relative Humidity – 50-60% (However, humidity should not be below 30% or exceed 70% at any given point of time).

Lighting with 12 hours light and dark cycle. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. Mating procedure should be carried out in cages suitable for the purpose.

Age and weight: The Parental animals of all test groups should be of about 5 to 9 weeks old & of uniform weight at the starting of dosing. The females should be mated with males of the same species and strain, and the mating of the siblings should be avoided.

Acclimatization: Young Healthy animals acclimatized to the laboratory conditions for at least 5 days prior to the test.

Number of test animals

Each test and control group should contain a sufficient number of animals to yield preferably not less than 20 pregnant females at or near parturition.

Dose group

At least three dose levels and a concurrent control should be used.

Route of administration: The test substance is normally administered orally by diet, drinking water or gavage.

Limit Test

If no observable toxic effects are encountered at a dose level of 1000mg/kg body weight per day and the toxicity is not expected based upon data from structurally related compounds then the higher doses need not to be tested except when human exposure indicates the need for a higher dose level to be used.

Procedure

1. Daily dosing of Parental (P) males and females should begin when they are 5 to 9 weeks old and should continue for 10 weeks before mating period, during the 2 weeks mating period. Then, males should be humanely killed and examined when they are no longer needed for assessment of reproduction effects whereas females should be continued with dosing throughout pregnancy and up to weaning of the F1 offspring. Treatment of the P males and females should continue until termination (when no longer needed for assessment of reproduction effects).
2. Daily dosing of F1 generation animals should be begin at weaning and continued for 10 weeks before mating period, during the 2 weeks mating period. Then, males should be humanely killed and examined when they are no longer needed for assessment of reproduction effects whereas females should be continued with dosing throughout pregnancy and up to weaning of the F2 offspring. Treatment of the F1 males and females should continue until termination (when no longer needed for assessment of reproduction effects).
3. F1 offspring not selected for mating and all F2 offspring should be humanely killed after weaning.
4. Mating procedure: Each female should be placed with a single male from the same dose level (1:1 mating) until copulation occurs or 2 weeks have elapsed. Each day, the females should be examined for the presence of sperm or vaginal plugs. The day a vaginal plug or sperms are found should be considered as day 0 of pregnancy. Mating pair should be clearly identified. If no mating, swap with other animals to determine if male or female is at fault. Mating of siblings should be avoided.

Observations

Clinical observations

Behavioral changes, sign of difficult or prolonged parturition and all signs of toxicity should be recorded. An additional or more detailed examination of each animal should be conducted on at least a weekly basis. All animals should be observed for morbidity and mortality.

Body weight and food/water consumption of parent animals

Parental animals (P & F1) shall be weighed on the first day of dosing and at least weekly thereafter. Parental females (P & F1) shall be weighed at a minimum on gestation days 0, 7, 14 and 20 or 21, and during lactation on the same days as the weighing of litters and on the day the animals were killed. During the pre-mating and gestation periods food consumption shall be measured weekly at a minimum. Water consumption shall be measured weekly at a minimum if the test substance is administered in the water.

Oestrous cycle

Oestrous cycle length and normality are evaluated in P and F1 females by vaginal smears prior to mating, and optionally during mating, until evidence of mating is found.

Sperm parameters

For all P and F1 males at termination, testis and epididymis weight shall be recorded and one of each organ reserved for histopathological examination. Of a subset of at least ten males of each group of P and F1 males, the remaining testes and epididymides should be used for enumeration of homogenization-resistant spermatids and cauda epididymis sperm reserves, respectively. For this same subset of males, sperm from the cauda epididymides or vas deferens should be collected for evaluation of sperm motility and sperm morphology.

The total number of homogenization-resistant testicular spermatids and cauda epididymal sperm should be enumerated. Cauda sperm reserves can be derived from the concentration and volume of sperm in the suspension used to complete the qualitative evaluation, and the number of sperm recovered by subsequent mincing and/or homogenizing of the remaining cauda tissue. In these instances, the controls and high dose group may be analysed first. If no treatment related effects (e.g. effects on sperm count, motility or morphology) are seen the other dose groups need not be analysed. When treatment-related effects are noted in the high dose group, then the lower dose groups should also be evaluated.

A morphological evaluation of an epididymal (or vas deferens) sperm sample should be performed. Sperm (at least 200 per sample) should be examined as fixed, wet preparation and classified as either normal or abnormal.

Offspring

Each litter should be examined as soon as possible after delivery (lactation day 0) to establish the number and sex of pups, stillbirths, live births, and the presence of gross anomalies.

Physical development of the offspring should be recorded mainly by body weight gain. Other physical parameters (e.g. ear and eye opening, tooth eruption, hair growth) may give supplementary information, but these data should preferably be evaluated in the context of data on sexual maturation (e.g. age and body weight at vaginal opening or balano-preputial separation. The age of vaginal opening and preputial separation should be determined for F1 weanling selected for mating. Anogenital distance should be measured at postnatal day 0 in F2 pups if triggered by alterations in F1 sex ratio or timing of sexual maturation.

Gross necropsy

At the time of termination or death during the study, all parental animals (P & F1), all pups with external abnormalities or clinical signs, as well as at least one randomly selected pups/sex/litter from both the F1 and F2 generation, shall be examined macroscopically for any structural abnormalities or pathological changes.

Organ weights

At the time of termination, body weight and the weight of the following organs of all P and F1 parental animals shall be determined (paired organs should be weighed individually):

- Uterus, ovaries
- Testes, epididymis (total and cauda)
- Prostate
- Seminal vesicles with coagulating glands and their fluids
- Brain, liver kidney, spleen, pituitary thyroid and adrenal glands and known target organs

Histopathology

Parental animals

The following organs and tissues of parental (P and F1) animals, or representative samples thereof, shall be fixed and stored in a suitable medium for histopathological examination.

- Vagina, uterus with cervix, and ovaries (preserved in an appropriate fixative)
- One testis (preserved in bouin's or comparable fixative), one epididymis, seminal vesicles, prostate and coagulating gland
- Previously identified target organs from all P and F 1 animals selected for mating.

Full histopathology of the preserved organs and tissue listed above should be performed for all high dose and control P and F1 animals selected for mating..

Detailed testicular histopathological examination (e.g. using bouin's fixative, paraffin embedding and transverse section of 4-5 µm thickness) should be conducted in order to identify treatment-related effects such as retained spermatids, missing germ cell layers or types, multinucleated giant cells or sloughing of spermatogenic cells into the lumen.

Weanlings

Grossly abnormal tissue and target organs from all pups with external abnormalities or clinical signs shall be fixed and stored in a suitable medium for histopathological examination.

Result assessment

Identification of separate NOAEL and LOAEL for toxicity parameters as well as for reproduction, parturition, lactation, pre- and postnatal developmental including growth and sexual development.

(Reference: OECD TEST GUIDELINE: 416, 2001)

EXTENDED ONE GENERATION REPRODUCTION TOXICITY STUDY

Objective

The objective of the study is to evaluate specific life stages not covered by other types of toxicity studies and test for effects that may occur as a result of pre-and postnatal chemical exposure. The Extended One-Generation Reproductive Toxicity Study then serves as a test for reproductive endpoints that require the interaction of males with females, females with conceptus, and females with offspring and the F1 generation until after sexual maturity.

Principle of the test

The test substance is administered continuously in graduated doses to several groups of sexually-mature males and females. This parental (P) generation is dosed for a defined pre-mating period (selected based on the available information for the test substance; but for a minimum of two weeks) and a two-week mating period. P males are further treated at least until weaning of the F1. They should be treated for a minimum of 10 weeks. Treatment of the P females is continued during pregnancy and lactation until termination after the weaning of their litters (i.e. 8-10 weeks of treatment). The F1 offspring receive further treatment with the test substance from weaning to adulthood. Clinical observation and pathological examinations are performed on all animals for the signs of toxicity with special emphasis on effects on the integrity and performance of their reproductive systems and the health, growth, and development and function of the offspring.

Test animals: The rat is the preferred species.

Age & weight: Both males and females should be studied and the females should be nulliparous and non-pregnant. The P animals should be sexually mature, of similar weight (within sex) at initiation of dosing, similar age (approximately 90 days) at mating, and representative of the species and strain under study.

Housing and feeding

Rats should be housed at temperature $22(\pm 3)^{\circ}\text{C}$

Relative Humidity – 50-60% (However, humidity should not be below 30% or exceed 70% at any given point of time).

Lighting with 12 hours light and dark cycle. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. Mating procedure should be carried out in cages suitable for the purpose.

Acclimatization: Young Healthy animals acclimatized to the laboratory conditions for at least 5 days prior to the test.

Number of test animals

Each test and control group should contain a sufficient number of mating pairs to yield at least 20 pregnant females per dose group.

Dose group: At least three dose levels and a concurrent control should be used.

Route of administration

The test substance is normally administered orally by diet, drinking water or gavage. It can be modified for administration by other routes like dermal, inhalation, depending on the characteristics of the compound and the information required.

Limit Test

If no observable toxic effects are encountered at a dose level of 1000mg/kg body weight per day in repeat dose study and the toxicity is not expected based upon data from structurally related compounds then the higher doses need not to be tested except when human exposure indicates the need for a higher dose level to be used. In such cases, the Extended One-Generation Reproductive Toxicity Study could be conducted using a control group and a single dose of at least 1000 mg/kg body weight/day. If evidence for reproductive or developmental toxicity be found at this limit dose, further studies at lower dose levels will be required to identify a NOAEL.

Procedure

1. Dietary exposure is the preferred method of administration. If gavage studies are performed, the pups will normally only receive test substance indirectly through the milk, until direct dosing commences for them at weaning. When the test substance is administered by gavage, the volume of liquid administered at one time should not normally exceed 1 mL/100 g body weight (0.4 mL/100 g body weight is the maximum for oil, e.g.corn oil). In diet or drinking water studies, the pups will additionally receive test substance directly when they commence eating for themselves during the last week of the lactation period.
2. The duration of the pre-mating treatment in the Extended One-Generation Reproductive Toxicity Study is aimed at the detection of effects on functional changes that may interfere with mating behaviour and fertilisation. The pre- mating treatment should be sufficiently long to achieve steady-state exposure conditions in P males and females. A 2-week pre-mating treatment for both sexes is considered adequate in most cases. (For females, this covers 3-4 complete oestrous cycles and should be sufficient to detect and adverse effects on cyclicity. For males, this is equivalent to the time required for epididymal transit of maturing spermatozoa and should allow the detection of post-testicular effects on sperm, during the final stages of spermiation and epididymal sperm maturation, at mating). At the time of termination, when testicular and epididymal histopathology and analysis of sperm parameters are scheduled, the P and F1 males, will have been exposed for at least one entire spermatogenic process.

3. Once the pre-mating dosing period is established, the animals should be treated with the test substance continuously on a 7-days/week basis until necropsy. Dosing should continue during the 2-week mating period and, for P females, throughout gestation and lactation up to the day of termination after weaning. Males should be treated in the same manner until termination at the time when the F1 animals are weaned. For necropsy, priority should be given to females which should be necropsied on the same day of lactation. Necropsy of males can be spread over a larger number of days, depending on laboratory facilities.
4. Each P female should be placed with a single, randomly selected, unrelated male from the same dose group (1:1 pairing) until evidence of copulation is observed or 2 weeks have elapsed. If there are insufficient males, for example due to male death before pairing, then male(s) which have already mated may be paired (1:1) with a second female(s) such that all females are paired. Day 0 of pregnancy is defined as the day on which mating evidence is confirmed (a vaginal plug or sperm are found). Animals should be separated as soon as possible after evidence of copulation is observed. If mating has not occurred after 2 weeks, the animals should be separated without further opportunity for mating. Mating pairs should be clearly identified in the data.
5. On day 4 after birth, the size of each litter may be adjusted by eliminating extra pups by random selection to yield, as nearly as possible, five males and five females per litter.
6. At weaning (around PND 21) pups from all available litters up to 20 per dose and control group are selected for further examinations and maintained until sexual maturation (unless earlier testing is required)

Observations

1. For the P and the selected F1 animals, a general clinical observation is made once a day. In the case of gavage dosing, the timing of clinical observations should be prior to and post dosing (for possible signs of toxicity associated with peak plasma concentration). Pertinent behavioural changes, signs of difficult or prolonged parturition and all signs of toxicity are recorded. Twice daily, during the weekend once daily, all animals are observed for severe toxicity, morbidity and mortality. In addition, a more detailed examination of all P and F1 animals (after weaning) is conducted on a weekly basis.
2. P animals are weighed on the first day of dosing and at least weekly thereafter. In addition, P females are weighed during lactation on the same days as the weighing of the pups in their litters. All F1 animals are weighed individually at weaning (PND21) and at least weekly thereafter. Body weight is also recorded on the day when they attain puberty (completion of preputial separation or vaginal patency). All animals are weighed at sacrifice. During the study, food and water consumption (in the case of test substance administration in the drinking water) are recorded at least weekly on the same days as animal body weights (except during cohabitation).
3. Normally the assessment of oestrous cyclicity (by vaginal cytology) will start at the beginning of the treatment period and continue until confirmation of mating or the end of the 2-week mating period. If females have been screened for normal oestrous cycles before treatment, then it is useful to continue smearing as treatment starts.

4. In addition to the standard endpoints (e.g. body weight, food consumption, clinical observations including mortality/morbidity checks), the dates of pairing, the date of insemination and the date of parturition are recorded and the precoital interval (pairing to insemination) and the duration of pregnancy (insemination to parturition) are calculated. The P females should be examined carefully at the time of expected parturition for any signs of dystocia. Any abnormalities in nesting behaviour or nursing performance should be recorded.
5. Each litter should be examined as soon as possible after parturition (PND 0 or 1) to establish the number and sex of pups, stillbirths, live births, and the presence of gross anomalies (externally visible abnormalities, including cleft palate; subcutaneous haemorrhages; abnormal skin colour or texture; presence of umbilical cord; lack of milk in stomach; presence of dried secretions). In addition, the first clinical examination of the neonates should include a qualitative assessment of body temperature, state of activity and reaction to handling. Pups found dead on PND 0 or at a later time should be examined for possible defects and cause of death. Live pups are counted and weighed individually on PND 0 or PND1, and regularly thereafter, e.g. at least on PND 4, 7, 14, and 21.
6. The anogenital distance (AGD) of each pup should be measured on at least one occasion from PND 0 through PND 4. Pup body weight should be collected on the day the AGD is measured and the AGD should be normalized to a measure of pup size, preferably the cube root of body weight. The presence of nipples/areolae in male pups should be checked on PND 12 or 13. All selected F1 animals are evaluated daily for balano-preputial separation of vaginal patency for male/female respectively commencing before the expected day for achievement of these endpoints to detect if sexual maturation occurs early. Any abnormalities of genital organs, such as persistent vaginal thread, hypospadias or cleft penis, should be noted. Sexual maturity of F1 animals is compared to physical development by determining age and body weight at balano-preputial separation or vaginal opening for male/female respectively.
7. Systemic effects should be monitored in P animals. Fasted blood samples from a defined site are taken from ten randomly-selected P males and females per dose group at termination, stored under appropriate conditions and subjected to partial or full-scale haematology, clinical biochemistry, assay of T4 and TSH or other examination suggested by the known effect profile of the test substance. The following haematological parameters should be examined: haematocrit, haemoglobin concentration, erythrocyte count, total and differential leukocyte count, platelet count and blood clotting time/potential. Investigations of plasma or serum should include: glucose, total cholesterol, urea, creatinine, total protein, albumin and 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 and bile acids may provide useful information under certain circumstances. In addition, blood from all animals may be taken and stored for possible analysis at a later time to help clarify equivocal effects or to generate internal exposure data. If a second mating of P animals is not intended, the blood samples are obtained just prior to, or as part of, the procedure at scheduled sacrifice. In the case animals are retained, blood samples should be collected a few days before the animals are mated for the second

time. Unless existing data from repeated-dose studies indicate that the parameter is not affected by the test substance, urinalysis should be performed prior to termination and the following parameters evaluated: appearance, volume, osmolality or specific gravity, pH, protein, glucose, blood and blood cells, cell debris. Urine may also be collected to monitor excretion of test substance and/or metabolite(s).

8. Sperm parameters should be measured in all P generation males unless there is existing data to show that sperm parameters are unaffected in a 90-day study.

Gross necropsy

At the time of termination or premature death, all P and F1 animals are necropsied and examined macroscopically for any structural abnormalities or pathological changes. Special attention should be paid to the organs of the reproductive system. Pups that are humanely killed in a moribund condition and dead pups should be recorded and, when not macerated, examined for possible defects and/or cause of death and preserved. For adult P and F1 females, a vaginal smear is examined on the day of necropsy to determine the stage of the oestrous cycle and allow correlation with histopathology in reproductive organs. The uteri of all P females (and F1 females, if applicable) are examined for the presence and number of implantation sites, in a manner which does not compromise histopathological evaluation.

Organ weights

At the time of termination, body weights and wet weights of the organs listed below from all P animals and all F1 adults are determined as soon as possible after dissection to avoid drying. These organs should then be preserved under appropriate conditions. Unless specified otherwise, paired organs can be weighed individually or combined, consistent with the typical practice of the performing laboratory.

- Uterus (with oviducts and cervix), ovaries
- Testes, epididymides (total and cauda for the samples used for sperm counts)
- Prostate (dorsolateral and ventral parts combined). Care should be exercised when trimming the prostate complex to avoid puncture of the fluid filled seminal vesicles. In the event of a treatment- related effect on total prostate weight, the dorsolateral and ventral segments should be carefully dissected after fixation, and weighed separately.
- Seminal vesicles with coagulating glands and their fluids (as one unit)
- Brain, liver, kidneys, heart, spleen, thymus, pituitary, thyroid (post-fixation), adrenal glands and known target organs or tissues.

In addition to the organs listed above, samples of peripheral nerve, muscle, spinal cord, eye plus optic nerve, gastrointestinal tract, urinary bladder, lung, trachea (with thyroid and parathyroid attached), bone marrow, vas deferens (males), mammary gland (males and females) and vagina should be preserved under appropriate conditions.

Histopathology

Full histopathology of the organs is performed for all high-dose and control P animals. Organs demonstrating treatment-related changes should also be examined in all animals at the lower dose groups to aid in determining a NOAEL. Additionally, reproductive organs of all animals suspected of reduced fertility, e.g. those that failed to mate, conceive, sire, or deliver healthy offspring, or for which oestrous cyclicity or sperm number, motility, or morphology were affected, and all gross lesions should be subjected to histopathological evaluation.

Result assessment

Assess reproductive/developmental end points.

(Reference: OECD TEST GUIDELINE: 443, 2012)

GENOTOXICITY STUDIES

Bacterial Reverse Mutation Test (Ames Test)

In-Vitro Mammalian Chromosome Aberration Test

In- Vitro Mammalian Cell Gene Mutation Test

In-Vitro Mammalian Cell Micronucleus Test

Mammalian Bone Marrow Chromosome Aberration Test In-Vivo

Mammalian Spermatogonial Chromosome Abberation Test

Mammalian Erythrocyte Micronucleus Test In-Vivo

Un-Scheduled Dna Synthesis (Uds) Test With Mammalian Liver Cells In-Vivo

Mouse Heritable Translocation Assay

Rodent Dominant Lethal Test

BACTERIAL REVERSE MUTATION TEST (AMES TEST)

Objective

The bacterial reverse mutation test is commonly employed as an initial screen for genotoxic activity and, in particular, for point mutation-inducing activity.

Principle of the test

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 synthesize 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.

Methodology Preparations 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 culture

temperature is 37°C. At least five strains of bacteria should be used. These should include four strains of *S. typhimurium* (TA1535; TA1537 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 they may not detect certain oxidizing 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:

1. *S. typhimurium* TA1535, and
2. *S. typhimurium* TA1537 or TA97 or TA97a, and
3. *S. typhimurium* TA98, and
4. *S. typhimurium* TA100, and
5. *E. coli* WP2 uvrA, or *E. coli* WP2 uvrA (pKM101), or *S. typhimurium* TA102.

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.

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 or a combination of phenobarbitone and β naphthoflavone. 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.

Test substance

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.

Test conditions 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 bacteria 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.

Exposure concentrations

The recommended maximum test concentration for soluble non-cytotoxic substances is 5 mg/plate or 5 microlitre/plate. For non-cytotoxic substances that are not soluble at 5 mg/plate or 5 microlitre/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 microlitre/plate should be tested up to a cytotoxic concentration.

The precipitate should not interfere with the scoring.

Number of concentrations

At least five different concentrations should be tested with adequate intervals between test points.

Controls

Concurrent strain specific, positive and negative(solvent or vehicle) controls, both with and without metabolic activation, should be included in each assay.

Chemical and CAS Number for assays employing a metabolic activation system

9,10-Dimethylanthracene [CAS no. 781-43-1]

7,12-Dimethylbenzanthracene [CAS no. 57-97-6]

Congo Red [CAS no. 573-58-0] (for the reductive metabolic activation method)

Benzo(a)pyrene [CAS no. 50-32-8]

Cyclophosphamide (monohydrate) [CAS no. 50-18-0 (CAS no. 6055-19-2)]

2-Aminoanthracene [CAS no. 613-13-8]

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.

For assays performed without metabolic activation system, examples of strain-specific positive controls are:

Chemical with CAS Number and Strain for assays performed without metabolic activation system

(a) Sodium azide [CAS no. 26628-22-8] TA1535 and TA100

(b) 2-Nitrofluorene [CAS no. 607-57-8] TA98

- (c) 9-Aminoacridine [CAS no. 90-45-9] or ICR191 [CAS no. 17070-45-0] TA1537, TA97 and TA97a
- (d) Cumene hydroperoxide [CAS no. 80-15-9] TA102
- (e) Mitomycin C [CAS no. 50-07-7] WP2 uvrA and TA102
- (f) N-Ethyl-N-nitro-N-nitrosoguanidine [CAS no. 70-25-7] or 4-nitroquinoline 1-oxide [CAS no. 56-57-5] WP2, WP2 uvrA and WP2 uvrA (pKM101)
- (g) Furfurylamine (AF-2) [CAS no. 3688-53-7] plasmid-containing strains

Other appropriate positive control reference substances may be used. The use of chemical class-related positive control chemicals may 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.

Procedure

Treatment with test substance

For the plate incorporation method, without metabolic activation, usually 0.05 ml or 0.1 ml of the test solutions, 0.1 ml of fresh bacterial culture (containing approximately 10⁸ 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 1.5 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. 28. For the pre incubation method the test substance/test solution is pre incubated with the test strain (containing approximately 10⁸ 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.

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.

Data and Reporting 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 preincubation), and metabolic activation conditions.

Result assessment

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. Biological relevance of the results should be considered first. 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.

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 a substance induces point mutations by base substitutions or frame shifts 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.

(Reference: OECD Test guidelines 471, 1997)

IN VITRO MAMMALIAN CHROMOSOME ABERRATION TEST

Objective

In vitro chromosome aberration test is used to identify agents that cause structural chromosome aberrations in cultured mammalian cells. The *in vitro* chromosomal aberration test may employ cultures of established cell lines or primary cell cultures of human or rodent origin. The cells used should be selected on the basis of growth ability in culture, stability of the karyotype (including chromosome number) and spontaneous frequency of chromosomal aberrations.

Principle of the test

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. colchicine), harvested, stained and metaphase cells are analysed microscopically for the presence of chromosome aberrations.

Methodology Preparations 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). The choice of the cell lines used should be scientifically justified.

Media and culture conditions

Appropriate culture media and incubation conditions (culture vessels, CO₂ 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.

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 culture medium containing a mitogen (e.g. phytohemagglutinin) and incubated at 37°C.

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 co-factor supplemented post-mitochondrial fraction (S9) prepared from the livers of rodents treated with enzyme-inducing agents such as Aroclor 1254, or a combination of phenobarbitone and β -naphthoflavone. The S9 fraction typically is used at concentrations ranging from 1 to 2% (v/v) but may be increased to 10% (v/v) in the final test medium. The use of products that reduce the mitotic index, especially calcium complexing products (31) should be avoided during treatment. The choice of type and concentration of exogenous metabolic activation system or metabolic inducer employed may be influenced by the class of substances being tested.

Test substance

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.

Test conditions 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.

Measuring cell proliferation and cytotoxicity and choosing treatment concentration.

Exposure concentrations

Among the criteria to be considered when determining the highest concentration, concentrations that have the capability of producing artifactual positive responses, such as are cytotoxicity, precipitation in the culture medium, solubility in the test system, and changes in pH or osmolality. Measurements of cell proliferation are made to assure that a sufficient number of treated cells have reached mitosis during the test and that the treatments are conducted at appropriate levels of cytotoxicity. 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.

While the evaluation of cytotoxicity in an initial test may be useful to better define the concentrations to be used in the main experiment, an initial test is not mandatory. If performed, it should not replace the measurement of cytotoxicity in the main experiment.

Relative Population Doubling (RPD) or Relative Increase in Cell Count (RICC) for cell lines, mitotic index (MI) for primary culture of lymphocytes are appropriate methods for the assessment of cytotoxicity in cytogenetic tests.

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 concentration intervals of approximately 2-3 fold will be appropriate.

If the maximum concentration is based on cytotoxicity, the highest concentration should aim to achieve $55 \pm 5\%$ cytotoxicity using the recommended cytotoxicity parameters (*i.e.* reduction in RICC and RPD for cell lines and reduction in MI for primary cultures of lymphocytes to $45 \pm 5\%$ of the concurrent negative control). Care should be taken in interpreting positive results only to be found in the higher end of this $55 \pm 5\%$ cytotoxicity range.

At the time 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. If no precipitate or limiting cytotoxicity is observed, the highest test concentration should correspond to 10 mM, 2 mg/mL or 2 µl/mL, whichever is the lowest. When the test chemical is not of defined composition *e.g.* substance of unknown or variable composition, complex reaction products or biological materials. For Relatively non-cytotoxic compounds the maximum concentration should be 5 mg/ml.

If no precipitate or limiting cytotoxicity is observed, the highest test concentration should correspond to 10 mM, 2 mg/mL or 2 µl/mL, whichever is the lowest (39) (40) (41). When the test chemical is not of defined composition *e.g.* substance of unknown or variable composition, complex reaction products or biological materials

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 (turbidity) 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.

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. Provided it is done concurrently with the non-activated test using the same treatment duration, this single positive control response will demonstrate both the activity of the metabolic activation system and the responsiveness of the test system. Long term treatment (without S9) should however have its own positive control as the treatment duration will differ from the test using metabolic activation.

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	Chemical and CAS No
Absence of exogenous metabolic activation	Methyl methanesulphonate [CAS no. 66-27-3]
	Mitomycin C [CAS no. 50 -07-7]
	4-Nitroquinoline-N-Oxide [CAS no. 56-57-5]
	Cytosine arabinoside (CAS no. 147-94-4)
Presence of exogenous metabolic activation	Benzo(a)pyrene [CAS no. 50-32-8]
	Cyclophosphamide [CAS no. 50-18-0]

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.

Procedure

Treatment with test substance

Proliferating cells are treated with the test chemical in the presence and absence of a metabolic activation system.

Culture harvest time

First short treatment for 3-6 hrs (with and without metabolic activation) and sampling at a time equivalent to 1.5 normal cell cycle length. If results are negative then an additional experiment with successive treatments for duration of 1.5 cell cycle length without metabolic activation. If successive treatment for longer than 1.5 cycle is required for specimens preparation with metabolic activation , conduct confirmation studies as necessary.

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. 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.

Chromosome preparation

Cell cultures are treated with 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.

Acceptability criteria:

- The concurrent negative control is considered acceptable for addition to the laboratory historical negative control database.
- Concurrent positive controls should induce responses that are compatible with those generated in the historical positive control data base and produce a statistically significant increase compared with the concurrent negative control.
- Cell proliferation criteria in the solvent control should be fulfilled.
- All three experimental conditions were tested unless one resulted in positive results .
- Adequate number of cells and concentrations are analysable .
- The criteria for the selection of top concentration are consistent.

Result assessment

All slides, including those of the positive and negative controls, should be independently coded before microscopic analysis for chromosomal aberrations.

Cells with structural chromosomal aberration(s) including and excluding gaps should be scored.

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. The laboratory should establish:

- A historical positive control range and distribution,
- A historical negative (untreated, solvent) control range and distribution.

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.

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. Providing that all acceptability criteria are fulfilled, a test chemical is considered to be clearly positive if, in any of the experimental conditions examined.

- a) at least one of the test concentrations exhibits a statistically significant increase compared with the concurrent negative control,

- b) the increase is dose-related when evaluated with an appropriate trend test,
- c) any of the results are outside the distribution of the historical negative control data (e.g. Poisson-based 95% control limits;

When all of these criteria are met, the test chemical is then considered able to induce chromosomal aberrations in cultured mammalian cells in this test system.

Providing that all acceptability criteria are fulfilled, a test chemical is considered clearly negative if, in all experimental conditions examined:

- a) none of the test concentrations exhibits a statistically significant increase compared with the concurrent negative control,
- b) there is no concentration-related increase when evaluated with an appropriate trend test,
- c) all results are inside the distribution of the historical negative control data (e.g. Poisson-based 95% control limits;

The test chemical is then considered unable to induce chromosomal aberrations in cultured mammalian cells in this test system. A test substance for which the results do not meet the above criteria is considered nonmutagenic 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.

(Reference: OECD Test guidelines 473, 2014)

IN VITRO MAMMALIAN CELL GENE MUTATION TEST

Objective

The *in vitro* mammalian cell gene mutation test can be used to detect gene mutations induced by chemical substances.

Principle of the test

Cells in suspension or monolayer culture are exposed to the test substance, both with and without metabolic activation, for a suitable period of time (3-6 hours), and subcultured to determine cytotoxicity and to allow phenotypic expression prior to mutant selection. 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 (**typically a minimum of 7-9 days**). 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. Suitable cell lines include L5178Y mouse lymphoma cells, the CHO, AS52 and V79 lines of Chinese hamster cells, and TK6 human lymphoblastoid cells.

Methodology Preparations Cells

The minimal number of viable cells surviving treatment and used at each stage in the test should be based on the spontaneous mutation frequency.

Media and culture conditions

Appropriate culture media and incubation conditions (culture vessels, temperature, CO₂ 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.

Preparation of cultures

Cells 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.

Metabolic activation

Exogenous metabolising systems should be used when employing cells which have inadequate endogenous metabolic capacity. The most commonly used system is a co- factor supplemented post-mitochondrial fraction (S9) prepared from the livers of rodents treated with enzyme-inducing agents such as Aroclor 1254 or a combination of phenobarbitone and D-naphthoflavone. **The S9 fraction typically is used at concentrations ranging from 1 to 2% (v/v) but may be increased to 10% (v/v) in the final test medium.** The choice of the type and concentration of exogenous metabolic activation system or metabolic inducer employed may be influenced by the class of substances being tested

Test substance

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. **Gaseous or volatile test chemicals should be tested by appropriate modifications to the standard protocols, such as treatment in sealed culture vessels.** Fresh preparations should be employed unless stability data demonstrate the acceptability of storage.

Test conditions 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.

Measuring cytotoxicity and choosing exposure concentrations

Among the criteria to be considered when determining the highest concentration are cytotoxicity and solubility in the test system and changes in pH or osmolality. Concentration selection is based on cytotoxicity and other considerations. While the evaluation of cytotoxicity in an initial test may be useful to better define the concentrations to be used in the main experiment, an initial test is not required. Even if an initial cytotoxicity evaluation is performed, the measurement of cytotoxicity for each culture is still required in the main experiment.

Cytotoxicity should be evaluated using RS, i.e. cloning efficiency (CE) of cells plated immediately after treatment, adjusted by any loss of cells during treatment, based on cell count, as compared with adjusted cloning efficiency in negative controls (assigned a survival of 100%). 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 root 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 compounds the maximum concentration should be 5 mg/ml, 5 microliter / ml, or 0.01 M, whichever is the lowest.

Relatively insoluble substances should be tested up to or beyond their limit of solubility (turbidity) 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 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

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 one that requires activation to give a mutagenic response. **Examples of positive controls include :**

Metabolic Activation condition	Locus	Substance and CAS No.
Absence of exogenous metabolic activation	<i>Hprt</i>	Ethylmethanesulfonate [CAS no. 62-50-0] Ethylnitrosourea [CAS no. 759-73-9] 4-Nitroquinoline 1-oxide [CAS no. 56-57-5]
	<i>xprt</i>	Streptonigrin [CAS no. 3930-19-6] Mitomycin C [CAS no. 50-07-7]
Presence of exogenous metabolic activation	<i>Hprt</i>	3-Methylcholanthrene [CAS no. 56-49-5] 7,12-Dimethylbenzanthracene [CAS no. 57-97-6] Benzo[a]pyrene [CAS no. 50-32-8]
	<i>xprt</i>	Benzo[a]pyrene [CAS no. 50-32-8]

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.

Procedure

Treatment with test substance

Proliferating cells are treated with the test chemical in the presence and absence of a metabolic activation system. 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.

Phenotypic expression time and Measurement of survival, viability and mutant frequency

After the treatment period, cells are cultured to allow 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 (7-9 days) to allow near optimal phenotypic expression of newly induced mutants (HPRT and XPRT).

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.

Proficiency testing

A selection of positive control substances (see Table 1 in paragraph 25) should be investigated in the absence and in the presence of metabolic activation, in order to demonstrate proficiency to detect mutagenic substances, to determine the effectiveness of the metabolic activation system and to demonstrate the appropriateness of the cell growth conditions during treatment, phenotypic expression and mutant selection and of the scoring procedures. A range of concentrations of the selected substances should be chosen so as to give reproducible and concentration-related increases above the background in order to demonstrate the sensitivity and dynamic range of the test system.

The laboratory should establish:

- A historical positive control range and distribution,
- A historical negative (untreated, solvent) control range and distribution.
- Negative control data should consist of mutant frequencies from single or preferably replicate cultures. Concurrent negative controls should ideally be within the 95% control limits of the distribution of the laboratories historical negative control database.

Result assessment

The presentation of results should include all of the data needed to calculate cytotoxicity (expressed as RS) and the mutant frequency.

There are several criteria for determining a positive result, such as a concentration-related, or a reproducible increase in mutant frequency.

Acceptance of a test is based on the following criteria:

- The concurrent negative control is considered acceptable for addition to the laboratory historical negative control database.

- Concurrent positive controls should induce responses that are compatible with those generated in the historical positive control data base and produce a statistically significant increase compared with the concurrent negative control.
- Two experimental conditions (i.e. with and without metabolic activation) were tested unless one resulted in positive results.
- Adequate number of cells and concentrations are analyzable.
- The criteria for the selection of top concentration are consistent- The concurrent negative control is considered acceptable for addition to the laboratory historical negative control database.

Providing that all acceptability criteria are fulfilled, a test chemical is considered to be clearly positive if, in any of the experimental conditions examined:

- a) at least one of the test concentrations exhibits a statistically significant increase compared with the concurrent negative control,
- b) the increase is concentration-related when evaluated with an appropriate trend test,
- c) any of the results are outside the distribution of the historical negative control data (e.g. Poissonbased 95% control limit).

When all of these criteria are met, the test chemical is then considered able to induce gene mutations in cultured mammalian cells in this test system. Recommendations for the most appropriate statistical methods.

Providing that all acceptability criteria are fulfilled, a test chemical is considered clearly negative if, in all experimental conditions examined:

- a) none of the test concentrations exhibits a statistically significant increase compared with the concurrent negative control,
- b) there is no concentration-related increase when evaluated with an appropriate trend test,
- c) all results are inside the distribution of the historical negative control data (e.g. Poisson-based 95% control limit).

The test chemical is then considered unable to induce gene mutations in cultured mammalian cells in this test system.

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 nonmutagenic in this system. Although most studies will give clearly positive or negative results, in rare cases the dataset 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.

(Reference: OECD Test guidelines 476, 2016)

IN VITRO MAMMALIAN CELL MICRONUCLEUS TEST

Objective

The assay detects the activity of clastogenic and aneugenic chemicals in cells that have undergone cell division during or after exposure to the test substance.

Principle of the test

The *in vitro* micronucleus (MNvit) assay is a genotoxicity test for the detection of micronuclei (MN) in the cytoplasm of interphase cells. Micronuclei may originate from acentric chromosome fragments (*i.e.* lacking a centromere), or whole chromosomes that are unable to migrate to the poles during the anaphase stage of cell division. Cell cultures of human or mammalian origin are exposed to the test substance both with and without an exogenous source of metabolic activation unless cells with an adequate metabolizing capability are used.

Methodology Preparations

The mammalian cell MNvit test may employ cultures of cell lines or primary cell cultures, of human or rodent origin. Cultured primary human peripheral blood lymphocytes and a number of rodent cell lines such as CHO, V79, CHL/IU, and L5178Y cells may be used. The use of other cell lines and types should be justified based on their demonstrated performance in the assay. Because the background frequency of micronuclei will influence the sensitivity of the assay, it is recommended that cell types with a low, stable background frequency of micronucleus formation be used. When primary cell are used, for animal welfare reasons, the use of cells from human origin should be considered where feasible and sampled in accordance with the human ethical principles and regulations.

Human peripheral blood lymphocytes should be obtained from young (approximately 18-35 years of age), healthy, non-smoking individuals with no known recent exposures to genotoxic chemicals or radiation. If cells from more than one donor are pooled for use, the number of donors should be specified. **The micronucleus frequency increases with age and this trend is more marked in females than in males and this should be taken into account in the selection of donor cells for pooling.**

Media and culture conditions

Appropriate culture medium and incubation conditions (culture vessels, Humidified atmosphere of 5% CO₂ concentration, temperature of 37° C) should be used for maintaining cultures. Established cell lines and strains should be checked routinely for the stability of the modal chromosome number and the absence of mycoplasma contamination, and should not be used if contaminated or if the modal chromosome number has changed. The normal cell cycle time for the culture conditions used in the testing laboratory should be known. If the cytokinesis-block method is used then the concentration of the cytokinesis inhibitor should be optimised for the particular cell type and should be shown to produce a good yield of binucleate cells for scoring.

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 in monolayers, and suspension cultures will not reach excessive density before the time of harvest, and incubated at 37°C.

Lymphocytes: Whole blood treated with an anti-coagulant (*e.g.* heparin), or separated lymphocytes, are cultured in the presence of a mitogen *e.g.* phytohaemagglutinin (PHA) prior to exposure to the test substance and cytoB.

Metabolic activation: Exogenous metabolising systems should be used when using cells with inadequate endogenous metabolic capacity. The most commonly used system is a co-factor-supplemented post-mitochondrial fraction (S9) prepared from the livers of rodents treated with enzyme-inducing agents such as Aroclor 1254 or a combination of phenobarbitone and β -naphthoflavone.

The S9 fraction typically is used at concentrations ranging from 1 to 2% (v/v) but may be increased to 10% (v/v) in the final test medium. The use of products that reduce the mitotic index, especially calcium complexing products (62), should be avoided during treatment. The choice of type and concentration of exogenous metabolic activation system or metabolic inducer employed may be influenced by the class of substances being tested

Test substance

Solid test substances should be dissolved 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. Gases or volatile substances should be tested by appropriate modifications to the standard protocols, such as treatment in sealed vessels. Fresh preparations of the test substance should be used unless stability data demonstrate the acceptability of storage.

Test Conditions Solvents/vehicles

The solvent/vehicle should not react with the test substance, or be incompatible with the survival of the cells or with the maintenance of S9 activity at the concentration used. If other than well established solvent/vehicles (*e.g.* water, cell culture medium, dimethyl sulfoxide) are used, their use should be supported by data indicating their compatibility with the test substance and their lack of genetic toxicity. It is recommended that, wherever possible, the use of an aqueous solvent/vehicle should be considered first.

Use of cytoB as a cytokinesis blocker

One of the most important considerations in the performance of the MNvit assay is ensuring that the cells being scored have completed mitosis during the treatment or the post-treatment incubation period, if one is used. CytoB is the agent that has been most widely used to block cytokinesis because it inhibits actin assembly, and thus prevents separation of daughter cells after mitosis, leading to the

formation of binucleated cells. Micronucleus scoring, therefore, can be limited to cells that have gone through mitosis during or after treatment. The effect of the test substance on cell proliferation kinetics can be measured simultaneously. CytoB should be used as a cytokinesis blocker when human lymphocytes are used because cell cycle times will be variable within cultures and among donors and because not all lymphocytes will respond to PHA.

The appropriate concentration of cytoB should be determined by the laboratory for each cell type to achieve the optimal frequency of binucleated cells in the solvent/vehicle control cultures. The appropriate concentration of cytoB is usually between 3 and 6 µg/ml.

Measuring cell proliferation and cytotoxicity and choosing exposure concentrations

When determining the highest test substance concentration to be tested, concentrations that have the capability of producing artifactual positive responses, such as those producing excessive cytotoxicity, precipitation in the culture medium, and marked changes in pH or osmolality, should be avoided.

Measurements of cell proliferation are made to assure that the treated cells have undergone mitosis during the assay and that the treatments are conducted at appropriate levels of cytotoxicity. Cytotoxicity should be determined with and without metabolic activation using an appropriate indication of cell death & growth. The measurement of Relative Increase in Cell Counts (RICC) or Relative Population Doubling (RPD) is recommended to estimate cytotoxic and cytostatic activity of a treatment. When cytoB is used, cytotoxicity can be determined using the replication index (RI). Treatment of cultures with cytoB, and measurement of the relative frequencies of mononucleate, binucleate, and multi-nucleate cells in the culture, provides an accurate method of quantifying the effect on cell proliferation and the cytotoxic or cytostatic activity of a treatment, and ensures that only cells that divided during or after treatment are scored.

In studies with cytoB, cytostasis/cytotoxicity can be quantified from the cytokinesis-block proliferation index (CBPI) or may be derived from the RI from at least 500 cells per culture. When cytoB is used to assess cell proliferation, a CBPI or RI should be determined from at least 500 cells per culture. These measurements among others can be used to estimate cytotoxicity by comparing values in the treated and control cultures.

In studies without cytoB, it is necessary to demonstrate that the cells scored in the culture have undergone division during or following treatment with the test substance, otherwise false negative responses may be produced. Methods that have been used for ensuring that divided cells are being scored include incorporation and subsequent detection of bromodeoxyuridine (BrdU) to identify cells that have replicated, the formation of clones when cells from permanent cell lines are treated and scored *in situ* on a microscope slide (Proliferation Index (PI)), or the measurement of Relative Population Doubling (RPD) or Relative Increase in Cell Count (RICC) or other proven methods.

Assessment of other markers for cytotoxicity or cytostasis (e.g. confluency, cell number, apoptosis, necrosis, metaphase counting) can provide useful information.

At least three analysable test concentrations should be evaluated. In order to achieve this, it may

be necessary to perform the experiment using a larger number of closely spaced concentrations and analyse micronucleus formation in those concentrations providing the appropriate range of cytotoxicities.

The highest concentration should aim to produce $55 \pm 5\%$ cytotoxicity. Higher levels may induce chromosome damage as a secondary effect of cytotoxicity. Where cytotoxicity occurs, the test concentrations selected should cover a range from that producing $55 \pm 5\%$ cytotoxicity, to little or no cytotoxicity.

If no cytotoxicity or precipitate is observed, the highest test concentration should correspond to 0.01 M, 5 mg/mL or 5 µl/mL, whichever is the lowest. The concentrations selected for analysis should, in general, be separated by a spacing of no more than $\sqrt{10}$. For test substances that exhibit a steep concentration-response curve, it may be necessary to more closely space the test substance concentrations so that cultures in the moderate and low toxicity ranges also will be scored.

When solubility is a limiting factor, or for poorly soluble test chemicals the maximum concentration, if not limited by cytotoxicity, should be the lowest concentration at which minimal precipitate is visible in cultures, provided there is no interference with scoring. Evaluation of precipitation should be done by methods such as light microscopy, noting precipitate that persists, or appears during culture (by the end of treatment).

Controls

Concurrent positive and solvent/vehicle controls both with and without metabolic activation should be included in each experiment.

Positive controls are needed to demonstrate the ability of the cells used, and the test protocol, to identify clastogens and aneugens, and to affirm the metabolic capability of the S9 preparation. The positive controls should employ known inducers of micronucleus formation at concentrations expected to give small, but reproducible increases over background, and demonstrate the sensitivity of the test system. Examples of positive Controls (Table 1) include:

Table 1

Metabolic activation condition	Chemical and CAS No
1. Clastogens active without metabolic activation	
	Methyl methanesulphonate [CAS no. 66-27-3]
	Mitomycin C [CAS no. 50 -07-7]
	4-Nitroquinoline-N-Oxide [CAS no. 56-57-5]
	Cytosine arabinoside (CAS no. 147-94-4)
2. Clastogens requiring metabolic activation	
	Benzo(a)pyrene [CAS no. 50-32-8]
	Cyclophosphamide () [CAS no. 50-18-0]

Metabolic activation condition	Chemical and CAS No
3. Aneugens	
	Cochicine (64-86-8)
	Vinblastine (143-76-9)

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.

A clastogen that requires metabolic activation (*e.g.* cyclophosphamide; benzo[a]pyrene) should be used to demonstrate both the metabolic competence and the ability of the test system to detect clastogens. Other positive control substances may be used if justified. Because some positive controls that need metabolic activation may be active without exogenous metabolic activation under certain treatment conditions or in certain cell lines, the need for metabolic activation, and the activity of the S9 preparation, should be tested in the selected cell line and at the selected concentrations.

At the present time, no aneugens are known that require metabolic activation for their genotoxic activity. Currently accepted positive controls for aneugenic activity are, for example, colchicine and vinblastine. Other substances may be used if they induce micronuclei solely, or primarily, through aneugenic activity. To avoid the need for two positive controls (for clastogenicity and aneugenicity) without metabolic activation, the aneugenicity control can serve as the positive control without S9, and the clastogenicity control can be used to test the adequacy of the metabolic activation system used. Positive controls for both clastogenicity and aneugenicity should be used in cells that do not require S9. The use of chemical class-related positive control chemicals may be considered, when suitable substances are available. All positive control substances used should be appropriate for the cell type and activation conditions.

Solvent/vehicle controls should be included for every harvest time. In addition, untreated negative controls (lacking solvent/vehicle) should also be used unless there are published or laboratory historical control data demonstrating that no genotoxic or other deleterious effects are induced by the chosen solvent at the concentrations used.

Procedure Treatment Schedule

In order to maximise the probability of detecting an aneugen or clastogen acting at a specific stage in the cell cycle, it is important that sufficient numbers of cells are treated with the test substance during all stages of their cell cycles.

All treatments should commence and end while the cells are growing exponentially and the cells should continue to grow up to the time of sampling. The treatment schedule for cell lines and primary cell cultures may, therefore, differ somewhat from that for lymphocytes which require mitogenic stimulation to begin their cell cycle.

Theoretical considerations, together with published data indicate that most aneugens and clastogens will be detected by a short term treatment period of 3 to 6 hrs in the presence and absence of S9, followed by removal of the test substance and a growth period of 1.5 – 2.0 cell cycles. Cells are

sampled at a time equivalent to about 1.5 – 2.0 times the normal (*i.e.* untreated) cell cycle length either after the beginning or at the end of treatment (See Table 2†). Sampling or recovery times may be extended if it is known or suspected that the test substance affects the cell cycling time (*e.g.* when testing nucleoside analogues).

Because of the potential cytotoxicity of S9 preparations for cultured mammalian cells, an extended exposure treatment of 1.5 – 2.0 normal cell cycles is used only in the absence of S9. In the extended treatment, options are offered to allow treatment of the cells with the test chemical in the absence or presence of cytoB. These options address situations where there may be concern regarding possible interactions between the test substance and cytoB.

The suggested cell treatment schedules are presented in Table 2. All treatments should commence and end while the cells are growing exponentially.

Table 2. Cell treatment and harvest times for the MNvit assay

Lymphocytes, primary cells and cell lines treated with cytoB	+ S9 Short term	Treat for 3-6 hrs in the presence of S9; remove the S9 and treatment medium; add fresh medium and cytoB; harvest 1.5 – 2.0 normal cell cycle lengths after the beginning of treatment.
	– S9 Short exposure	Treat for 3-6 hrs; remove the treatment medium; add fresh medium and cytoB; harvest 1.5 – 2.0 normal cell cycles lengths after the beginning of treatment later.
	– S9 Extended exposure	treat for 1.5 – 2.0 normal cell cycles lengths in the presence of cyto B; harvest at the end of the treatment period.; remove the test substance; add fresh medium and cytoB; harvest 1.5 – 2.0 normal cell cycles later.
Cell lines treated without cytoB (Identical to the treatment schedules outlined above with the exception that no cytoB is added)		

In monolayers, mitotic cells (identifiable as being round and detaching from the surface) may be present at the end of the 3-6 hr treatment. Because these mitotic cells are easily detached, they can be lost when the medium containing the test substance is removed. Care should be taken to collect these when cultures are washed, and to return them to the cultures, to avoid losing cells that are in mitosis, and at risk for micronuclei /chromosome aberration, at the time of harvest.

Cell harvest and slide preparation

Each culture is harvested and processed separately. Cell preparation may involve hypotonic treatment, but this step is not necessary if adequate cell spreading is otherwise achieved. Different techniques can be used in slide preparation provided that high-quality cell preparations for scoring are obtained. Cell cytoplasm should be retained to allow the detection of micronuclei and (in the cytokinesis-block method) reliable identification of binucleate cells.

The slides can be stained using various methods, such as Giemsa or fluorescent DNA specific dyes. The use of a DNA specific stain (*e.g.* acridine orange or Hoechst 33258 plus pyronin-Y) can eliminate some of the artifacts associated with using a non-DNA specific stain. Anti-kinetochore antibodies, FISH with pancentromeric DNA probes, or primed *in situ* labelling with pancentromere-specific primers, together with appropriate DNA counterstaining, can be used to identify the contents (chromosome/chromosomal fragment) of micronuclei if mechanistic information of their formation is of interest. Other methods for differentiation between clastogens and aneugens may be used if they have been shown to be effective.

Analysis

In cytoB-treated cultures, micronucleus frequencies should be analysed in at least 2000 binucleated cells per concentration (at least 1000 binucleated cells per culture; two cultures per concentration). If single cultures are used, at least 2000 binucleated cells per concentration should be scored from that culture. If substantially fewer than 1000 binucleate cells per culture, or 2000 if a single culture is used, are available for scoring at each concentration, and if a significant increase in micronuclei is not detected, the test should be repeated using more cells, or at less toxic concentrations, whichever is appropriate. Care should be taken not to score binucleate cells with irregular shapes or where the two nuclei differ greatly in size; neither should binucleate cells be confused with poorly spread multinucleate cells. Cells containing more than two main nuclei should not be analysed for micronuclei, as the baseline micronucleus frequency may be higher in these cells. Scoring of mononucleate cells is acceptable if the test substance is shown to interfere with cytoB activity.

In cell lines assayed without cytoB treatment, micronuclei should be scored in at least 2000 cells per concentration (at least 1000 cells per culture; two cultures per concentration). Where only one culture per concentration is used, at least 2000 cells should be scored from that culture.

When cytoB is used, a CBPI or an RI should be determined to assess cell proliferation using at least 500 cells per culture. When treatments are performed in the absence of cytoB, it is essential to provide evidence that the cells being scored have proliferated.

Acceptability criteria

A laboratory proposing to use the MNvit assay described in this Test Guideline should demonstrate its ability to reliably and accurately detect substances of known aneugenic and clastogenic activity, with and without metabolic activation, as well as known negative substances. As evidence of its ability to perform this test method correctly, the laboratory should provide evidence that the cells being scored for micronucleus formation have completed one nuclear division if the test is performed without the use of cytoB.

A selection of positive control substances (see Table 1) should be investigated with short and long treatments in the absence of metabolic activation, and also with short treatment in the presence of metabolic activation, in order to demonstrate proficiency to detect clastogenic and aneugenic substances, determine the effectiveness of the metabolic activation system and demonstrate the

appropriateness of the scoring procedures (microscopic visual analysis, flow cytometry, laser scanning cytometry or image analysis). A range of concentrations of the selected substances should be chosen so as to give reproducible and concentration-related increases above the background in order to demonstrate the sensitivity and dynamic range of the test system.

The laboratory should establish:

- A historical positive control range and distribution,
- A historical negative (untreated, solvent) control range and distribution.

Data from negative, solvent, and positive controls should be used to establish historical control ranges. These values should be used in deciding the adequacy of the concurrent negative/positive controls for an experiment

If minor changes to the protocol (e.g. use of automated instead of manual scoring techniques; use of a new cell type) are proposed for the assay, then the effectiveness of the change should be demonstrated before the modified protocol can be considered acceptable for use. Demonstration of effectiveness includes demonstration that the major mechanisms of chromosome breakage and gain or loss can be detected, and that appropriate positive and negative results can be achieved for the class of the individual substance, or the broad range of substances, to be tested.

If the cytokinesis-block technique is used, only the frequencies of binucleate cells with micronuclei (independent of the number of micronuclei per cell) are used in the evaluation of micronucleus induction. Concurrent measures of cytotoxicity and/or cytostasis for all treated, negative and positive control cultures should be determined (16). The CBPI or the RI should be calculated for all treated and control cultures as measurements of cell cycle delay when the cytokinesis-block method is used. Individual culture data should be provided.

There are several criteria for determining a positive result, such as a concentration-related increase or a statistically significant increase in the number of cells containing micronuclei

Acceptability of a test is based on the following criteria:

- The concurrent negative control is considered acceptable for addition to the laboratory historical negative control database.
- Concurrent positive controls should induce responses that are compatible with those generated in the laboratorys historical positive control data base and produce a statistically significant increase compared with the concurrent negative control.
- Cell proliferation criteria in the solvent control should be fulfilled.
- All experimental conditions were tested unless one resulted in positive results .
- Adequate number of cells and concentrations are analysable .
- The criteria for the selection of top concentration are consistent

Providing that all acceptability criteria are fulfilled, a test chemical is considered to be clearly positive if, in any of the experimental conditions examined.

- at least one of the test concentrations exhibits a statistically significant increase compared with the concurrent negative control .
- the increase is dose-related in at least one experimental condition when evaluated with an appropriate trend test.
- any of the results are outside the distribution of the historical negative control data (e.g. Poisson-based 95% control limits;

When all of these criteria are met, the test chemical is then considered able to induce chromosome breaks and/or gain or loss in this test system. Recommendations for the most appropriate statistical methods can also be found in the literature

Providing that all acceptability criteria are fulfilled, a test chemical is considered clearly negative if, in all experimental conditions examined.

- none of the test concentrations exhibits a statistically significant increase compared with the concurrent negative control,
- there is no concentration-related increase when evaluated with an appropriate trend test,
- all results are inside the distribution of the historical negative control data (e.g. Poisson-based 95% control limits;

The test chemical is then considered unable to induce chromosome breaks and/or gain or loss in this test system

Result assessment

The biological relevance of the results should be considered first. Consideration of whether the observed values are within or outside of the historical control range can provide guidance when evaluating the biological significance of the response. Appropriate statistical methods may be used as an aid in evaluating the test results. However, the results of statistical testing should be assessed with respect to dose-response relationship. Reproducibility and historical data should also be taken into consideration.

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

Positive results from the MNvit assay indicate that the test substance induces chromosome breakage loss, in cultured mammalian cells. Negative results indicate that, under the test conditions used, the test substance does not induce chromosome breaks and/or gain or loss in cultured mammalian cells.

(Reference: OECD Test Guideline 487, 2014)

MAMMALIAN BONE MARROW CHROMOSOME ABERRATION TEST IN-VIVO

Objective

The mammalian *in vivo* chromosome aberration test is used for the detection of structural chromosome aberrations induced by test compounds in bone marrow cells of animals, usually rodents.

Principle of the test

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). Chromosome preparations are then made from the bone marrow cells and stained, and metaphase cells are analysed for chromosome aberrations.

Methodology

Preparations

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 $\pm 20\%$ of the mean weight of each sex. The scientific justification for using species other than rats and mice should be provided in the report. If species other than rodents are used, it is recommended that the measurement of bone marrow chromosomal aberration be integrated into another appropriate toxicity test.

Housing and feeding conditions

The temperature in the experimental animal room should be 22°C ($\pm 3^{\circ}\text{C}$). Although the relative humidity should be at least 30% and preferably not exceed 70% other than during room cleaning, the aim should be 50-60%. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. The choice of diet may be influenced by the need to ensure a suitable admixture of a test substance when administered by this method. Animals may be housed individually, or be caged in small groups of the same sex.

Preparation of the animals

Healthy young adult animals (for rodents, 6-10 weeks old at start of treatment) 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 5 days.

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.

Test conditions 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.

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 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

Positive control substances include:

Ethyl methanesulphonate [CASRN 62-50-0],

Methyl methanesulphonate [CASRN 66-27-3]

Ethyl nitrosourea [CASRN 759-73-9]

Mitomycin C [CASRN 50-07-7]

Cyclophosphamide (monohydrate) [CASRN 50-18-0; (CASRN 6055-19-2)]

Triethylenemelamine [CASRN 51-18-3]

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. Negative control data should consist of the incidence of structural chromosomal aberration in each animal.

Procedure

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.

Treatment schedule

Test chemicals are normally administered as a single treatment, but may be administered as a split dose (i.e. two or more treatments on the same day separated by no more than 2-3 hours) to facilitate administering a large volume. 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. 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.

Dose levels

If a preliminary range-finding study is performed because there are no suitable data already available to aid in dose selection, it should be performed in the same laboratory, using the same species, strain, sex, and treatment regimen to be used in the main study. Substances that exhibit saturation of toxicokinetic properties, or induce detoxification processes that may lead to a decrease in exposure after long-term treatment may be exceptions to the dose-setting criteria and should be evaluated on a case-by-case basis.

If there is toxicity, three dose levels are used for the first sampling time.

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 compounds, 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.

Administration of doses

The test substance is usually administered by gavage using a stomach tube or a suitable intubation cannula, or by intraperitoneal injection.

Bone marrow and Chromosome preparations

A blood sample should be taken at appropriate time(s) in order to permit investigation of the plasma levels of the test chemicals for the purposes of demonstrating that exposure of the bone marrow occurred, where warranted and where other exposure data do not exist. Immediately after sacrifice, bone marrow is obtained from the femurs or tibias, exposed to hypotonic solution and fixed. The metaphase cells are then spread on slides and stained.

Result assessment

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.

The following criteria determine the acceptability of the test:

- a) The concurrent negative control data are considered acceptable for addition to the laboratory historical control database.
- b) The concurrent positive controls or scoring controls should induce responses that are compatible with those generated in the historical positive control database and produce a statistically significant increase compared with the negative control
- c) The appropriate number of doses and cells has been analysed;
- d) The criteria for the selection of highest dose are consistent

Providing that all acceptability criteria are fulfilled, a test chemical is considered clearly positive if:

- a) At least one of the treatment groups exhibits a statistically significant increase in the frequency of cells with structural chromosomal aberrations (excluding gaps) compared with the concurrent negative control,
- b) This increase is dose-related at least at one sampling time when evaluated with an appropriate trend test, and
- c) Any of these results are outside the distribution of the historical negative control data (e.g. Poisson-based 95% control limits).

Providing that all acceptability criteria are fulfilled, a test chemical is considered clearly negative if in all experimental conditions examined:

- a) None of the treatment groups exhibits a statistically significant increase in the frequency of cells with structural chromosomal aberrations (excluding gaps) compared with the concurrent negative control,

- b) There is no dose-related increase at any sampling time when evaluated by an appropriate trend test,
- c) All results are inside the distribution of the historical negative control data (e.g. Poisson-based 95% control limits), and
- d) Bone marrow exposure to the test substance(s) occurred

In order to establish sufficient experience with the conduct of the assay prior to using it for routine testing, the laboratory should have demonstrated the ability to reproduce expected results from published data (e.g. (6)) for chromosomal aberration frequencies with a minimum of two positive control substances.

During the course of the proficiency investigations, the laboratory should establish:

- A historical positive control range and distribution, and
- A historical negative control range and distribution.

Any changes to the experimental protocol should be considered in terms of their impact on the resulting data remaining consistent with the laboratory's historical control database.

General clinical observations of the test animals should be made and clinical signs recorded at least once a day, preferably at the same time(s) each day and considering the peak period of anticipated effects after dosing. At least twice daily during the dosing period, all animals should be observed for morbidity and mortality.

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. 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.

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

Although most experiments will give clearly positive or negative results, in rare cases the data set will preclude making a definite judgment 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.

(Reference: OECD Test Guideline 475, 2016)

MAMMALIAN SPERMATOGONIAL CHROMOSOMAL ABERRATION TEST

Objective

The purpose of the *in-vivo* Mammalian Spermatogonial Chromosomal Aberration Test is to identify those chemicals that cause structural aberrations in mammalian spermatogonial cells. Structural aberrations may be of two types, chromosome and chromatid. This test measures chromosomal aberrations in spermatogonial germ cells and is, therefore, expected to be predictive of induction of heritable mutations in these germ cells.

Principle of the test

Animals are exposed to the test chemical by an appropriate route of exposure and are euthanized at appropriate times after treatment. Prior to euthanasia, animals are treated with a metaphase-arresting agent (e.g., colchicine or Colcemid®). Chromosome preparations are then made from germ cells and stained, and metaphase cells are analyzed for chromosome aberrations.

Test Animal: Healthy young adult Male mice should be used.

Age/weight: Healthy young adult animals should be of age between 8 to 12 weeks at the start of treatment. The weight variation of animal used should be minimal and not exceeding $\pm 20\%$ of the mean weight of each sex.

Housing & Feeding

The temperature in the animal room should be 22°C ($\pm 3^{\circ}\text{C}$). Although the relative humidity ideally should be 50-60%, it should be at least 30% and preferably not exceed 70% other than during room cleaning. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water.

Acclimatization: The animals should be acclimatized to the laboratory conditions for at least 5 days by keeping in individual cages prior to dosing.

Dose Range Finding study

If a preliminary range-finding study is performed because there are no suitable data already available to aid in dose selection, it should be performed in the same laboratory, using the same species, strain, and treatment regimen to be used in the main study, according to recommendations for conducting dose range-finding studies. The range-finding study should be based on the same dosing regimen as the main test, and aim to identify the maximum tolerated dose (MTD), defined as the dose inducing slight toxic effects relative to the duration of the study period (for example, abnormal behaviour or reactions, minor body weight depression or hematopoietic system cytotoxicity) but not death or evidence of pain, suffering or distress necessitating humane euthanasia.

Main Study

In the main study, in order to obtain dose response information, a complete study should include a minimum of three appropriately spaced dose levels and negative vehicle/solvent control group. If the test chemical does produce toxicity in a range-finding study, or based on existing data, the MTD and two lower dose levels generally separated by a factor of 2, but by no greater than 4, should be selected. If the test chemical does produce toxicity, the limit dose plus two lower doses should be selected.

Limit Test

The limit dose for an administration period of 14 days or more is 1000 mg/kg bodyweight/day, and for administration periods of less than 14 days, the limit dose is 2000 mg/kg/body weight/day.

Number of Animals: Minimum of 5 male animal per group.

Preparation of doses

Solid test chemicals should be dissolved or suspended in appropriate solvents or vehicles or admixed in diet or drinking water prior to dosing of the animals. Liquid test chemicals may be dosed directly or diluted prior to dosing. For inhalation exposures, test materials can be administered as gas, vapour, or a solid/liquid aerosol, depending on their physicochemical properties. Fresh preparations of the test chemical should be employed unless stability data demonstrate the acceptability of storage and define the appropriate storage conditions.

The solvent/vehicle should not produce toxic effects at the dose levels used, and should not be capable of chemical reaction with the test substances.

Administration of Doses

The test chemical is administered orally by gavage using a stomach tube or a suitable intubation cannula. Other routes of exposure (such as, drinking water, subcutaneous, intravenous, topical, inhalation, intratracheal, dietary, or implantation) may be acceptable when it leads to an exposure relevant to the expected route of human exposure. 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.

Positive & Negative Controls

Concurrent positive and negative control animals should always be used. The positive control chemicals should be known to produce structural chromosomal aberrations in vivo in spermatogonial cells under the same conditions used for the test. 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.

Examples of positive control substances include

Cyclophosphamide (monohydrate) [CAS no. 50-18-0 (CAS no. 6055-19-2)] (9)

Cyclohexylamine [CAS no. 108-91-8] (7)

Mitomycin C [CAS no. 50-07-7] (6)

Monomeric acrylamide [CAS 79-06-1] (10)

Triethylenemelamine [CAS 51-18-3] (8)

Procedure

Test chemicals are usually administered once (i.e. as a single treatment).

In the highest dose group two sampling times after treatment are used. Since cell cycle kinetics can be influenced by the test chemical, one early and one late sampling time approximately 24 and 48 hours after treatment are used.

In addition, other sampling times may be used.

A repeat dose treatment regimen can be used, such as in conjunction with a test on another endpoint that uses a 28 day administration period. However, additional animal groups would be required to accommodate different sampling times.

Prior to euthanasia, animals are injected intraperitoneally with an appropriate dose of a metaphase arresting chemical (e.g. Colcemid® or colchicine). Animals are sampled at an appropriate interval thereafter. For mice this interval is approximately 3 -5 hours.

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 should be observed for morbidity and mortality. In experiments using a prolonged treatment regimen, all animals should be weighed at least once a week, and at euthanasia. Measurements of food consumption should be made at least weekly. If the test chemical is administered via the drinking water, water consumption should be measured at each change of water and at least weekly. Animals exhibiting non-lethal indicators of excess toxicity should be euthanized prior to completion of the test period.

Chromosome Preparation

Immediately after euthanasia, cell suspensions are obtained from one, or both, testes, exposed to hypotonic solution and fixed. The cells are then spread on slides and stained.

Analysis

At least 200 well spread metaphases should be scored for each animal. Staining methods that permit the identification of the centromere should be used. 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 not less than $2n \pm 2$, where n is the haploid number of chromosomes for that species. Chromosome and chromatid-type aberrations should be recorded separately and classified by sub-types (breaks, exchanges).

Acceptability criteria

The following criteria determine the acceptability of a test:

- a) Concurrent negative control is consistent with published norms for historical negative control data, or the laboratory's historical control data.
- b) Concurrent positive controls fulfil the positivity criteria.
- c) Adequate number of cells and doses have been analyzed.
- d) If both mitosis and meiosis are 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. If only mitosis is observed, the mitotic index should be determined in at least 1000 cells for each animal.

Result assessment

At least three treated dose groups should be analysed in order to provide sufficient data for dose-response analysis. Providing that all acceptability criteria are fulfilled, a test chemical is considered a clear positive if at least one of the test doses exhibits a statistically significant increase compared with the concurrent negative control, and the increase is dose-related in at least one experimental condition. The test chemical is then considered able to induce chromosomal aberrations in spermatogonial cells of the test animals. Statistical tests used should consider the animal as the experimental unit.

If all acceptability criteria are fulfilled, a test chemical is considered a clear negative if none of the test dose exhibits statistically significant increase compared with the concurrent negative control, and there is no dose-related increase in any experimental condition. The test chemical is then considered unable to induce chromosomal aberrations in the spermatogonial cells of the test animals.

If the response is not clearly negative or positive, and in order to assist in establishing the biological relevance of a result (e.g. a weak or borderline increase), the data should be evaluated by expert judgment and/or further investigations using the existing experimental data.

For each animal the number of cells with structural chromosomal aberration(s) and the number of chromosome aberrations per cell should be evaluated. Chromatid- and chromosome-type aberrations classified by sub-types (breaks, exchanges) should be listed separately with their numbers and frequencies for experimental and control cultures. Gaps should be recorded, but not considered, when determining whether a compound induces significant increases in the incidence of cells with chromosomal aberrations along with the frequencies of polyploid cells and cells with endoreduplicated chromosomes when these events are seen.

(Reference: OECD Test Guideline: 483, 2016)

MAMMALIAN ERYTHROCYTE MICRONUCLEUS TEST

Objective

The micronucleus test is used to identify substances that cause cytogenetic damage induced by the test chemical to the chromosomes or the mitotic apparatus of erythroblasts which results in the formation of micronuclei containing lagging chromosome fragments or whole chromosomes.

Principle of the test

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. When peripheral blood is used, the blood is collected at appropriate times after treatment and smear preparations are made and stained. For studies with peripheral blood, as little time as possible should elapse between the last exposure and cell harvest. Preparations are analyzed for the presence of micronuclei. The test evaluates micronucleus formation in erythrocytes sampled either in the bone marrow or peripheral blood cells of animals, usually rodents.

Methodology Preparations

Selection of animal species

Mice, rats, or any appropriate mammalian species may be used. Young healthy animals with consistent body weight not exceeding a variation within 20 %.

Housing and feeding conditions

The temperature in the experimental animal room should be 22 °C (± 3 °C). Although the relative humidity should be at least 30% and preferably not exceed 70% other than during room cleaning, the aim should be 50-60%. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. The choice of diet may be influenced by the need to ensure a suitable admixture of a test substance when administered by this route. Animals may be housed individually, or caged in small groups of the same sex.

Preparation of the animals

Healthy young adult animals (for rodents, ideally 6-10 weeks old at start of treatment, though slightly older animals are also acceptable) 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 minimized.

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. For inhalation exposures, test chemicals can be administered as a gas, vapour, or a solid/liquid aerosol, depending on their physicochemical properties. Fresh preparations of the test substance should be employed unless stability data demonstrate the acceptability of storage.

Test conditions 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.

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 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. Positive control substances should reliably produce a detectable increase in micronucleus frequency over the spontaneous level. It is acceptable that the positive control be administered by a route different from the test substance and sampled at only a single time. Set a negative control for which a vehicle is used.

Examples of positive control substances include:

Ethyl methanesulphonate [CASRN 62-50-0]

Methyl methanesulphonate [CASRN 66-27-3]

Ethyl nitrosourea [CASRN 759-73-9]

Mitomycin C [CASRN 50-07-7]

Cyclophosphamide (monohydrate) [CASRN 50-18-0 (CASRN 6055-19-2)]

Triethylenemelamine [CASRN 51-18-3]

Colchicine [CASRN 64-86-8] or Vinblastine [CASRN 865-21-4] – as aneugens

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 interanimal 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. Negative control data should consist of the incidence of micronucleated immature erythrocytes in each animal. Concurrent negative controls should ideally be within the 95% control limits of the distribution of the laboratory's historical negative control database.

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.

Procedure

Number and sex of animals

Each treated and control group must include at least 5 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 between sexes in toxicity, then testing in a single sex will be sufficient.

Treatment schedule

Test substances may also be administered in a suitable vehicle or solvent control 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 mice or rats in one of three 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 interval(s) between samples. The use of sampling times earlier than 24 hours after treatment should be justified. When a positive response is recognized 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 .
- c) If three or more daily treatments are used (e.g. three or more treatments at approximately 24 hour intervals), bone marrow samples should be collected no later than 24 hours after the last treatment and peripheral blood should be collected no later than 40 hours after the last treatment. This treatment option accommodates combination of the comet assay (e.g. sampling 2-6 hours after the last treatment) with the micronucleus test, and integration of the micronucleus test with repeated-dose toxicity studies.

Other sampling times may be used in addition, when relevant and scientifically justified and to facilitate integration with other toxicity tests..

Dose levels

Dose range finding studies is performed if there is no suitable data available The study should aim to identify the maximum tolerated dose (MTD), defined as the highest dose that will be tolerated without evidence of study-limiting toxicity, relative to the duration of the study period (for example, by inducing body weight depression or hematopoietic system cytotoxicity, but not death or evidence of pain, suffering or distress necessitating humane euthanasia).

If there is toxicity, three dose levels are used for first sampling time. And should cover range from maximum to little or no toxicity. At later sampling time, only highest dose needs to be used. The highest dose may also be defined as a dose that produces 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 of more than 50%, but to not less than 20% of the control value).

In order to obtain dose response information, a complete study should include a negative control group and a minimum of three dose levels generally separated by a factor of 2, but by no greater than 4.

Limit Test

If the test chemical does not produce toxicity in a range-finding study or based on existing data, the highest dose for an administration period of 14 days or more should be 1000 mg/kg body weight/day, 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.

Administration of doses

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/kg body weight

In order to establish sufficient experience with the conduct of the assay prior to using it for routine testing, the laboratory should have demonstrated the ability to reproduce expected results from published data for micronucleus frequencies with a minimum of two positive control substances.

During the course of the proficiency investigations, the laboratory should establish:

- A historical positive control range and distribution, and
- A historical negative control range and distribution.

Observations:

General clinical observations of the test animals should be made and clinical signs recorded at least

once a day, preferably at the same time(s) each day and considering the peak period of anticipated effects after dosing. At least twice daily during the dosing period, all animals should be observed for morbidity and mortality.

Target tissue exposure:

A blood sample should be taken at appropriate time(s) in order to permit investigation of the plasma levels of the test substances for the purposes of demonstrating that exposure of the bone marrow occurred, where warranted and where other exposure data do not exist.

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. Small volumes of peripheral blood can be obtained, according to adequate animal welfare standards, either using a method that permits survival of the test animal, such as bleeding from the tail vein or other appropriate blood vessel, or by cardiac puncture or sampling from a large vessel at animal euthanasia. Blood cells are immediately stained supravitaly or smear preparations are made and then stained. The use of a DNA specific stain [e.g. acridine orange or Hoechst 33258 plus pyronin-Y] 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] can also be used provided that these systems have been shown to adequately work for micronucleus preparation in the laboratory.

Analysis :

All slides or samples for analysis, including those of positive and negative controls, should be independently coded before any type of analysis and should be randomised so the manual scorer is unaware of the treatment condition; such coding is not necessary when using automated scoring systems which do not rely on visual inspection and cannot be affected by operator bias. The proportion of immature among total (immature + mature) erythrocytes is determined for each animal by counting a total of at least 500 erythrocytes for bone marrow and 2000 erythrocytes for peripheral blood (42). At least 4000 immature erythrocytes per animal should be scored for the incidence of micronucleated immature erythrocytes.

Result assessment

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.

- a) The concurrent negative control data are considered acceptable for addition to the laboratory historical control database

- b) The concurrent positive controls or scoring controls should induce responses that are compatible with those generated in the historical positive control database and produce a statistically significant increase compared with the concurrent negative control.
- c) The appropriate number of doses and cells has been analysed.
- d) The criteria for the selection of highest dose are consistent.

Providing that all acceptability criteria are fulfilled, a test chemical is considered clearly positive if:

- a) At least one of the treatment groups exhibits a statistically significant increase in the frequency of micronucleated immature erythrocytes compared with the concurrent negative control,

This increase is dose-related at least at one sampling time when evaluated with an appropriate trend test, and If only the highest dose is examined at a particular sampling time, a test chemical is considered clearly positive if there is a statistically significant increase compared with the concurrent negative control and the results are outside the distribution of the historical negative control data.

Providing that all acceptability criteria are fulfilled, a test chemical is considered clearly negative if, in all experimental conditions examined:

- a) None of the treatment groups exhibits a statistically significant increase in the frequency of micronucleated immature erythrocytes compared with the concurrent negative control,
- b) There is no dose-related increase at any sampling time when evaluated by an appropriate trend test,
- c) Bone marrow exposure to the test substance(s) occurred.

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. 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 judgment 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 a 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.

(Reference: OECD Test Guideline 474, 2016)

UNSCHEDULED DNA SYNTHESIS (UDS) TEST WITH MAMMALIAN LIVER CELLS IN VIVO

Objective

The purpose of the unscheduled DNA synthesis (UDS) test with mammalian liver cells *in vivo* is to identify substances that induce DNA repair in liver cells of treated animals.

Principle of the test

The test is based on the incorporation of ³H-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 ³H TdR is usually determined by autoradiography, since this technique is not as susceptible to interference from S-phase cells as, for example, liquid scintillation counting.

Methodology

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.

Housing and feeding conditions

The temperature in the experimental animal room should be 22°C (±3°C). Although the relative humidity should be at least 30% and preferably not exceed 70% other than during room cleaning the aim should be 50-60%. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. The choice of diet may be influenced by the need to ensure a suitable admixture of a test substance when administered by this method. Animals may be housed individually, or be caged in small groups of the same sex.

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 and kept in their cages for at least five days prior to the start of the study to allow for acclimatization to the laboratory conditions.

Test substance

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.

Test conditions 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.

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. 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:

N-Nitrosodimethylamine [CAS no. 62-75-9]

N-2-Fluorenylacetamide (2-AAF) [CAS no. 53-96-3]

Other appropriate positive control substances may be used. It is acceptable that the positive control may be administered by a route different from the test substance.

Procedure

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.

Treatment schedule

Test substances are generally administered as a single treatment.

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).

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.

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 2ml/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.

Preparation of liver cells

Liver cells 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.

Determination of UDS

Freshly isolated mammalian liver cells are incubated usually with medium containing 3HTdR 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"). For more prolonged incubation times, cold chase may not be necessary. The cells are then rinsed, fixed and dried. 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.

Result assessment

Examples of criteria for positive/negative responses include:

Positive

- (i) NNG value(s) above a pre-set threshold which is justified on the basis of laboratory historical data; OR
- (ii) NNG value(s) significantly greater than concurrent control.

Negative

- (i) NNG value(s) within/below historical control threshold; OR
- (ii) NNG value(s) 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.

(Reference: OECD Test Guideline 486, 1997)

MOUSE HERITABLE TRANSLOCATION ASSAY

Objective

The purpose of mouse heritable translocation test is to detect structural and numerical chromosome changes in mammalian germ cells as recovered in first generation progeny.

Principle of the test

The types of chromosome changes detected in this test system 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 F1 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 F1 males or male offspring of F1 females. The XO-females are cytogenetically identified by the presence of only 39 chromosomes in bone marrow mitoses.

Test Animal: Sexually mature male mice should be used. No specific mouse strain is required. However, the average litter-size of the strain should be greater than 8 and be relatively constant.

Housing and Feeding

The temperature in the animal room should be 22°C ($\pm 3^\circ\text{C}$). Although the relative humidity ideally should be 50-60%, it should be at least 30% and preferably not exceed 70% other than during room cleaning. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water.

Acclimatization: The animals should be acclimatized to the laboratory conditions for at least 5 days by keeping in individual cages prior to dosing.

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 analysis of male F1 progeny. Large numbers of animals, of the order of 500 F1 males per dose level, are required.

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. Non-toxic substances should be tested up to 5 g/kg when using a single dose regimen or up to 1 g/kg/day when using a repeated dose regimen or, if these dosages are not practicable, at the highest dose attainable.

Controls

Adequate control data, derived from concurrent and historical controls, should be used.

Administration of Doses: Routes of administration are usually oral intubation or intraperitoneal injection.

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 7 days/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:

- A) Fertility testing of F1 progeny and subsequent verification of possible translocation carriers by cytogenetic analysis;
- B) Cytogenetic analysis of all male F1 progeny without prior selection by fertility testing

A) Fertility testing

Reduced fertility of an F1 individual can be established by litter size observation and/or analysis of uterine contents of female mates.

Litter size observation: F1 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 F2 progeny are recorded at birth and litters are discarded thereafter. If female F1 progeny are tested, the F2 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 F1 animals are eliminated from further testing if the test F2 litter reaches or exceeds a predetermined normal value, otherwise a second or third F2 litter is observed. F1 animals that cannot be classified as normal after observation of up to three F2 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. F1 males to be tested are mated to 2-3 females each. Conception is established by daily inspection for vaginal plugs in the morning. Females are sacrificed 14-16 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 2 cells with multivalent association constitutes the required evidence that the tested animal is a translocation carrier.

If no breeding selection has been performed, all F1 males are inspected cytogenetically. A minimum of 25 diakinesis metaphase I cells per male must be scored microscopically. Examination of mitotic metaphases, spermatogonia or bone-marrow, is required in F1 males with small testes and meiotic breakdown before diakinesis or from F1 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.

Result assessment

The mean litter size and sex ratio from parental matings at birth and weaning are reported for each mating interval.

- For fertility assessment of F1 animals, the mean litter size of all normal matings and the individual litter sizes of F1 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 F1 translocation carriers are reported.
- For cytogenetic analysis of diakinesis-metaphase I, the number and types of multivalent configurations and the total number of cells are listed for each translocation carrier.
- For sterile F1 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 F2 progeny and cytogenetic analysis results are reported. Where possible, F1 translocation carriers are preselected by fertility tests.
- There are several criteria for determining a positive result statistically:
 - one of which is a statistically significant increase in the number of translocations observed for at least one test point.
 - Another criterion may be based on the detection of a statistically significant dose related increase in the number of translocations observed.

Note: A test substance producing neither a statistically significant increase in the number of translocations observed for at least one test point nor a statistically significant dose-related increase in the number of translocations observed is considered non-mutagenic in this system.

(Reference: OECD Test Guideline: 485, 1986)

RODENT DOMINANT LETHAL TEST

Objective

The purpose of the Dominant lethal (DL) test is to investigate whether chemicals produce mutations resulting from chromosomal aberrations in germ cells. In addition, the dominant lethal test is relevant to assessing genotoxicity because, although they may vary among species, factors of *in vivo* metabolism, pharmacokinetics and DNA-repair processes are active and contribute to the response. Induction of a DL mutation after exposure to a test chemical indicates that the chemical has affected germinal tissue of the test animal. Dominant lethal (DL) effects cause embryonic or foetal death. Induction of a dominant lethal event after exposure to a test substance indicates that the substance has affected germinal tissue of the test species.

A DL assay is useful for confirmation of positive results of tests using somatic *in vivo* endpoints, and is a relevant endpoint for the prediction of human hazard and risk of genetic diseases transmitted through the germline.

Principle of the test

Dominant lethals are generally accepted to be the result of chromosomal aberrations (structural and numerical anomalies), but gene mutations and toxic effects cannot be excluded. A dominant lethal mutation is one occurring in a germ cell which does not cause disfunction of the gamete but which is lethal to the fertilised egg or developing embryo.

Test Animal and age

Mice (commonly used) or Rats are recommended as the test species. Healthy, sexually mature animals are randomized and assigned to treatment and control groups. Strains with low background dominant lethality, high pregnancy frequency and high implant numbers.

Housing and feeding conditions

The environmental conditions should meet the needs of the test species in accordance with good animal husbandry. The temperature in the animal room should be 22°C ($\pm 3^\circ\text{C}$). Although the relative humidity ideally should be 50-60%, it should be at least 30% and preferably not exceed 70% other than during room cleaning. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water.

Number of Animal

An adequate number of animals should be used, taking into account the spontaneous variation of the biological characteristics being evaluated. The number chosen should be based on the predetermined sensitivity of detection and power of significance. For example, in a typical experiment, the number of males in each group should be sufficient to provide between 30 and 50 pregnant females per mating interval.

Preparations Test substances

Solid test chemicals should be dissolved or suspended in appropriate solvents or vehicles or admixed in diet or drinking water prior to dosing of the animals. Liquid test chemicals may be dosed directly or diluted prior to dosing. For inhalation exposures, test materials can be administered as gas, vapour, or a solid/liquid aerosol, depending on their physicochemical properties. Fresh preparations of the test chemical should be employed unless stability data demonstrate the acceptability of storage and define the appropriate storage conditions.

Where appropriate, test substances should be dissolved or suspended in water or isotonic saline. Chemicals insoluble in water may be dissolved or suspended in appropriate vehicles. Normally, freshly prepared solutions or suspensions of the test substance should be employed.

Methodology

Description of the Test Procedure

Several treatment schedules are available. The most widely used requires single administration of the test substance. Other treatment schedules, such as treatment on five consecutive days, may be used if justified by the investigator.

Individual males are mated sequentially to virgin females at appropriate predetermined intervals (weekly intervals). The number of matings following treatment is governed by the treatment schedule and should ensure that germ cell maturation is adequately covered. Females are sacrificed in the second half of pregnancy, and the uterine contents are examined to determine the total number of implants and the number of live and dead embryos.

The dominant lethality of a test chemical is determined by comparing the live implants per female in the treated group with the live implants per female in the vehicle/solvent control group. The increase of dead implants per female in the treated group over the dead implants per female in the control group reflects the test-chemical-induced post-implantation loss.

Test Conditions

Route of administration

The usual routes of administration are oral subcutaneous, intravenous, topical, inhalation, oral (by gavage), or implantation may be chosen as justified. Intra-peritoneal injection is not normally recommended.

Dose level

If a preliminary range-finding study is performed because there are no suitable data already available to aid in dose selection, 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 study should aim to identify the maximum tolerated dose (MTD), defined as the highest dose that will be tolerated without evidence of study-limiting toxicity, relative to the duration of the study period (for example, abnormal

behaviour or reactions, minor body weight depression or hematopoietic system cytotoxicity), but not death or evidence of pain, suffering or distress necessitating humane euthanasia.

A complete study should include a negative control group and three dose levels generally separated by a factor of 2 but not greater than 4 should be used. The highest dose for a single administration should be 2000mg/kg body weight produce signs of toxicity (e.g. slightly reduced fertility). However, in an initial assessment of dominant lethality a single high dose may be sufficient.

For not-toxic substances, the limit dose for an administration period of 14 days or more is 1000 mg/kg body weight/day, and for administration periods of less than 14 days the limit dose is 2000 mg/kg body weight/day.

Control

Positive and negative (vehicle) controls should be included in each experiment. When acceptable positive control results are available from experiments conducted recently (within the last 12 months) in the same laboratory these results can be used instead of a concurrent positive control. Positive control substances should be used at a dose which demonstrates the test sensitivity. The positive control substances should be known to produce DLs under the conditions used for the test. Except for the treatment, animals in the control groups should be handled in an identical manner to animals in the treated groups.

The following are examples of the type of substance which might be used as a positive control

- triethylenemelamine
- cyclophosphamide
- ethylmethanesulphonateMonomeric Acrylamide
- Chlorambucil

Negative control animals, treated with solvent or vehicle alone, and otherwise treated in the same way as the treatment groups, should be included for every sampling time

Test performance

Competence in this assay should be established by demonstrating the ability to reproduce dominant lethal frequencies from published data with positive control substances and vehicle controls and obtaining negative control frequencies that are consistent acceptable range of data or with the laboratory's historical control distribution.

Individual males are mated sequentially at appropriate predetermined intervals to one or two virgin females. Females should be left with the males for at least the duration of one estrus cycle or alternatively 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 germ cell maturation is adequately covered.

General clinical observations of the test animals should be made and clinical signs recorded at least once a day, preferably at the same time(s) each day and considering the peak period of anticipated effects after dosing. At least twice daily during the dosing period, all animals should be observed for morbidity and mortality.

Females are sacrificed in the second half of pregnancy and uterine contents are examined to determine the number of implants and live and dead embryos. The ovaries may be examined to determine the number of corpora lutea

Data and Reporting Treatment of Results

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. The mating interval, dose level for males, and the numbers of live implants and dead implants should be enumerated for each female. The post-implantation loss is calculated by determining the ratio of dead to total implants from the treated group compared to the ratio of dead to total implants from a control group. Pre-implantation loss can be calculated as the difference between the number of corpora lutea and the number of implants or as a reduction in the average number of implants per female in comparison with control matings. Where pre-implantation loss is estimated, it should be reported.

The Dominant Lethal factor is estimated as: $(\text{post-implantation deaths} / \text{total implantations per female}) \times 100$.

Data are evaluated by appropriate statistical methods.

Differences among animals within the control and treatment groups should be considered before making comparisons between treated and control groups.

Results assessment

There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of dominant lethals.

- a) Concurrent negative control is consistent with published norms for historical negative control data, and the laboratory's historical control data if available.
- b) Concurrent positive controls induce responses that are consistent with published norms for historic positive control data, or the laboratory's historical positive control database, if available, and produce a statistically significant increase compared with the negative control.
- c) Adequate number total implants and doses have been analysed.
- d) The criteria for the selection of top dose are consistent

Providing that all acceptability criteria are fulfilled, a test chemical is considered a clear positive if:

- a) at least one of the test doses exhibits a statistically significant increase compared with the concurrent negative control;
- b) the increase is dose-related in at least one experimental condition (e.g. a weekly mating interval) when evaluated with an appropriate test; and,
- c) any of the results are outside of the acceptable range of negative control data, or the distribution of the laboratory's historical negative control data.

The test chemical is then considered able to induce dominant lethal mutations in germ cells of the test animals.

Providing that all acceptability criteria are fulfilled, a test chemical is considered a clear negative if:

- a) none of the test doses exhibits a statistically significant increase compared with the concurrent negative control;
- b) there is no dose-related increase in any experimental condition; and
- c) all results are within acceptable range of negative control data, or the laboratory's historical negative control data.

The test chemical is then considered unable to induce dominant lethal mutations in germ cells of the test animals.

A test substance which does not produce a statistically significant dose-related increase in the number of dominant lethals is considered non- mutagenic in this system. Both biological and statistical significance should be considered together in the evaluation.

Interpretation of results

A positive dominant lethal assay suggests the possible genotoxicity of a test substance in the germ cells of the treated sex of the test species.

A negative result suggests that, under the conditions of the test, the test substance may not be genotoxic in the germ cells of the treated sex of the test species.

(Reference: OECD Test guideline 478, 2016)

METABOLISM

(ADME - ABSORPTION DISTRIBUTION METABOLISM AND EXCRETION)

Metabolism in Rat

Feeding Studies in Livestock (Goat/Cow & Hen/Poultry) Including Metabolism in Livestock

METABOLISM IN RAT

Objective

To obtain information on Absorption, Distribution, Biotransformation & Excretion to aid in understanding its mechanism of toxicity.

Test animal: Rat

Age: Young healthy adult animals (6-12 weeks) should be used. The weight variation of individual animals should not exceed $\pm 20\%$ of the mean weight of the test group.

Number/sex: Minimum four animals of one sex per dose. The use of both sexes (4M+ 4F) should be considered if there is evidence to support significant sex related differences.

Acclimatization: Animals should generally be housed individually during the testing period in the appropriate metabolism cages.

Housing and feeding condition

Rats should be housed at temperature $22 \pm 3^\circ \text{C}$ and relative humidity 50 to 60% with 12 hours light and dark cycle. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water.

Test substance

Radiolabelled compound - The radiolabel should be located in a core portion of the molecule which is metabolically stable. For complex molecules subject to cleavage more than one label site should be considered to ensure adequate identification of metabolites

Route: Preferably Oral, if any other route (dermal/ inhalation) is used justification should be given .

Administration

Test substance should be dissolved or suspended homogenously in the same vehicle employed for the other oral gavage toxicity studies performed with the test substance. Rationale for the choice of vehicle should be provided. Below are the different routes for administration of dose:

1. **For oral:** The maximum volume of liquid to be administered by oral gavage at one time depends on the size of the test animals, the type of dose vehicle, and whether or not feed is withheld prior to administration of the test substance. The rationale for administering or restricting food prior to dosing should be provided. Normally the volume should be kept as low as practical for either aqueous or non-aqueous vehicles. Dose volumes should not normally exceed 10 mL/kg body weight for rodents.
2. **For Dermal:** One or more dose levels for the test substance should be used in the dermal treatment. The test substance (e.g., neat, diluted or formulated material containing the test chemical which is applied to the skin) should be the same (or a realistic surrogate) as that to which humans or other potential target species might be exposed.
3. **For inhalation:** A single concentration (or more if needed) of test substance should be used. Inhalation treatments are to be conducted using a “nose-cone” or “head-only” apparatus to prevent absorption by alternate routes of exposure.
4. **For Intravenous:** Intravenous (IV) administration of the test substance and measurement of the test substance in blood and/or excreta may be used to establish bioavailability or relative oral absorption. For the IV study, a single dose (usually equivalent to but not to exceed the lower oral dose – see dose selection) of test substance is administered using an appropriate vehicle. This material should be administered in a suitable volume (e.g. 1 mL/kg bw) at the chosen site of administration to at least four animals of the appropriate sex. A fully dissolved or suspended dose preparation is necessary for IV administration of the test substance.

Pilot Study

It is done for the selection of experimental parameters for the toxicokinetics studies (e.g. metabolism, mass balance, analytical procedures, dose finding, exhalation of CO₂ etc.)

Usually a single oral dose is administered. The dose should be non-toxic, but high enough to allow for metabolite identification in excreta (and plasma, if appropriate) as well as to meet the stated purpose.

Main Study Dosage

Minimum two doses should be used. Both doses should be high enough to allow for metabolite identification in excreta (and plasma, if appropriate). Lower dose should be some fraction of the higher dose and have some relevance to the NOAELs in repeat dose studies. Information from available toxicity data should also be considered for dose selection. In some circumstances, repeated dose administration may be needed to address fully the potential for accumulation and/or

persistence or changes in TK (i.e. for instance; enzyme induction & inhibition). For test substances of **low** toxicity, a maximum dose of 1000 mg/kg body weight should be used.

Measurements

Mass balance

It is determined by summation of the percent of the administered (radioactive) dose excreted in urine, faeces and expired air, and the percent present in tissues, residual carcass and cage wash.

Absorption

An initial estimation of absorption can be achieved by excluding the percentage of dose in the gastro- intestinal (GI) tract and/or faeces from the mass balance determination. If a biliary excretion study is undertaken, the oral route of administration is typically used. In this study, the bile ducts of at least four animals of the appropriate sex (or of both sexes, if warranted) should be cannulated and a single dose of the test substance should be administered. Following administration of the test substance, excretion of radioactivity/test substance in bile should be monitored as long as necessary to estimate the percentage of the administered dose that is excreted via this route. For the calculation of percent absorption:

Percent absorption = (Amount in bile + urine + expired air+ carcass without GI tract contents)/ amount administered X 100

Note: If a significant amount is in the bile within an hour or so of dosing it will not have been systemically available to organs other than the liver and to an extent blood cells.

Bioavailability

The purpose of these studies is to

- A) Obtain estimates of basic TK parameter [e.g. C max, Tmax, half life ($t_{1/2}$), AUC] for the test substances. These studies may be conducted at one dose or, more likely, at two or more doses. Dose setting should be determined by the nature of the experiment and/or the issue being addressed. Kinetic data may be needed to resolve issues such as substances bioavailability and/or to clarify the effect of dose on clearance (e.g. to clarify whether clearance is saturated in a dose- dependent fashion).

For these studies a minimum of four animals of one sex per dose group should be used should be considered if there is evidence to support significant sex-related differences in toxicity.

Following administration of the test substance (radiolabelled), blood samples should be obtained from each animal at suitable time points using appropriate sampling methodology. Samples should be analyzed for each individual animal. In some circumstances (e.g., metabolite characterization), it might be necessary to pool samples from more than one animal. If a radiolabelled substance is used, analysis of total radioactivity present might be adequate. If so, total radioactivity should be analyzed in whole blood and plasma or plasma and red blood cells to allow calculation of the blood/plasma ratio.

B) Obtain course information to address questions related to issues such as toxic mode of action, bioaccumulation and biopersistence via determination of levels of test substances in various tissues.

C) Reasons for performing other tissue kinetic studies might include:

1. Evidence of extended blood half-life, suggesting possible accumulation of test substance in various tissues or
2. Interest in seeing if a steady state level has been achieved in specific tissues (e.g. in repeated dosing studies, even though an apparent blood steady state level of test substances may have been achieved, there may be interest in ascertaining that a steady state level has also been attained in target tissues)

For these types of time-course studies, an appropriate oral dose of test substances should be administered to a minimum of four animals per dose per time point and the time course of distribution monitored in selected tissues. Only one sex may be used, unless gender specific toxicity is observed. Whether total radioactivity or parent substance and/or metabolism are analyzed will also depend on the issue being addressed. Assessment of tissue distribution should be made using appropriate techniques.

D) Studies addressing the possible effects of enzyme induction/inhibition or biotransformation of test substance under may be needed under one or more of the following cases:

1. Available evidence indicated a relationship between biotransformation of test substance and enhanced toxicity;
2. The available toxicity data indicate a non-linear relationship between dose and metabolism;
3. The results of metabolite identification studies identification of a potentially toxic metabolite that might have been produced by an enzyme pathway induced by the test substances;
4. In explaining effects which are postulated to be linked to enzyme induction phenomena;
5. If toxicologically significant alterations in the metabolic profile of the test substances are observed through either in vitro or in vivo experiments with different species or conditions, characterization of the enzyme(s) involved may be needed (e.g. Phase I enzymes such as isoenzymes of the Cytochrome P450-dependent mono-oxygenase system, Phase II enzymes such as isoenzymes of sulfotransferase or uridine diphosphate glucuronosyl transferase, or any other relevant enzymes). This information might be used to evaluate the pertinence of species to species extrapolations.

Tissue Distribution

Knowledge of tissue distribution of a test substance and/or its metabolites is important for the identification of target tissues, and understanding of the underlying mechanisms of toxicity, and in order to get information on the potential for test substance and metabolite accumulation and persistence. The percent of the total (radioactive) dose in tissues as well as residual carcass should at a minimum be measured at the termination of the excretion experiment (e.g typically up to 7 days post dose or less depending on the test substance specific behavior).

Tissues like liver, fat, GI Tract, kidney, spleen whole blood, residual carcass, target organ tissues and any other tissues like thyroid, erythrocytes, reproductive organs, skin, eye should be given significance in the toxicological evaluation of test substance. Analysis of additional tissues at the same time points should be considered to maximize utilization of animals and in the event that target organ toxicity is observed in sub-chronic or chronic toxicity studies. The (radioactive) residue concentration and tissue-to-plasma blood ratios should also be reported.

For routes of exposure other than oral, specific tissues should be collected and analyzed, such as lungs in inhalation studies and skin in dermal studies.

When no or very low levels of radiolabel / substance is detected in tissues at study termination , care should be taken in order to prevent misinterpretation of the data. In this type of situation, tissue distribution should be investigated at the time of test substance (and/or metabolite) peak plasma/blood concentration (T_{max}) or peak rate of urinary excretion, as appropriate. Furthermore, tissue collection at additional time points may be needed to determine tissue distribution of the test substance and/or its metabolites, to evaluate time dependency (if appropriate), to aid in establishing mass balance.

Note: Data is needed on key tissues when there is a significant amount of compound present T_{max} or $2 \times T_{max}$, not when 95% has been excreted on day 7.

Metabolism

Excreta (and plasma, if appropriate) should be collected for identification and quantification of unchanged test substance and metabolites. Compounds which have been characterized in excreta as comprising 5% or greater of the administered dose should be identified by exact structural determination of the components.

Pooling of excreta to facilitate metabolite identification within a given dose group is acceptable. Profiling of metabolites from each time period is recommended. However, if lack of sample and/or radioactivity precludes this, pooling of urine and faeces across several time points is acceptable but pooling across sexes or doses is not acceptable.

Identification is accomplished either by co-chromatography of the metabolite with known standards using two dissimilar system or by techniques capable of positive structural identification such as mass spectrometry, nuclear magnetic resonance (NMR).

Excretion

The rate and extent of excretion of the administered dose should be determined by measuring the percent recovered (radioactive) dose from urine, faeces and expired air.

Each animal is to be placed in a separate metabolic unit for collection of excreta (urine, faeces and expired air). At the end of each collection period, the metabolic units should be rinsed with appropriate solvent (this is known as the “cage wash”) to ensure maximum recovery of the test substance (radioactivity). Collection of excreta should be terminated at 7 days, or at least 90% of the administered dose had been recovered, whichever occurs first.

The total quantities of substance (radioactivity) in urine are to be determined for at least two time points on day 1 of collection, one of which should be at 24 hr post dosing, and daily thereafter until study termination. The selection of more than two sampling points on day one (e.g 6,12 and 24 hr) is encouraged. The result of pilot studies should be analysed for information on alternate or additional time points for collection. A rationale should be provided for the collection schedules.

The total quantities of test substance (radioactivity) in faeces should be determined on a daily basis beginning at 24 hr post-dosing until study termination, unless pilot studies suggest alternate or additional time points for collection. A rationale should be provided for alternative collection schedules.

The collection of expired CO₂ and other volatile materials may be discontinued when less than 1% of the administered dose is found in the exhaled air during a 24-h collection period.

Result assessment

All data should be summarized and tabulated with appropriate statistical evaluation. In addition, the following information is to be included if applicable:

1. Quantity and percent recovery of radioactivity in urine, faeces, expired air, and urine and faeces cage wash.
2. Tissue distribution reported as percent of administered dose and concentration, and tissue to blood or tissue to plasma ratios;
3. Material balance developed from each study involving the assay of body tissues and excreta;
4. Expect a certain minimum recovery (90 – 110%) or require justification. Plasma concentration and toxicokinetic parameter (bioavailabilities, AUC, C_{max}, T_{max}, clearance, half-life) after administration by the relevant routes of exposure;
5. Rate and extent of absorption of the test substance after administration by the relevant routes of exposure.
6. Quantities of the test substance and metabolites (reported as percent of the administered dose) collected in excreta;
7. Individual animal data for all measurement end point (e.g dose administration, percent recovery, concentration, TK parameters etc)
8. A figure with the proposed metabolic pathways and the molecular structure of the metabolites.

(Reference: OECD Test Guideline 417, 2010)

FEEDING STUDIES IN LIVESTOCK INCLUDING METABOLISM

Objective

Study used to determine the qualitative (Identification of major components of the residue in the edible tissues) and quantitative (Estimate of total residue in the edible livestock commodities as well as the excreta) metabolism and/or degradation of the active ingredient resulting from pesticide use in feedstuffs, direct application to livestock. The study provides-

- Provide an estimate of total residues in the edible livestock commodities, as well as the excreta.
- Identify the major components of the terminal residue in the edible tissues, thus indicating the components to analyzed in residue quantification studies (i.e., the residue definitions for both risk assessment and enforcement).
- Elucidate a metabolic pathway for the pesticide in ruminants and poultry.
- Provide evidence whether or not a residue should be classified as fat soluble.

Test Animals: Livestock metabolism studies are generally carried out in ruminants (cows or Lactating goats) and poultry (chickens-Laying hens) This study in India should be carried out on lactating goats/and chicken laying hen/swine.

Number of test animals: Ruminant metabolism study can be carried out on a single animal. For poultry, the use of ten birds per experiments (or dose) is recommended.

Acclimatization: The acclimatization period should be such to ensure that the livestock maintain good levels of milk and egg production prior to dosing in the study.

Test Substance: Stably positioned radiolabelled (preferably ^{14}C radioisotope) active ingredients should be used so that all significant moieties or degradation product can be tracked.

Dosage

The minimum dosage used in livestock oral metabolism studies should approximate the level of exposure expected from feeding of treated crops with highest observed residues. Exaggerated dosage are usually needed to obtain sufficient residue in the tissues for characterization and/or identification. Livestock should be dosed orally at least at a level of 10mg/kg in the diet. Ruminants should be dosed daily for at least five days, and poultry for at least seven days.

Dose administration: Livestock should be dosed orally via a balling gun, capsule or gavage to ensure complete administration of the active ingredient. Rational for use of vehicle should be given, if any.

Time of Sacrifice

Animals should preferentially be sacrificed at 6-12 hour after the last dose. However, under no circumstances should the time of sacrifice be later than 24 hours after the last dose.

Sampling of animal parts

Excreta, milk and eggs should be collected twice daily (if applicable). Tissues to be collected should include at least muscle (loin and flank muscles in ruminant and leg and breast muscle in poultry), liver (whole organ for the goat and poultry), kidney (ruminants only), and fat (renal, omental, subcutaneous). The Total radioactive residue (TRR) should be quantified for all tissues, excreta, milk, and eggs. For milk, the fat fraction should be separated from the aqueous portion by physical means and the TRR in each fraction quantified. Characterization and identification of the residue in urine and feces frequently facilitates characterization of the lower levels of residue found in tissue, but is not required. The radioactivity in the different muscle and fat types should be quantified separately. If the concentration of radioactivity is similar within tissue type, the samples may be pooled before metabolite analysis. Gross pathology of the collected organs should be performed. Abnormalities should be recorded and reported.

Analytical Phase

In the analytical phase of a livestock metabolism study, the animal parts to be analysed are sampled, chopped or homogenized and the TRR determined. Full accountability of all radioactivity must be ensured.

Extractable Residues

Samples are extracted with a series of solvents and/or solvent systems (including aqueous) with various polarities and other characteristics depending on the nature of the expected residues. These initially obtained residues are defined as extractable residues. The strategy for characterization and/or identification of extractable residues are summarized in table 1.

Identification: It refers to the exact structural determination of components of the total radioactive residue. Typically, identification is accomplished either by co-chromatography of the metabolite with known standards using two dissimilar systems or by technique capable of positive structural identification such as MS, NMR etc. Identification by co-chromatography should be obtained using two dissimilar, analytically independent systems such as reverse and normal phase thin layer chromatography (TLC) and high performance layer chromatography (HPLC).

Characterization: Refer to elucidation of the general nature/characteristics of the radioactive residue short of metabolite identification. Terms used to characterize residue include organosoluble, water or aqueous soluble, neutral, acidic or basic, polar, nonpolar, nonextractable etc. Characterization may also involve description of chemicals moieties known to be present in the molecule based on conversion to a common structure or due to reactivity with particular reagents. The degree of characterization refers to how close the assignment comes to structural identification.

Table 1. Strategy for Identification and Characterization of Extractable Residues from Metabolism in Livestock Studies

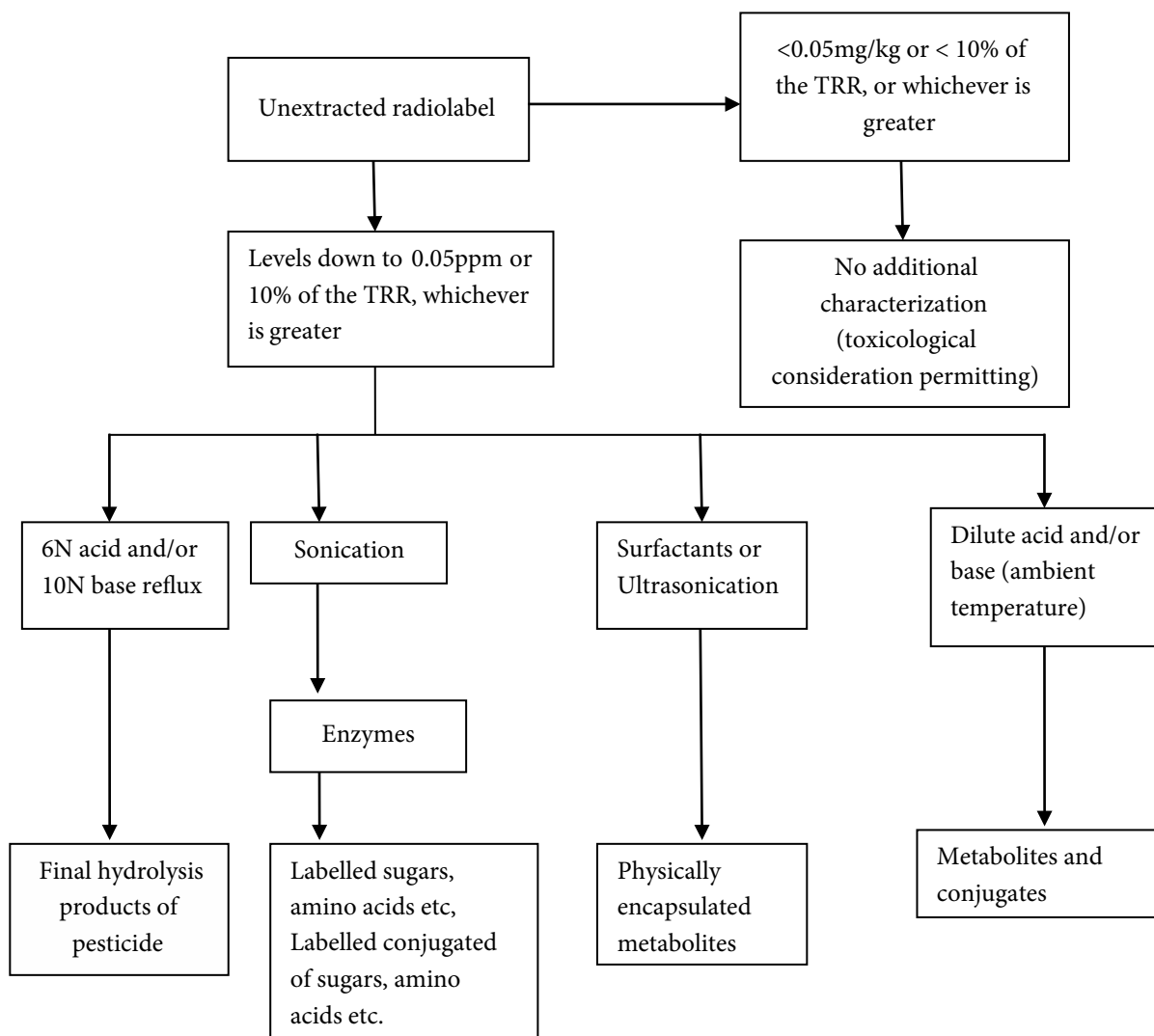
Relative amount (%)	Concentration (mg/kg)	Required Action
< 10	< 0.01	No action if no toxicological concern.
< 10	0.01 – 0.05	Characterize, Only attempt to confirm identity if straightforward, e.g., a reference compound is available or the identification is known from a previous study.
< 10	> 0.05	Characterization/identification needs to be decided on a case-by- case basis taking into account how much has been identified.
> 10	< 0.01	Characterize. Only attempt to confirm identity if straightforward, e.g., a reference compound is available or the identification is known from a previous study.
> 10	0.01 – 0.05	Significant attempts to identify should be made especially if needed to establish a pathway, ultimately characterization might be accepted.
> 10	> 0.05	Identify using all possible means.
> 10	> 0.05 Unextracted radiolabel	Unextractable radiolabel

Unextractable residues

Three situation in which radioactive residues are observed to be non-extractable are given below:

- Incorporation into biomolecules (i.e., amino acids, sugars, etc.).This occurs when the test compound is degraded into small (usually one or two) carbon units which enter the carbon pool of endogenous compounds used in the biosynthesis of new cell constituents by the animal.
- Chemical reaction or physico-chemical tight-binding with appropriate moieties in biomolecules to form bound residues, which can be released via other chemical reactions (e.g., enzymatic or acid/base hydrolysis).
- Physical encapsulation (trapping) or integration of radioactive residues into livestock matrices. Release of residues in this situation may require solubilisation of the tissue, usually by drastic treatment with base, although use of surfactants may allow the radioactive residue to be released under less severe conditions.

Characterization and identification of non-extractable and bound residues as shown in figure below:



Storage Stability

Determination as to whether sample integrity was maintained during collection, sample preparation, and storage should be made. Such analysis should show that the basic profile of radiolabelled residue has not changed through out the duration of the study.

Storage stability data are not normally necessary for samples analyzed within six months of collection, provided evidence is given that attempts were made to limit degradation of residues by appropriate storage of matrices and extracts during the analytical portion of the study. In those cases where a metabolism study cannot be completed within six months of sample collection, evidence should be provided that the identity of the residue did not change during the period between collection and final analysis. This can be done by analysis of representative substrates early in the study and at its completion. The substrate should be the item stored, i.e. if the matrix extract is used through out the study and the matrix is not extracted later in the study, the stability of extract should be shown.

Ideally metabolism samples should be stored at/or below -18°C. Storage under any other condition needs to be recorded and justified.

Result assessment

- The routes or pathways, mechanisms involved and extent or degree of metabolism observed in animals.
- The nature, amount, and distribution of the TRR in the sampled tissues, eggs and milk at the time of sample collection.
- Based on the results of studies conducted on radiolabelled animal samples to prove the extraction efficiency, the capability of developed and available enforcement analytical methodology to determine the identified components of the terminal residue, whether free or non-extractable/conjugated, and the capability of the same or modified analytical methodology to determine all components of the ROC, whether free or non-extractable/conjugated in the animal tissue, milk or eggs.

Note: Non-ruminant (swine) metabolism studies maybe necessary if the rat metabolism is significantly different than the ruminant or poultry metabolism.

(Reference: OECD Test guidelines 503, 2007)

IMMUNOTOXICITY

Immunotoxicity Study

IMMUNOTOXICITY STUDY

Objective

To evaluate the ability of the test substance to induce dysfunction or inappropriate suppressive or stimulatory responses in components of the immune system of the test animal that may likely to arise from subchronic exposure of the test substance.

Principle of the test

The test substance is administered in graduated doses to several groups of experimental animals, one dose level per group, for a period of at least 30 days. Animals are observed daily to detect any signs of clinical toxicity. Animals are sacrificed at the end of dosing period and parameters relating to immune system are examined. In some tests, sensitization of animals with an appropriate immunogen is required.

Test animals: Both mouse and rat are the preferred species.

Age/Sex: Young healthy adult animals free of parasites and pathogens of single sex preferably female (Nulliparous and non-pregnant) should be used. Other sex can be used if sex related sensitivity is anticipated. The weight variation of the animals used should not exceed $\pm 20\%$ of the mean weight at the commencement of the study.

Housing and feeding

Rats should be housed at temperature $22(\pm 3)^{\circ}\text{C}$ and relative humidity 30 to 70% with 12 hours light and dark cycle.

Acclimatization: Test animals to be acclimatized to the laboratory conditions for at least 7 days prior to the test.

Number of test animals

At least 10 animals should be used at each dose level and in control group for each immunological parameters. A satellite control group of at least 20 animals, treated with the test substance at the high dose for 30 days, should be included for possible observation of reversibility, persistence, or delayed occurrence of immunotoxic effects. Inclusion of positive control group of 5 animals per assay, dosed with known immunosuppressant is required to verify assay sensitivity.

Dose group

At least three dose levels and a concurrent control should be used. The highest dose level should not produce significant stress, malnutrition, or fatalities, but ideally should produce some measureable sign of general toxicity. The lowest dose level ideally should not produce any evidence of immunotoxicity.

Route of administration: The test substance should be normally administered for at least 30 days orally, by gavages or via diet or drinking water.

Observations

- All animals should be observed for at least 30 days. Animals in satellite group should be kept for further 30 days without treatment to detect recovery from or persistence of immunotoxic effects, or for the detection of delayed occurrence of immunotoxic effects.
- A careful cageside examination should be made on each animal for at least once each day. Clinical signs of toxicity should be recorded as they are observed, including the time of onset, degree and duration. Cageside observation should include, but not be limited to, changes in: skin & fur, eyes & mucus membranes, respiratory system, autonomic & central nervous system, circulatory system, somatomotor activity, behavior pattern, resistance to infection.
- Food and water consumption should be determined weekly.
- Animals should be weighed just prior to dosing, weekly thereafter, and just prior to sacrifice.
- All moribund and dead animals should be euthanized and necropsy should be conducted.

Clinical examination

At the end of test period, 10 control and 10 animal per dose group should be fasted overnight, prior to sacrifice and subjected to the following examination-

- Hematology determination: Hemoglobin concentration, Hematocrit, Erythrocyte count, Total and differential count, Platelets count
- Clinical biochemistry determination: glucose, serum glutamic-pyruvic transaminase, urea nitrogen, albumin, total serum protein, globulin, RFT, LFTetc

Gross necropsy

All animals should be subjected to a limited gross necropsy which includes body weight determination, wet weight determination of thymus and spleen as soon as possible after dissection to avoid drying. Absolute and relative Organ weight of Thymus, spleen, popliteal lymph nodes liver, lungs, Brain kidneys, bone marrow, lymph nodes, Gut Associated Lymphoid Tissue (GALT), adrenals, pituitary, ovary or testes and all gross lesions from 10 animals for each dose level and control group animal should be preserved in a suitable medium for possible histopathological examination.

Immunotoxicity tests

Tier 1: Components of the immune system, and the studies suggested to screen the potential of a test substance to effect them are:

1. Humoral immunity: The aspect of immunity that is mediated by macromolecules found in extracellular fluids such as secreted antibodies, complement proteins and certain antimicrobial peptides. Humoral immunity is so named because it involves substances found in the humour or body fluids. One of the following assays should be done to assess humoral immune response:
 - (i) Antibody plaque-forming cell (PFC) assay: It is used to demonstrate the effects of subchronic exposure to a test substance on antibody producing cell enumerated from spleen. The T cell-dependent antigen, sheep red blood cell (SRBC) , should be injected intravenously, usually at 26 days after the first dosing with the test substance. Animal should be evaluated for the optimum day for PFC formation after immunization. The activity of each new batch of complement, spleen cell viability should be determined.
 - (ii) Immunoglobulin quantification: Effects of test substance on the antibody response to antigen should be determined. Test animals are immunized with an appropriate thymus-dependent antigen, followed by a secondary challenge with antigen at an appropriate time. IgG & IgM titers in serum of each test animals should then be determined using sufficiently sensitive technique like enzyme-linked immunosorbent assay (ELISA).
2. Specific cell-mediated immunity: It is an immune response that does not involve antibody but rather involves the activation of antigen-specific cytotoxic T- lymphocyte, and the release of various cytokines in response to an antigen. One of the following three assays is required for an assessment of subchronic (30 day) exposure to a test substance on specific cell-mediated immunity.
 - (i) One-way mixed lymphocyte culture (MLC) assay: it is used to demonstrate the effect of subchronic exposure to a test substance on lymphocyte blastogenesis as stimulated by allogenic lymphocytes. Lymphocyte blastogenesis is measured by incorporation of radiolabel into DNA.
 - (ii) Delayed-type hypersensitivity (DTH) reaction: It is In-vivo assay useful in demonstrating the effect of test substance on an induced DTH reaction in test animals. In general, test animals are sensitized , later challenge with an appropriate thymus- dependent antigen. At 24-48 hours after challenge, DTH reactions in treated animals are compared to DTH reactions in control group animals.
 - (iii) Cytotoxic T-lymphocyte (CTL) assay: demonstrating the effects of subchronic exposure to a test substance on the generation of cytotoxic T- lymphocytes. In this assay, an appropriate allogenic tumor is used for CTL induction (either in-vivo or in- vitro). Splenocytes from treated and untreated test animals are then incubated with ⁵¹Cr-labeled allogeneic tumor cells. The amount of radiolabel released from the target cells after incubation with the effector cells for four hours is used as a measure of T- lymphocyte cytotoxicity.

3. Non-specific cell-mediated immunity: Immune response which involves activation of phagocytes like Macrophages and Natural Killer cells. Following assays are involved in assessing the non-specific cell-mediated immunity:

- (i) Natural killer cell activity: The microculture method of Reynolds and Herberman is recommended to demonstrate effects of subchronic (30 day) exposure to a test substance on spontaneous cytotoxic activity. In this assay, splenocytes from treated and untreated test animals are incubated with ⁵¹Cr-labeled YAC-1 lymphoma cells. The amount of radiolabel released from the target cells after incubation with the effector cells for four hours is used as a measure of natural killer cytotoxicity.
- (ii) Macrophages: Assays should be performed at 30 days after dosing to evaluate effects of subchronic (30 day) exposure of test animals to a test substance on macrophage numbers and on macrophage phagocytosis. It should include total and differential count of resident peritoneal cell numbers, an evaluation of phagocytosis of particles (e.g., fluorescent latex beads) by peritoneal cells, in the presence and absence of augmentation factors (e.g., gamma interferon or bacterial lipopolysaccharide).

Tier 2: If dysfunction or impairment of the components of the immune system are indicated in any of the Tier I immunotoxicity tests, or if the data from the Tier I tests cannot be definitively interpreted, or if data from other sources indicate that the test substance, or structurally related substances (including metabolites and degradation products), are immunotoxic. then the applicable Tier II immunotoxicity studies shall be required. Tier 2 investigations are required to:

- (i) Provide information on the time-course of recovery from each significant adverse immunotoxic effect observed in tier I studies.
- (ii) Indicate whether the observed effects may result in impaired host resistance to infectious microbial agents and/or to tumor cell challenge.
- (iii) To provide additional information essential for a full evaluation of potential risks associated with the immunotoxicity of a test substance.

Selection of the appropriate tests to be done in tier II studies will depend on:

- The particular test or tests in tier I in which significant immunotoxic effects were observed.
- The availability of data from other sources which indicate that the test substance, or structurally-related substances, are immunotoxic.
- Whether data from initial testing in tier II indicate that expanded testing is required, using additional tests in this tier.

Other immunotoxicological studies: Additional tests may be required if considered necessary for a full evaluation of potential risks associated with the immunotoxicity of the test substance. These include, but are not necessarily limited to, available tests that are designed to evaluate effects of a substance on:

- Lymphoid organs and tissues (using enzyme and immune histochemistry).

- Serum complement.
- Antibody response to T-independent antigens.
- Enumeration of subpopulations of T- and B- lymphocytes.
- Granulocyte function.
- Bone marrow function.
- Lymphokines.
- Plaque-forming cell response to T-independent antigens.
- Mitog stimulation of lymphocyte blastogenesis.
- In vivo popliteal lymph node enlargement after injection of allogeneic lymphocytes.
- Hormones.
- Immune system development.
- Macrophage development, activation and function.
- Induction of autoimmunity.

For Developmental Immunotoxicity

Objective

To assesses the potential impact of chemical exposure on the developing immune system.

Procedure

Total of 20 pups per group (10 males and 10 females per group; one per litter, where possible). Additional pups may be required from the control group to act as positive control animals in the T- cell dependant antibody response assay (TDAR) at PND 56 ± 3 , in case of specific concern, e.g. if a chemical is suspected to be a neurotoxicant, immunotoxicant or reproductive toxicant. These pups may be used for examinations at different time points or for the evaluation of supplementary endpoints.

Assessment of potential developmental immunotoxicity

At PND 56 (± 3 days), 10 male and 10 female from each treatment group (1 male or 1 female per litter; all litters represented by at least 1 pup; randomly selected) should be used in a T-cell dependant antibody response assay, i.e. the primary IgM antibody response to a T-cell dependent antigen, such as Sheep Red Blood Cells (SRBC) or Keyhole Limpet Hemocyanin (KLH), consistent with current immunotoxicity testing procedures. The response may be evaluated by counting specific plaque-forming cells (PFC) in the spleen or by determining the titer of SRBC- or KLH-specific IgM antibody in the serum by ELISA, at the peak of the response. Responses typically peak four (PFC response) or five (ELISA) days after intravenous immunization. If the primary antibody response is assayed by

counting plaque-forming cells, it is permissible to evaluate subgroups of animals on separate days, provided that: subgroup immunization and sacrifice are timed so that PFCs are counted at the peak of the response; that subgroups contain an equal number of male and female offspring from all dose groups, including controls; and that subgroups are evaluated at approximately the same postnatal age. Exposure to the test substance will continue until the day before collecting spleens for the PFC response or serum for the ELISA assay. Terminated pups are subjected to gross necropsy. For up to 10 pups per sex per group, from as many litters as possible, brain, spleen, and thymus should be weighed and retained under appropriate conditions.

Histopathology

- **animals:** Gross abnormalities and target tissues should be saved for possible histological examination. Following parameters viz.
- Serum IgM antibody titres (sensitization to SRBC or KLH), or splenic IgM PFC units (sensitization to SRBC);
- Performance of the TDAR method should be confirmed as part of the optimisation process by laboratory setting up the assay for the first time, and periodically (e.g. yearly) by all laboratories;
- Discussion of the overall interpretation of the data based on the results, including a conclusion of whether or not the chemical caused developmental immunotoxicity and the NOAEL.

Suppression or enhancement of immune function as assessed by TDAR (T-cell dependent antibody response), should be evaluated in the context of all observations made. Significance of the outcome of TDAR may be supported by other effects on immunologically-related indicators (e.g. bone marrow cellularity, weight and histopathology of lymphoid tissues, lymphocyte subset distribution). Effects established by TDAR may be less meaningful in case of other toxicities observed at lower exposure concentrations.

(Reference: OPPTS880.3550 Immunotoxicity, February 1996; OECD Test Guideline 407, October, 2008 ; & OECD Test Guideline 443, 2011, 2012)

ECO TOXICITY STUDIES

Acute Avian Toxicity

Repeated Dose Avian Toxicity

Avian Reproduction Toxicity

Acute Toxicity – Fish

Acute Toxicity – Honey Bee

Acute Toxicity - Earthworm

ACUTE AVIAN TOXICITY

Objective

To estimate acute oral toxicity of test substance to birds.

Principle of the test

Limit dose test & LD₅₀ slope test (Sequential testing consisting of stages that refer to a period during an experiments in which birds are orally dosed simultaneously and observed for a period of time) are adopted to estimate median lethal dose (LD₅₀) along with slope of dose response curve and the confidence interval for LD₅₀. Sequential testing procedures target the placement of doses and match the precision of the endpoint with the precision required. These sequential procedures were designed to minimise the numbers of birds used.

Test Animals: Any species among Bobwhite quail, Mallard duck, Japanese quail, Pigeon, Chicken can be used. However, it should be ensured that at least one flying and one crawling bird are included.

Age/sex: Reproductively quiescent single sex birds in mature plumage but not in breeding condition should be used. If sensitivity due to sex is suspected, testing should be performed for each sex.

Housing & feeding condition

Birds should be housed in individual cages at temperature within the range of 15-27°C (suitable for quail and duck) and for others as required. Ventilation should be sufficient to supply at least ten changes of air per hour with photoperiod of eight hour light and 16 hour dark cycle for quail and mallard. For other species, it may be necessary to increase the light phase to ten hours. Fresh food and water should be provided *ad libitum*.

Acclimatization: Birds should be acclimatized to the test conditions and diet prior to dosing for at least 14 days for cage-reared birds. Normally, wild caught birds need longer acclimatization periods.

Dosage

Selection of dosage initially should be based on prior knowledge e.g study on rodent, toxicity of structurally related chemicals and preferably limit dose test results and for subsequent stages, it should be based on observed mortality and toxicity signs.

Dose administration

Birds should be fasted for 12-15 hours overnight immediately prior to dosing. Fasting overnight prior to dosing for larger birds such as northern bobwhite and Japanese quail is commended. Shorter fasting periods of 2 hours are suitable for birds weighting around 50 gm or less. The test substance of volume not more than 10 ml/kg body weight is administered orally in a single dose by gavage or capsules. If Regurgitation happens, it should be properly recorded and possibly be reduced as it compromises the evaluation of toxicity.

Procedure

Limit dose test

This is the preferred test when toxicity is expected to be low and lethality is unlikely at the limit dose. The limit dose must be adequate for assessment purposes, and it is usually 2000 mg/kg body weight. Procedure shown below in figure 1.

The limit dose test design consists of dosing five animals simultaneously to sham-dosing the control birds. Control birds will be weighed prior to dosing and on days 3, 7, and 14. Sham dosing will be performed on the same day as the first dosing with test substance (either with the limit dose test if it is performed, and/or with the opening stage (Stage 1 or Stage 2) of the sequential test). Birds are then observed for 14 days.

If no mortality in the dosed birds occurs for 14 days after dosing, it can be concluded at the 95% confidence level, that the LD50 is above the limit dose. A failed limit dose test moved to either Stage 1 or Stage 2 of the sequential test.

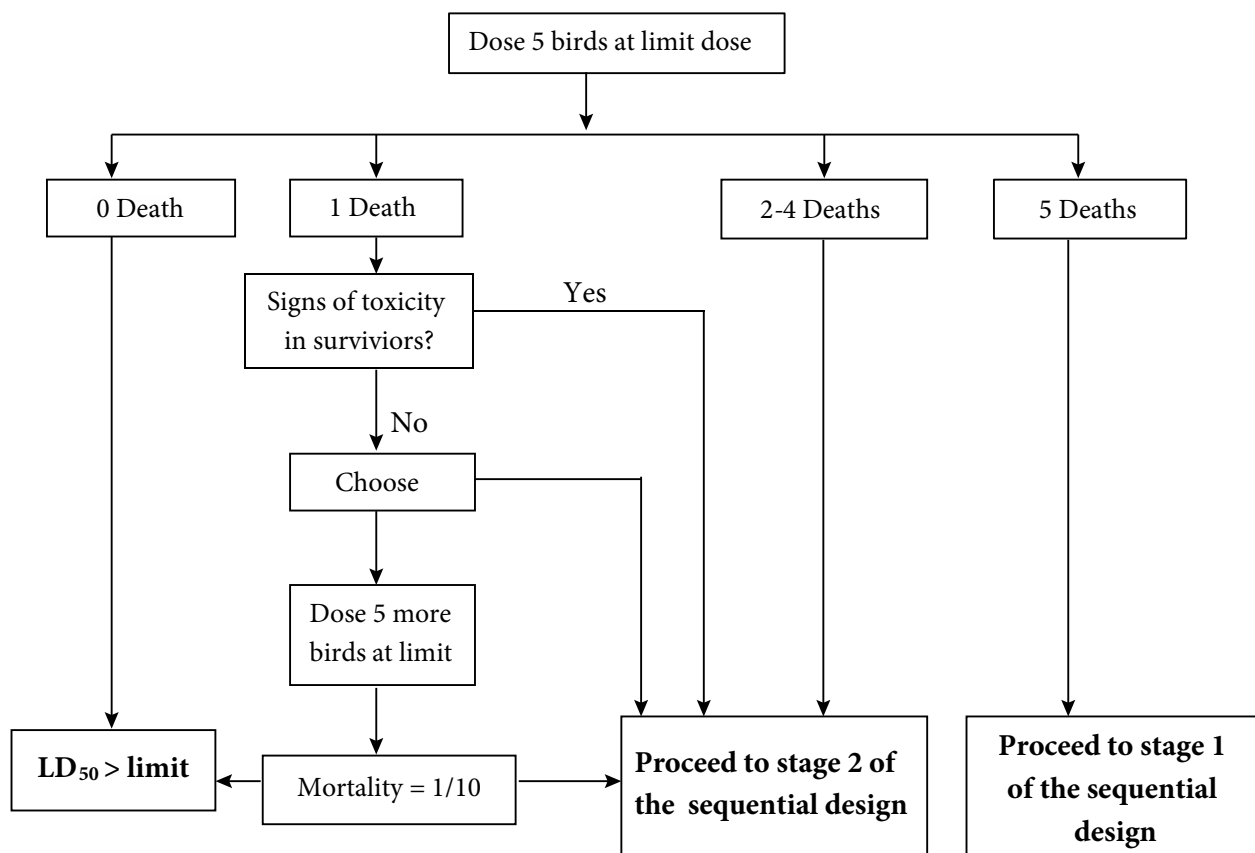
Additional control birds may also be required based on outcomes in the initial control group (The test is invalid if there is one non-incidental death or more than one death from any other cause.). A pool of birds should be held for this purpose.

If one treatment related death is observed, and no signs of toxicity are observed in other birds, then five more birds may be dosed at the limit, the test may proceed to Stage 2 of the sequential design.

If the observed treatment related mortality is one out of five birds and there are signs of toxicity in other birds or if there are two to four mortalities among five birds, or if there are two or more mortalities among ten birds, use the sequential design.

If mortality is complete (*i.e.*, all birds have died, including euthanized birds), use the sequential design shown in Figure 2 starting with Stage 1.

Figure 1: Limit dose test procedure



Sequential Test Procedures (LD_{50} – Slope)

This is the preferred test when the slope of the dose-response curve and/or the confidence interval is required in addition to an estimate of the LD_{50} . This is a 3 or 4 stage test with 24 or 34 birds in addition to a control group. Details of dosage for each stage of the sequential design are described in figure 2.

The test is divided into a number of discrete stages. At each stage, birds are simultaneously given a dose (mg/kg-bwt) of the test substance into the crop or proventriculus. Depending on the test stage, individual birds may receive different doses or more than one bird may receive the same dose. If toxicity is expected, the recommended strategy is to use a sequential design rather than the limit dose approach. Stages 1 and 2 require non-replicated doses, while Stages 3 and 4 require replicated doses.

After dosing, the birds are observed for a 14-day period or more, if required in order to measure mortality. Calculation of the working estimate on Day 3 of a test stage, allows the test and all dosing to be completed over a shorter time frame.

The initial estimate of the LD₅₀ can be based on prior knowledge of the toxicity of the chemical (e.g. mammalian toxicity tests) or of other compounds in same chemical class. Based on the initial estimate of LD₅₀, doses and steps are calculated for each stage. Determination of Partial kills (Instances when multiple birds are given single dose, and the mortality is between 0 and 100% at that dose) and Reversals (Instances when percent mortality is lower at the next higher dose than percent mortality at the given dose) are essential to identify the subsequent stages of the test correctly.

If an LD₅₀-slope test is being conducted and the time between stage starts is lengthy or changes appreciably (e.g., from three days to 10 days), birds should be sham-dosed and started on test in the middle of the sequential study (e.g., at the start of Stage 2 or Stage 3). Additional control birds may also be required based on outcomes in the initial control group.

Observations

- Birds are observed continuously during first two hours after dosing for regurgitation and for the onset of clinical signs, on at least three evenly spaced additional occasions during the first 24 hours for clinical signs, and at least daily thereafter for a total of 14 days.
- Observations made on each individual include regurgitation, signs of intoxication and remission, abnormal behavior, bodyweight, mortality and time of death.
- Observations of deaths that are clearly not treatment related (e.g physical injury) should be excluded from calculation.
- Birds should be weighed before dosing. Weight change and changes in water consumption should also be determined by measuring them on 3rd, 7th and 14th day or no later after dosing (or later depending on the duration of the study). Food consumption should be measured daily until day 3, then for the periods 3-7 and 7-14 days after dosing.
- Gross pathology should be undertaken on all birds from each treatment group at the end of the test (including control and bird that did not died during the study) to help identify incidental mortalities and obvious symptoms of toxicity.
- During the test, birds obviously in pain or showing signs of severe distress should be euthanized.

Result assessment

LD₅₀ in mg/kg body weight along with dose-response curve & confidence interval.

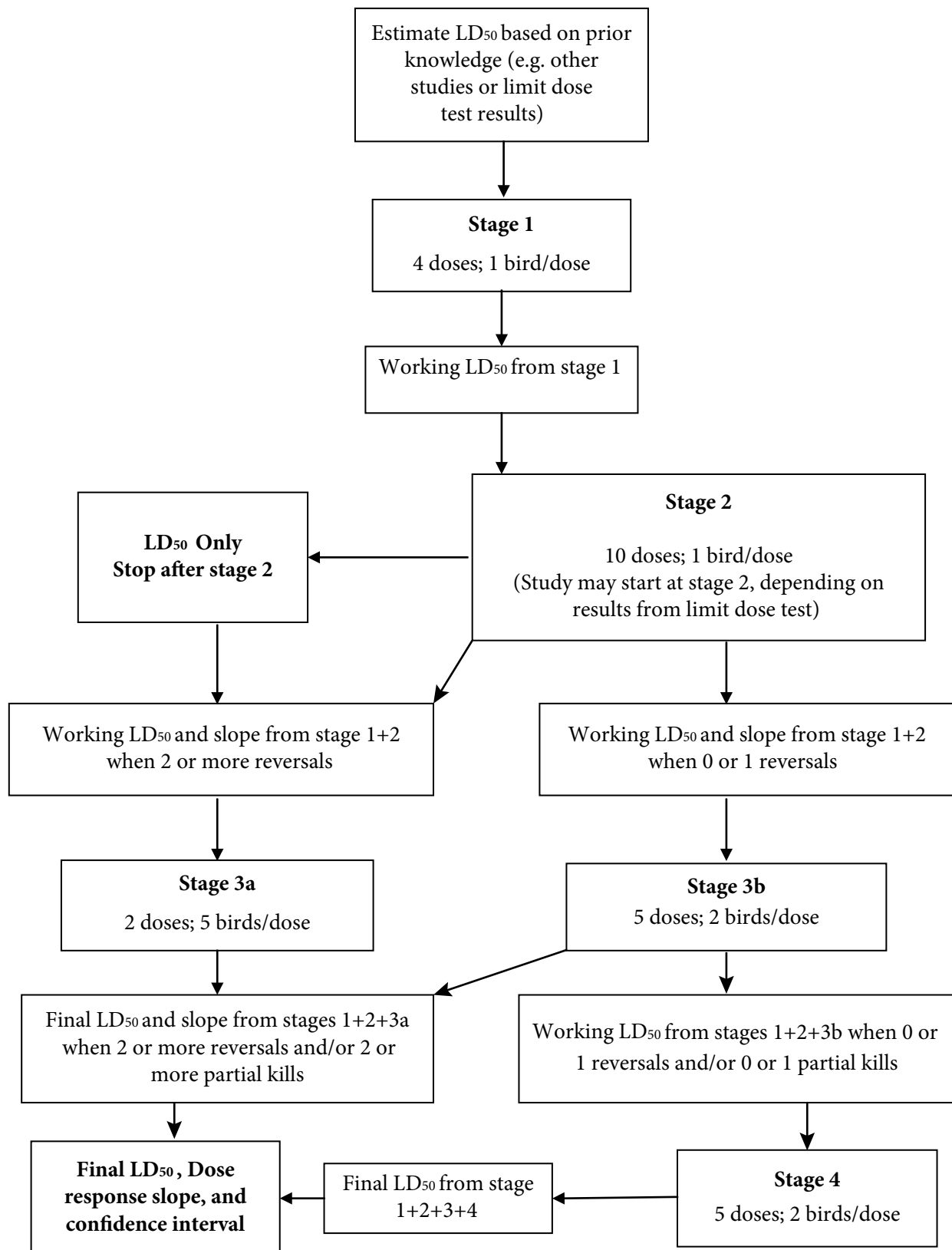
Toxic Endpoint

Mortality is the primary endpoint.

Note: It may be necessary to test additional species to develop a distribution of species sensitivity. In addition to quail, the mallard duck (*Anas platyrhynchos* (Anseriform)), feral pigeon (*Columbalivia*(Collumbiform)), zebra finch (*Poephila guttata* (Passeriform)), and budgerigar (*Melopsittacus undulatus* (Psittaciform)) may be used. This list is not intended to limit the recommended species.

(Reference: OECD Test guidelines 223, 2010)

Figure 2: Sequential design procedure



REPEATED DOSE AVIAN TOXICITY

Objective

To determine the effects of test substance administered in diet to birds.

Principle of the test

The test substance is fed in diet at a range of concentrations to birds for a period of five days followed by administration of the basal diet free of the test substance, for a minimum of three additional days. Mortalities and signs of toxicity are recorded daily.

Test Animals: Any species among Bobwhite quail, Mallard duck, Japanese quail, Pigeon, can be used. However, it should be ensured that the test is performed in at least one flying and one crawling bird species.

Age/sex: 10-17 days for all species except pigeon for which it is 56-70 days. The birds should be in good health and free from any apparent malformations. All test and control birds should be within one day of age of each other.

Housing & feeding condition

General environmental conditions should be maintained as 12 to 6 hours of light per day; 5 or 10 birds per pen, except pigeons which should be housed individually; and good ventilation. Basal diet should be provided *ad libitum*.

Acclimatization: Birds should be acclimated for a minimum of seven days to facilities and basal diet. Suitable facilities for holding birds indoors includes mechanisms for temperature, humidity and light control as required, as well as pens of suitable capacity for rearing the birds. They should be randomly assigned to Pens, and the pens randomly assigned to concentration levels that will be used. Basal diet should be available *ad libitum*. The population may be accepted for testing only if there is less than 5% mortality during acclimation period, otherwise reject the entire group. The mortality in control should not exceed 10% at the end of the test.

Dosage

A minimum of five test concentrations prepared in diets, is required to be tested. Each level should be separated by a constant factor preferably not exceeding 2.0. The lowest treatment level should not result in compound-related mortality or other observable toxic effects.

Dose administration

Diets containing the required amount of the test substance are prepared by uniformly mixing the appropriate amount of the test substance with the prescribed basal diet for young birds. Uniform

distribution of the test substance in the food is the criterion for selecting the method of mixing. If necessary, a carrier of low toxicity to birds may be used to ensure uniform distribution. Carriers should not exceed 2 per cent by weight of the diet and when used should also be added to the diets of the birds in the control. The diet should be analysed to ensure that the concentration of test substance is maintained in diet (at least 80% of nominal concentration).

Limit Test

If a test concentration of at least 5000 ppm in the diet, using the procedures described for the study, produces no compound-related mortality or other observed toxic effects, then a full study using five dose levels may not be necessary.

Procedure

Two control groups and one treatment group for each of the, at least, five dietary levels of the test substance should be used. Each group consists of 10 birds. Diets containing the test substance or control diets should be available *ad libitum*.

The birds should be on the test diet for five days (1-5 day) followed by a minimum of three days (6-8 day) on normal diet. If mortalities occur on days 7 or 8, or if signs of toxicity remain on day 8 and are not clearly in remission, the test duration should be extended beyond eight days until two successive days pass without a mortality or until 21 days (0-21) whichever comes first.

Observations

The observations should be made for:

- Signs of intoxication and other abnormal behaviour: twice on day 1, daily thereafter
- Mortality: twice on day 1, daily thereafter
- Body weights: day 0, 5, 8, and end of test (if extended beyond 8 days)
- Food consumption: days 0-5, 5-8, and 8-end of test (if extended)

Data interpretation & Result

The median lethal concentration (LC 50) can be determined by probit analysis, and statistical methods (e.g. Finney, DJ, 1971; Litchfield and Wilcoxon, 1949) or graphically.

(Reference: OECD Test guidelines 205, 1984)

AVIAN REPRODUCTION TOXICITY

Objective

To determine the effects of test substance administered in diet, on the health and reproductive performance of egg-laying adults in one generation reproduction test.

Principle of the test

The study is undertaken with birds that are already in egg production. The birds are fed a diet containing the test substance for a period of at least six weeks. The reproductive data are collected from all birds prior to initiation of treatment to ensure that only proven breeders are used in the test and to account pre-treatment differences when comparing treatment and control groups. The no observed effect concentration (NOEC) and possibly the low observed effect concentration (LOEC) are determined for adult health and reproductive parameters.

Test Animals: Japanese quail (*Coturnix japonica*) or the Northern bobwhite (*Colinus virginianus*). Birds should be healthy and free of abnormalities or injuries and should not receive any medication beginning one week prior to start of pre-treatment period.

Age/sex: Japanese quail generally start laying eggs at or above six weeks of age. Once egg laying has begun, it will take about two to three weeks for birds to reach full egg production. Depending on the strain, eggs will be fertile when birds are about eight to ten weeks of age. Northern bobwhite should be at least six months old at the onset of egg laying. Once egg laying has begun, it will take about five weeks for birds to reach full egg production.

Housing & feeding condition

Temperature, ventilation and light controlled facilities are needed throughout the test. Recommended ventilation is about 8 to 15 air changes per hour.

Artificial lighting should approximate the daylight visual spectrum and be automatically controlled. The photoperiod for adult birds, from the start of acclimation onwards, and chicks is 16 or 17 hours of light and 7 or 8 hours of darkness. Birds should be exposed to light intensity of at least 10 lux, measured at the level of the feeder.

Adult birds should be housed in pairs (one male/one female). When male and female quails are housed separately, pairs should be placed together at least for half an hour per day for five days a week.

Acclimatization: The acclimation period should begin at least two weeks prior to the start of the study. The test begins with the start of pre-treatment period. Diet and drinking water are provided *ad libitum*. Prior to and during the pre-treatment period, adult birds are fed a basal diet. During the treatment period, birds are fed basal diet mixed with the test substance at specified test concentrations. Parental mortality during last two weeks of acclimation should not exceed 3%.

In Japanese Quail, If necessary, birds must be photostimulated either before or at the start of the acclimation period. Onset of egg laying can take place during the acclimation period. The test begins with the start of the pre-treatment period. The birds should be at peak egg production at the start of the pre-treatment period.

In Northern bobwhite, Photo stimulation should take place, prior to acclimation, about six weeks prior to the scheduled start of the pre-treatment period. This is to ensure that the treatment period will fall in the six weeks period in which egg production is least variable.

Dosage

If multiple test concentrations are to be tested, at least three different dosage groups should be tested and the highest concentration should be chosen at a level that is expected to reveal significant effects on adult health or reproductive parameters but should not cause mortality or other severe signs of parental toxicity that preclude the reproductive parameters evaluation; or 1000 mg/kg diet (limit test dose). Any intermediate concentrations generally should be geometrically spaced between the highest and lowest doses.

Dose administration

To prepare the test diets, the required amounts of test substance are homogeneously mixed into the diet. If required a non-toxic (not more than 2% by weight of diet) vehicle like water, corn oil may be used for homogeneous distribution. The diet with test substance should be prepared at appropriate frequency so as the actual concentration of test substance does not fall below 80% of the initial concentration. Stability analysis under test conditions must be performed prior to the start of the test. A sufficient number of samples should be taken to account for variability.

Limit Test

If a limit test with concentration as indicated under dosage does not reveal any toxic effects when tested according to procedures described in this guideline, no additional concentrations need to be tested.

Procedure

Birds are weighed and randomly allocated to pens and treatment groups. The number of pairs allocated to each group (for example 20) should be sufficient to ensure that there are 16 breeding pairs in the control group at the end of the treatment period. Pairs allocated to the study should have laid at least one egg during the last week prior to start of pre-treatment.

The pre-treatment period lasts two weeks. There should preferably be no mortality among control pairs. If mortality leads to a loss of more than 15% of control pairs between start of pre-treatment and the end of the treatment period this must lead to serious reconsideration of husbandry conditions during the treatment period.

During the treatment period, the birds are exposed to the test substance for a period of six weeks. At least three groups each receive a different test concentration. The control group is fed the same diet and carrier without the test substance. At the end of the treatment period all adult birds are necropsied.

Observations Adult birds

- Toxic signs and health conditions should be evaluated at least once daily, during the acclimation, stabilisation, pre-treatment and treatment periods. Observations should include mortality and clinical signs of toxicity such as lethargy, depression, wing droop, ruffled feathers, lacrimation, etc. Any injuries sustained and subsequent treatment should also be recorded.
- Food consumption (per pair) should be recorded at least weekly, during pre-treatment and treatment, as often as food is replaced in the feeders. Any apparent food spillage should be noted.
- Body weights should be determined at least at the start of pre-treatment, at the start of treatment and at the end of the treatment period.

Pathology

The surviving adult birds till the end of treatment period are humanely sacrificed and undergo necropsy and gross pathology assessment. The wet weight of the liver, spleen and testes are recorded as soon as possible after death. Adult birds that die or are killed during the course of the treatment period, will be subjected to the same procedure.

Offspring- egg

Prior to start of pre-treatment, egg production of all pair of birds available for the study (including potential replacements) should be recorded so as to exclude pairs that do not lay eggs from the study.

During the pre-treatment period and treatment period, all eggs, with the exception of those that are cracked, broken or abnormal or used for eggshell measurements, are set, artificially incubated and allowed to hatch. All offspring are maintained on untreated diet until 14 days after hatching.

Eggs are collected at least once daily, numbered according to pen of origin and stored; broken eggs are numbered, recorded and then discarded. The total number of eggs should be accounted for at the end of the study. The eggs are stored in a cold storage facility, for a maximum of one week, prior to setting in the incubator. Before placing the eggs in the incubator, they are candled to check for abnormalities and fine cracks that can be identified only by candling. After removing all cracked and abnormal eggs, the remaining eggs are equilibrated to room temperature and set in the incubator. All cracked and abnormal eggs should be recorded. From the second day of incubation onwards, eggs should be turned at least three times per day.

During the pre-treatment and treatment periods one egg per pen is collected from odd numbered pens in odd numbered weeks and from even numbered pens during even numbered weeks. These eggs are used for measurements of eggshell thickness or eggshell strength. Eggshell strength is measured using a strength tester. To determine eggshell thickness, each egg will be cut open around the equator and washed out; subsequently the shells are left to dry with the membrane intact for at least 48 hours at room temperature. Eggshells are measured to at least 0.01 mm and the mean value calculated per egg. Both eggshell strength and eggshell thickness will be recorded as part of the validation process, after which a decision will be made which parameter to choose.

Fertility and embryo viability are checked by removing the eggs from the incubator and candling them. This is done after approximately 8 days for Japanese quail or 11 days for Northern bobwhite. All eggs that appear to contain live embryos are placed back into the incubator.

Fertility, infertility, viability and embryonic death are recorded. Approximately 2 or 4 days before hatching for Japanese quail and Northern bobwhite, respectively, the eggs are candled again for viability of embryos; all live embryos are transferred to a hatcher and recorded as late viable embryos. Those embryos that are dead are discarded and recorded as late embryonic dead. During candling, eggs should not be allowed to cool to room temperature, since this may delay embryonic development. After hatching, chicks should be dry before they are taken out of the hatcher. No assistance should be given to chicks during hatching.

Chicks

After hatching, chicks are identified and weighed individually or by pen of origin. They may be housed together, in groups of approximately equal number, by week and preferably by treatment group. Chicks are observed daily for 14 days for clinical signs. After 14 days the chicks are weighed again and killed humanely. Chicks in severe distress will be killed in extremis.

Result assessment

Numerical data should be presented in tabular form for the pre-treatment and treatment periods and the weeks in which the data were collected.

Measurements of endpoints made on adult birds and reproductive parameters will be evaluated by comparing values obtained from birds in treated groups with values obtained from control birds through statistical analysis e.g. Duncan MacLeod report, 1994.

In addition for reproductive parameters pre-treatment differences may be compensated while comparing treatment and control groups during or at the end of the treatment period. Methods that compare individual performance pre and post dose using a covariate analysis e.g. Springer et al, 1999.

It must be noted that under this guideline, where exposure to the test substance starts after egg-laying has begun, an effect may occur in the course of the six-week period after one or more gamete cycles have taken place. In such cases the results should be evaluated in one or two week increments.

A “No Observed Effect Concentration” (NOEC) expressed in mg/kg diet and mg/kg body weight per day should be determined for all health and reproductive parameters evaluated. The mg/kg/day value should be presented as the mean and range for each treatment group.

Toxic Endpoint

The number of 14-day old survivors per hen per day is the integrated biological endpoint including egg production, fertility, embryonic mortality, hatchability and chick survivorship.

(Reference: OECD Test guidelines 206, 1984)

ACUTE TOXICITY – FISH

Objective

To estimate acute toxicity (LC₅₀) of test substance to fish. The purpose of the acute toxicity test with fish species is to help in the assessment of possible risk to similar species in natural environments, as an aid in determination of possible water quality criteria for regulatory purposes, and for use in correlation with acute testing of other species for comparative purposes.

Principle of the test

The goal is to determine concentration-response curves for fish mortality, the LC₅₀'s, and the 95 percent confidence intervals for each species tested in a static, static-renewal, or flow-through system. Fish are exposed to test substance for 96 hours and mortalities are recorded at 24, 48, 72 and 96 hours. The concentration which kills 50% of fish (LC₅₀) is determined.

Test Animals

Fresh water fish (< 8 weeks age) viz. common carp, Guppy, Rainbow trout, zebra fish, Fathead Minnow, Rice fish bluegill, *Catla* (*Catla catla*), *Roghu* (*Labeo rohita*), Channel catfish, salmon

Marine water fish viz. smallmouth and largemouth bass, walleye, crappie and bream, warmouth, longear and green sunfish, sheeps-head minnow, atlantic silverside.

Age/size: Juvenile fish must be tested. Juvenile fish < 3.0 g should be used and the longest should not be more than twice the length of the shortest. The fish should be of normal size and appearance for their age. All fish must be of the same age.

Housing & feeding condition

Fishes preferably must be held in good quality natural water (clean surface or ground water), sea water (for estuarine or marine species) or reconstituted water with total hardness of between 10 and 250 mg CaCO₃ per liter, and with a pH 6 to 8.5 for at least seven days immediately before testing with the photoperiod of 12 to 16 hours daily, temperature as appropriate for species, oxygen concentration of at least 80% of air saturation value. Salinity should be 20±5 ppt for sea water fish. Feeding should be given three times per week or daily until 24 hours before the test is started.

Acclimatization: Fishes must be obtained and held in the laboratory for at least 12 days before used for testing. No feeding is permitted within 48 hr of test initiation. Mortalities must be recorded during acclimatization & the following criteria should be applied:

- Mortalities of greater than 10% of population in seven days: Rejection of the entire batch.
- Mortalities of between 5 and 10% of population: Acclimatization continued for seven additional days
- Mortalities of less than 5% of population: Acceptance of batch.

Number of test animal

At least seven fish per replicate must be used at each test concentration and in the controls. Two replicates per test concentration are preferred to avoid test repetition due to system failures, and to provide a stronger statistical baseline.

Test Solutions

Test solution of the chosen concentration are prepared by dilution of a stock solution preferably with water or vehicles can be used for test substance having low water solubility provided additional control for the same concentration of vehicle should also be performed.

Test concentrations

At least five concentration in a geometric series with a factor preferably not exceeding 2.2. Selection of appropriate test concentration range could be attained by conducting a proper range-finding test. Vehicles such as organic solvents, emulsifier or dispersants of low toxicity to fish may be used. The concentration of organic solvents, emulsifier or dispersants should not exceed 100 µg/l. Every test should include controls consisting of the same dilution water, conditions, procedures, and test population, except that no test substance is added. Solvent (carrier) controls are also required if a solvent was used.

Limit Test

If no mortality are encountered at a concentration of 100 mg (active ingredient)/l , then the LC₅₀ is greater than the limit test concentration & the higher concentration need not to be tested except when human exposure indicates the need for a higher concentration level to be used. Full study should be conducted if any mortality occur.

Procedure

All fishes are exposed to their respective test concentration preferably for 96 hours. Maximum loading of 1.0g fish/liter for static and semi-static test is recommended. Disturbance that may change the behavior of the fish should be avoided.

Observations

- Fishes are inspected at least after 24, 48, 72 and 96 hours. Observations at three and six hours after the start of test are desirable.
- Fishes are considered dead if there is no visible movement (e.g. gill movements) and if touching of the caudal peduncle produces no reaction. Dead fish are removed when observed and mortalities are recorded.
- Visible abnormalities like loss of equilibrium, swimming behavior, respiratory function, pigmentation etc. are to be recorded.
- Measurements of pH, dissolved oxygen and temperature should be carried out at least daily.

Result assessment

96 hours LC₅₀ value, with confidence limits.

(Reference: OECD Test guidelines 203, 1992 & OPPTS 850: 1075, 1996)

ACUTE TOXICITY – HONEY BEE

Objective

To estimate acute toxicity of test substance to honey bee.

Principle of the test

Adult worker honeybees are exposed to a range of doses of the test substance dissolved/dispersed in appropriate carrier/solvent. Justification should be provided if vehicle is used. The bees are fed 50% (w/v) sucrose solution for oral test and for contact test, applied directly to the thorax (droplets). Mortality is recorded daily during at least 48 hours and compared with control values. If the mortality rate is increasing between 24 and 48h whilst control mortality remains at an accepted level, i.e. $\leq 10\%$, it is appropriate to extend the duration of the test to a maximum of 96h. The results are analysed in order to calculate the LD50 at 24h and 48h and in case the study is prolonged, at 72h and 96h. The average mortality for total no. of controls must not exceed 10% at the end of the test.

Test Animals: *Apis indica* and *Apis mellifera* are the preferred species. Healthy Adult worker honeybees of similar age should be used. It should be collected in the morning of use or in the evening before test and kept under test conditions to the next day. Bees treated with chemical substances such as antibiotics etc. should not be used for toxicity test for four weeks from the time of the end of the last treatment.

Housing & feeding condition

Honey bees should be housed in easy to clean and well-ventilated cages of appropriate material which should be held in dark experimental room at a temperature of $25 \pm 2^\circ \text{C}$ & relative humidity of 50- 70%. Sucrose solution in water with a final concentration of 500g/l should be used as food. Food should be provided *ad libitum*.

Acclimatization: The collected bees are randomly allocated to test cages, which are randomly placed in the experimental room. For oral study, the bees must be starved for up to 2 hours before the initiation of the test. For contact studies, the collected bees may be anaesthetized with carbon dioxide or nitrogen for application of the test substance. The amount of anaesthetic used and times of exposure should be minimised. Moribund bees should be rejected and replaced by healthy bees before starting the test.

Number of test animal: 10 honeybees should be used at each dose level per replicate.

Preparation of doses

For oral exposure: If the test substance is a water miscible compound this may be dispersed directly in 50 per cent sucrose solution. For technical products and substances of low water solubility,

vehicles such as organic solvent, emulsifiers or dispersants of low toxicity to bees may be used (e.g. acetone, dimethylformamide, dimethylsulfoxide). Two separate control groups should be used: a solution in water, and a sucrose solution with the solvent/carrier at the concentration used in dosing solutions.

For contact exposure: The test substance is to be applied as solution in an organic solvent or watersolution with a wetting agent. Two separate control groups should be used, one treated with water, and another treated with the solvent/dispersant.

Note: If an organic solvent or a wetting agent is used three additional control batches of each ten bees for the solvent or the wetting agent have to be included

Dosage

Five dose level in a geometric series, with a factor not exceeding 2.2 and covering range of LD₅₀ should be used along with minimum three replicate test groups. A minimum of three control batch, each of ten bees including one control for vehicle, should be included in addition to the test series.

Toxic Standard

Dimethoate should be used. However, other toxic standard like Endosulphan or parathion would be acceptable where sufficient data can be provided to verify the expected dose response. At least three doses each containing ten bees should be selected to cover the expected LD₅₀ value.

Administration of doses

Each test group of bees should be provided with 100-200 µl of 50% sucrose solution in water, containing the test substance at the appropriate concentration. Once consumed (usually within 3-4 hours or 6 hours for some test substance or at higher concentration), the feeder should be removed from the cage and replaced with one containing sucrose solution alone. For topical application, anaesthetized bees are used. The bees are randomly assigned to the different test doses and controls. A volume of **1 µl** of solution containing the test substance at the suitable concentration should be applied with a microapplicator to the dorsal side of the thorax of each bee. Other volumes may be used, if justified. After administration, the bees are allocated to test cages and supplied with sucrose solutions *ad libitum*.

Limit Test

In case it is expected that pesticide has low bee toxicity, limit test can be preferred using the same procedure with dose level of 100µg a.i /bee. If no mortality are encountered at a dose of 100 µg (active ingredient)/bee, then the LD₅₀ is greater than the limit test dose & the higher dose need not to be tested except when human exposure indicates the need for a higher dose level to be used. Full study should be conducted if any mortality occur.

Duration

For oral test: The duration of the test is 48 hr after the test solution has been replaced with sucrose solution alone. If mortality continues to rise by more than 10% after the first 24 hr, the test duration should be extended to a maximum of 96 hr provided that control mortality does not exceed 10%.

For contact exposure: The duration of the test is 48h. If mortality increases by more than 10 per cent between 24h and 48h, the test duration should be extended up to a maximum of 96h provided that control mortality does not exceed 10 per cent.

Observations For oral test

- Mortality is recorded at 4 hr after start of the test and thereafter at 24 hr and 48 hr (i.e. after given dose). If a prolonged observation period is required, further assessments should be made at 24 hours intervals, upto a maximum of 96 hour, provided that the control mortality does not exceed 10%.
- The amount of diet consumed per group should be estimated. Comparison of the rates of consumption of treated and untreated diet within the given six hours can provide information about palatability of the treated diet.
- All abnormal behavioral effects observed during the testing period should be recorded.

For contact exposure

- Mortality is recorded at 4 h after dosing and thereafter at 24h and 48 h.
- If a prolonged observation period is required, further assessments should be made at 24 hours intervals, to a maximum of 96 hours, provided that the control mortality does not exceeding 10 per cent.
- All abnormal behavioural effects observed during the testing period should be recorded.

Result assessment

48 hours LD₅₀ value with Confidence limits

(Reference: OECD Test guidelines 213 & 214, 1998)

ACUTE TOXICITY- EARTHWORM

Objective

To estimate acute toxicity of test substance to Earthworm.

Principle of the test

The methods of testing toxicity of chemicals to earthworms are a paper contact toxicity test and an artificial soil test. A simple paper contact toxicity test is described as an optional initial screen to indicate those substances likely to be toxic to earthworms in soil and which will require further more detailed testing in an artificial soil. The simple contact test is easy to perform and gives reproducible results with the recommended species. The artificial soil test gives toxicity data more representative of natural exposure of earthworms to chemicals.

The screening test (filter paper contact test) involves exposing earthworms to test substances on moist filter paper in order to identify potentially toxic chemicals to earthworms in soil. The artificial soil test involves keeping earthworms in samples of a precisely defined artificial soil to which a range of concentrations of the test substance has been applied. Mortality is assessed 7 and 14 days after application. One concentration resulting in no mortality and one resulting in total mortality should be used.

Test Animals: The recommended test species is *Eisenia foetida* (Michaelson).

Age/weight: Worms should be adult (at least two months old with clitellum) with an individual weight of 300 to 600 mg. Its susceptibility to chemicals resembles that of true soil-inhabiting species, it has a short life cycle, hatching from cocoons in 3 to 4 weeks, and reaching maturity in seven to eight weeks at 20°C. It is very prolific, each worm producing two to five cocoons per week from each of which emerge several worms.

Housing and feeding

The test temperature is 20° ± 2°C. Testing is done in continuous light (to ensure that worms remain in the test medium throughout duration of test).

Materials required

Artificial soil test substrate, for example, 10 per cent sphagnum peat (as close to pH 5.5 to 6.0 as possible), 20 per cent kaolin clay (kaolinite content preferably above 30 per cent) and 70 per cent industrial sand (fine sand should be dominant with more than 50 per cent of the particles between 50 and 200 microns). The pH is adjusted to 6.0 ± 0.5 by addition of calcium carbonate. The complete mixture should be moist but not so wet that water appears when the artificial soil is compressed.

Other requisites include all glass test containers and an illuminated cabinet or chamber controllable to ± 2°C with a light intensity of 400 to 800 lux.

Number of test animal: 10 earthworms should be used at each dose level per replicate.

Preparation of doses For Filter Paper Test

The test substance is dissolved in water (if soluble up to a concentration of 1000 mg/l) or in a suitable organic solvent (e.g. acetone, hexane or chloroform), as appropriate, to give a range of known concentrations.

For artificial soil test

The artificial soil plus test substance should, whenever possible, be made up as follows: Immediately before the start of the test, an emulsion or dispersion of the test substance in deionised water is mixed with the artificial soil or sprayed evenly over it with a fine chromatographic or similar spray. If insoluble in water, the test substance can be dissolved in as small a volume as possible of a suitable organic solvent (e.g. hexane, acetone or chloroform). The solvent should be allowed to evaporate. If the test substance is not soluble, dispersible or emulsifiable, 10 g of a mixture of fine ground quartz sand and quantity of test substance corresponding to 750 g wet weight of artificial soil are mixed with 740 g wet artificial soil for each test container. Only agents which volatilise readily may be used to solubilise, disperse or emulsify the test substance. The test medium must be ventilated before use. The amount of water evaporated should be replaced. The control should receive the same quantity of any additive agent.

Reference Standard

The LC₅₀ of a reference substance should be determined occasionally as a means of assuring that the laboratory test conditions are adequate and have not changed significantly. A suitable reference substance is chloracetamide.

Duration

The test duration is 14 days (assessment of mortality at 7 and 14 days).

Dosage

Dose range finding study: A preliminary range-finding test may be done optionally prior to a more precise screening test. This could be done as follows:

For Filter Paper Test

Amount applied to filter paper	Concentration of solution applied
1.0 mg/cm ²	7×10^{-2} g/ml
0.1 mg/cm ²	7×10^{-3} g/ml
0.01 mg/cm ²	7×10^{-4} g/ml

Amount applied to filter paper	Concentration of solution applied
0.001 mg/cm ²	7×10^{-5} g/m
10.0001 mg/cm ²	7×10^{-6} g/ml

For artificial soil test

It could be based on treatments in the range 0.01, 0.1, 1.0, 10, 100, 1000 mg/kg (dry weight of artificial soil).

Procedure

For the main screening test five or more treatment levels in a geometric series should be used.

For Filter Paper Test

Flat-bottomed glass vials approximately 8 cm in length and 3 cm in diameter are recommended. Their sides are lined with filter paper cut to a suitable size so it does not overlap appreciably.

One ml of solution is pipetted into each vial and evaporated to dryness under a slow stream of filtered compressed air, the vial being rotated horizontally as it dries (for substances that are relatively insoluble in either water or organic solvents this may have to be repeated several times to obtain the larger deposits required). The control vial should be treated with 1 ml of deionised water or appropriate organic solvent. After drying, 1ml of deionised water is added to each vial to moisten the filter paper. Each vial is sealed with a cap or plastic film with a small ventilation hole.

For each treatment, ten replicates, each consisting of one worm per vial, are the minimum requirement. More than one worm in a vial should not be used because the death of one worm may have adverse effects on others in the same vial. The precision of the test can be increased by using 20 replicates. In each test a range of treatment levels and ten control vials are used.

Worms should be kept on moist filter paper for three hours before being placed in test vials so they can void their gut contents. They are then washed and dried before use. During the test, vials are laid on their sides on trays. Tests are done in the dark and for a period of 48 hours with a further optional mortality assessment after 72 hours.

Worms are classified as dead when they do not respond to a gentle mechanical stimulus to the front end. Any behavioural or pathological symptoms should be reported.

For artificial soil test

The artificial soil plus test substance should, whenever possible, be made up as follows:

Immediately before the start of the test, an emulsion or dispersion of the test substance in deionised water is mixed with the artificial soil or sprayed evenly over it with a fine chromatographic or similar spray. If insoluble in water, the test substance can be dissolved in as small a volume as possible of

a suitable organic solvent (e.g. hexane, acetone or chloroform). The solvent should be allowed to evaporate. If the test substance is not soluble, dispersible or emulsifiable, 10 g of a mixture of fine ground quartz sand and quantity of test substance corresponding to 750 g wet weight of artificial soil are mixed with 740 g wet artificial soil for each test container. Only agents which volatilise readily may be used to solubilise, disperse or emulsify the test substance. The test medium must be ventilated before use. The amount of water evaporated should be replaced. The control should receive the same quantity of any additive agent.

For each test, 750 g weight of the test medium is placed into each glass container and ten earthworms, which have been conditioned for 24 hours in an artificial soil and then washed quickly before use, are placed on the test medium surface. The containers are covered with perforated plastic film to prevent the test medium from drying and kept under the test conditions for 14 days.

Four replicates for each treatment are recommended. For each test, four control dishes, treated with the same solvent as that used in the test and containing ten worms, are used.

Observations

The mortality is assessed by emptying test medium onto a glass tray or plate, sorting worms from the medium and testing their reaction to a mechanical stimulus at the front end. After the 7 day assessment worms and medium are replaced in the test container. Any behavioural or pathological symptoms noted should be reported.

At the end of the test the moisture content of the test medium should be assessed and reported.

Result assessment

The mortality/concentration data should be plotted on log probability graph paper and the median lethal concentration (LC50) and its confidence limits estimated.

(Reference: OECD Test guidelines 207, April, 1984)

SPRAY OPERATOR EXPOSURE STUDY

Operator Exposure Modeling Study

Operator Exposure Modeling Study

The pesticides spray operator exposure risk assessment model is in the process of evolution and shall be finalized very soon. The details shall be issued as an addendum to the existing document.

TOXICITY DATA REQUIREMENTS FOR CHEMICAL PESTICIDES

This chapter will give information at a glance regarding the requirements of data as per the recommended guidelines for registration of first time introduction of pesticide in India. However, the data requirement under various other categories will be amended *mutatis mutandis*.

I. Acute Mammalian Toxicity Studies

S.No.	Toxicity Study	Technical	Formulation
01.	Acute Oral - Rat	R	R
02.	Acute Dermal – Rat / Rabbit	R	R
03.	Acute Inhalation	R ¹	R ¹
04.	Primary Skin Irritation	R ²	R ²
05.	Acute Eye Irritation	R ³	R ³
06.	Skin Sensitization*	R	R

R¹: Not required when :

- Vapour pressure is low i.e. $<1 \times 10^{-2}$ Pa under practical temperature conditions of India (up to 20⁰C). or
- Technical Grade Active Ingredient (TGAI) does not contain particles of diameter of $< 30\mu\text{m}$ ($> 1\%$ on w/w basis)

R²: The assessment of this endpoint shall be carried out according to a sequential testing strategy as follows:

- the assessment of dermal corrosivity using a validated in vitro test method;
- the assessment of dermal irritation using a validated in vitro test method;
- An In vivo dermal irritation study.

In vivo testing should not be conducted if:

- The substance or formulation is a strong acid ($\text{pH} \leq 2.0$) or base ($\text{pH} \geq 11.5$) and the available information indicates that it should be classified as skin corrosive. or
- the substance is classified as acutely toxic by the dermal route

R³: Testing need not be performed if the available information indicates that the substance should be classified as corrosive or severely irritating to the skin, e.g., if the substance is a strong acid ($\text{pH} < 2.0$) or base ($\text{pH} > 11.5$).

***In-vitro* study on case to case basis may be accepted.**

* : Buelher / GPMT / LLNA (Any one test will be accepted).

† The study carried out as per 425 will be preferred.

(Inhalation 62-63; PSI 65-69; Acute Eye Irritation 70-75)

II. Short Term Repeated Dose Toxicity Studies

S.No.	Toxicity Study	Technical	Formulation
01.	Repeated Dose Range Finding Oral Toxicity Study (upto 28 Days)	R ¹	NR
02.	Repeated Dose 90 Day Oral : a) Toxicity Study-Rodent b) Toxicity Study-Non Rodent (Dog)*	R	NR
03.	Repeated Dose Dermal Toxicity	R	NR
04.	Repeated Dose Inhalation Toxicity	R ²	NR

R¹ : This study is generally done to establish dose range but may not necessarily be conducted for a duration of 28 days.

R² Not required when :

- Vapour pressure is low i.e. $<1 \times 10^{-2}$ Pa under practical temperature conditions of India (up to 20°C), or
- Technical Grade Active Ingredient (TGAI) does not contain particles of diameter of $< 30 \mu\text{m}$ ($> 1\%$ on w/w basis)

*Published literature from reliable source maybe acceptable.

If a stakeholder is in possession of 52 week study of dog, the same shall be acceptable in place of sub-acute Oral Study

(Repeated Dose Inhalation 76-77)

III. Neurotoxicity Studies

S.No.	Toxicity Study	Technical	Formulation
01.	Acute Neurotoxicity - Rodent	R ¹	NR
02.	Repeated Dose Neuro toxicity - Rodent	R ²	NR
03.	Delayed Neurotoxicity – OP Compound – Acute Exposure	R	NR
04.	Delayed Neurotoxicity – OP Compound – Repeated Administration	R	NR
5.	Developmental Neurotoxicity	R ³	NR

R¹: Required only based on the WoE (weight of evidence) & Cause of Concern approach, considering the following :

- i) Compound if belongs to known neurotoxic class or with identified structural relationship with known neuro-toxicants.
- ii) Single dose acute / Repeated dose sub acute / sub chronic tox studies have not taken into account the Functional Observation Battery & Histopathology of relevant organs.

R²: Required only based on the WoE (weight of evidence) & Cause of Concern approach, considering the following :

- i) Compound if belongs to known neurotoxic class or with identified structural relationship with known neuro-toxicants.
- ii) No separate study is required if 90 days repeat dose sub chronic oral tox study has taken into account the Functional Observation Battery parameters, histopathology of relevant organs.

R³: required on case by case basis i.e. using WoE (weight of evidence) & Cause of concern approach by considering:

- i. The pesticide causes treatment-related neurological effects in adult animal studies (i.e., clinical signs of neurotoxicity, neuropathology, functional or behavioral effects).
- ii. The pesticide causes treatment-related neurological effects in developing animals (act as neuroteratogen).
- iii. The pesticide elicits a causative association between exposures and adverse neurological effects in human epidemiological studies.
- iv. The pesticide evokes a mechanism that is associated with adverse effects on the development of the nervous system.

(Neurotoxicity 78-80)

IV. Long Term Toxicity Studies

S.No.	Toxicity Study	Technical	Formulation
01.	Combined Carcinogenicity/chronic toxicity study- Rat	R	NR
02.	Carcinogenicity – Rat & Mice	R	NR
03.	Chronic toxicity – Rat	R	NR

Note: In case combined carcinogenicity / chronic toxicity in Rat is submitted, then there is no need to submit carcinogenicity study in rat and chronic toxicity in rat.

**(Combined Chronic toxicity / carcinogenicity 37, 81, carcinogenicity 82,
chronic toxicity 83)**

V. Development & Reproduction Toxicity Studies (DART)

S.No.	Toxicity Study	Technical	Formulation
01.	Developmental Toxicity Study – Rat & Rabbit	R	NR
02.	Two Generation* Reproduction Toxicity study	R	NR

*At this juncture One generation extended reproduction study was not accepted as it takes into consideration the outcome of immunitoxicity and neurotoxicity studies as well.

VI. Genotoxicity Study

S.No.	Toxicity Study	Technical	Formulation
01.	Bacterial Reverse Mutation Test (AMES Test)	R ¹	NR
02.	<i>In-Vitro</i> Mammalian Chromosome Aberration Test		
03.	<i>In- Vitro</i> Mammalian Cell Gene Mutation Test		
04.	<i>In-vitro</i> Mammalian Cell Micronucleus Test		
05.	<i>In-Vivo</i> Mammalian Bone Marrow Chromosome Aberration Test		
06.	Mammalian Spermatogonial Chromosome Abberation Test		
07.	<i>In-Vivo</i> Mammalian Erythrocyte Micronucleus Test		
08.	<i>In- Vivo</i> Un-Scheduled DNA Synthesis (UDS) Test with Mammalian Liver Cells		
09.	Mouse Heritable Translocation Assay		
10.	Rodent Dominant Lethal Test		

R¹: In addition to AMES test, a battery of in vitro and in vivo tests with specific end points viz. Point Mutation, Chromosomal aberration (chromatids can be seen separately) and DNA repair are to be submitted. 3 in vitro and 1 in vivo studies are required. Out of 3 in vitro studies, reverse mutation (AMES test) is mandatory and balance 2 in vitro and 1 in vivo tests can be chosen from enlisted battery of tests at different end points.

However, any positivity in any in vitro study, may trigger an additional corresponding in vivo test.

(Genotoxicity Studies 84 – 86; Bacterial Reverse Mutation Test 41; *In-Vitro* Mammalian Chromosome Aberration Test 44; *In-Vitro* Mammalian Cell Micronucleus Test 44; Mammalian Bone Marrow Chromosome Aberration Test 45; *In-Vivo* Mammalian Erythrocyte Micronucleus Test 47; *In-Vivo* Unscheduled DNA Synthesis (USS) Test With Mammalian Liver Cells 48; Mouse Heritable Translocation Assay 49; Rodent Dominant Lethal Cell 50)

VII. Absorption Distribution Metabolism and Excretion (ADME)

S.No.	Toxicity Study	Technical	Formulation
01.	Metabolism in Rat	R	NR
02.	Feeding studies in Livestock (Goat/Cow/Hen/Poultry) including Metabolism in Livestock	R ¹	NR

R¹: Data required in case of :

- a. Direct application on animal for treatment of ecto-parasites.
- b. Any crop or its part treated with pesticides is used as feed or fodder.
- c. In case of label expansion claim, requirement of study will be reassessed. In such a case the applicant needs to inform the RC. This should be made part of the guidelines for Label Expansion by bioefficacy experts and RC should be apprised appropriately in their agenda.

Note: Toxicity studies required: 1. Acute Oral Rat, 2. AMES Test, 3. 28-day sub-acute and Rat study on toxic metabolism

(Toxicokinetics 51, Metabolism 87)

VIII. Immunotoxicity

S.No.	Toxicity Study	Technical	Formulation
01.	Immunotoxicity study	R ¹	NR

R¹: Required case to case, based on Weight of Evidence (WoE) and sound scientific, and chemical-specific rationale i.e. specific study is not required if immunotoxicity gets addressed through different treatment related adverse effects, which are observed in different toxicity studies like :

- Haematology : Total and differential white blood cell counts, globulins and albumin/globulin ratio
- Immune system organ weights: Alteration in weight of thymus and spleen
- Detailed and extensive histopathology of primary and secondary immune system tissues: thymus, spleen, several alteration in lymphocytes population and presence of triggering factors in bone marrow and lymph nodes

(Immunotoxicity 88- 91)

IX. Eco-toxicity Study

S.No.	Toxicity Study	Technical	Formulation
01.	Acute Avian Toxicity	R ¹	R ¹
02.	Repeated Dose Avian Toxicity (one species)	R	NR
03.	Avian Reproduction Toxicity (one species)	R	NR
04.	Acute toxicity to fresh water fish (one species)	R	R
05.	Acute Toxicity – Honey Bee (Oral & Contact)	R	R
06.	Acute Toxicity – Earthworm	R	R

R¹: Technical : 2 bird species (Technical) and 1 bird species (Formulations)

However, if formulation is observed to be more toxic than technical (in acute oral rat study) then data on one additional avian species will be required for the formulations.

No wild caught bird to be used for a study.

(Ecotox studies 92, 94, Bird amendments 54, 93, Fish 95, 96)

GENERAL RECOMMENDATIONS

1. Principle of 3Rs (Replacement, Reduction and Refinement) : Pursuant to Chapter IV, section 17(2)(d) of the Prevention of Cruelty to Animals Act, 1960, which provides that “experiments on animals are avoided wherever it is possible to do so,” the principles of 3Rs of animal use shall be taken into account in the fulfillment of registration requirements, taking particular account of the timely use of new validated 3Rs methods recognized by OECD and other regulatory authorities (with robust regulatory framework). Tests on vertebrate animals shall be undertaken judiciously. Alternative approaches to be considered shall include *in vitro* tests and non-testing approaches such as waivers, read-across (data bridging) and valid *in silico* models subject to **satisfaction** of the Registration Committee.
2. Acute Dermal & Skin Irritation studies may be combined
3. OECD 436 will be also accepted besides OECD 403 for Acute Inhalation
4. To undertake risk assessment, data in brief on metabolism in plant/soil/water needs to be submitted with toxicology dossier.
5. In case of short and long term study, if there is some appreciable toxic effect for which known mechanism of action does not exist, applicant may be requested to generate some mechanistic study to support their data.
6. In case, major (>10 %) metabolites are detected in plant/soil/water metabolism studies, other than found in rat metabolism study additional relevant toxicity study are required.
7. The pesticides spray operator exposure risk assessment will be done as per the recommendation of the Registration Committee

Note: This guidance document is not a static one and shall be subject to modification on the basis of newer scientific developments in future. Any such amendment to the guidance document shall be issued as an addendum as and when necessary.

BIBLIOGRAPHY

1. OECD Test Guideline 423, Acute Toxic Class Method, adopted on 17th December, 2001
2. BMDP Statistics Software, Inc. BMDP Statistical Software Manual. W.J. Dixon, Chief Ed. University of California Press, Berkeley, CA, USA, 1990.
3. OECD Test Guideline 425, Up and Down Procedure, adopted on 3rd October, 2008
4. OECD Test Guideline 420, Fixed Dose Procedure, adopted on 17th October, 2001
5. Litchfield, J.T. and Wilcoxon, F., J. Pharmacol. Exp. Ther., 96, 99-113, 1949.
6. Bliss, C.I., Quart. J. Pharm. Pharmacol., 11, 192-216, 1938.
7. Finney, D.G., Probit Analysis. (3rd Ed.) London, Cambridge University Press, 1971.
8. Finney D.J. (1977), Probit Analysis, 3rd ed. Cambridge University Press, London/New York
9. Weil, C.S., Biometrics, 8, 249-263, 1952.
10. Thompson, W., B act. Rev., 11, 115-141, 1947.
11. Miller, L.C. and Tainter, M.L., Proc. Soc. Exp. Biol. Med. NY, 57, 261-264, 1944.
12. OECD Test guideline 402, Acute Dermal, adopted on 24th February, 1987
13. OECD Test guideline 403, Acute Inhalation, adopted on 07th September , 2009
14. OECD, Test Guideline 404, Acute Dermal Irritation/Corrosion, OECD Guidelines for the Testing of Chemicals 28th July 2015
15. OECD Test guideline 439, In-vitro Skin Irritation, adopted on 26th July, 2015
16. OECD Test guideline 405, Acute Eye Irritation, adopted on 2nd October, 2012
17. US EPA Health Effects Test Guidelines OPPTS 870.2400 Acute Eye Irritation EPA 712-C-98-195 August 1998
18. Chanter, D.O. and Heywood, R. The LD₅₀ test: some considerations of precision. *Toxicol Letters* 10:303-307, 1982.
19. Finney, D.J. The Median Lethal Dose and Its Estimation, *Archives of Toxicology* 56:215-218 (1985).
20. OECD Test guideline 429, Local Lymph Node assay (LLNA), adopted on 22nd July 2010
21. OECD Test guideline 406, Guinea Pig Maximisation test (GPMT), adopted on 17th July 1992
22. OECD Test guideline 406, Buehler's Test Method, adopted on 17th July 1992

23. OECD Test guideline 407, Repeated dose 28 day oral toxicity study – Rodent, adopted on 3rd October, 2008
24. OECD Test guideline 408, Repeated dose 90 day oral toxicity study – Rodent, adopted on 21st September, 1998
25. Guidelines for Collection of Blood from Experimental Animals, research animal resources, university of Minnesota, 2011
26. The University of Pennsylvania Institutional Animal Care and Use Committee (IACUC), Office of Regulatory Affairs, 1995.
27. OECD Test guideline 409, Repeated dose 90 day oral toxicity study – Non Rodent, adopted on 21st September, 1998
28. OECD Test guideline 410, Repeated dose dermal 21 day toxicity study , adopted on 12th May, 1981.
29. OECD Test guideline 412, Sub-acute Inhalation 28 day toxicity study, adopted on 07th September, 2009.
30. US EPA Health Effects Test Guidelines OPPTS 870.6100 Acute and 28-Day Delayed Neurotoxicity of Organophosphorus Substances, August, 1998.
31. OECD Test guideline 424, Neurotoxicity study in Rodents adopted on 21st July, 1997.
32. OECD Test guideline 418, Delayed Neurotoxicity of OP compound – acute exposure, adopted on 27th July, 1995.
33. OECD Test guideline 419, Delayed Neurotoxicity of OP compound – Repeated exposure, adopted on 27th July, 1995.
34. OECD Test guideline 426, Developmental Neurotoxicity study, adopted on 16th October, 2007.
35. OECD Test guideline 451, Carcinogenicity, adopted in 7th September, 2009.
36. OECD Test guideline 452, Chronic Toxicity, 7th September, 2009
37. OECD Test guideline 453, Combined Chronic Toxicity and Carcinogenicity, Adopted in 2009
38. OECD Test guideline 414, Prenatal Developmental Toxicity study, Adopted in 22th January, 2001.
39. OECD Test guideline 416, Two-Generation Reproduction Toxicity study, Adopted in 22th January, 2001.
40. OECD Test guideline 443, Extended One-Generation Reproductive Toxicity study, Adopted in 28th July, 2011. Figure1 corrected 2nd October, 2012.

41. OECD Test guideline 471, Bacterial Reverse Mutation Test (AMES Test), Adopted in 21st July, 1997.
42. OECD Test guideline 473, In-vitro Mammalian chromosome aberration test, Adopted in 26th September, 2014.
43. OECD Test guideline 476, In-vitro Mammalian Cell Gene Mutation Test, Adopted in 29th July, 2016.
44. OECD Test guideline 487, In-vitro Mammalian Cell Micronucleus Test, Adopted in 26th September, 2014.
45. OECD Test guideline 475, Mammalian Bone Marrow chromosome aberration test In-Vivo, Adopted in 29th July, 2016.
46. OECD Test guideline 483, Mammalian Spermatogonial chromosome aberration test In- Vivo, adopted in 29th July, 2016.
47. OECD Test guideline 474, Mammalian Erythrocyte Micronucleus Test In-vivo, Adopted in 29th July, 2016.
48. OECD Test guideline 486, Unscheduled DNA Synthesis (UDS) Test with Mammalian Liver Cells *In Vivo*, Adopted in 21st July, 1997.
49. OECD Test guideline 485, Mouse Heritable Translocation Assay, Adopted in 23rd October, 1986.
50. OECD Test guideline 478, Rodent Dominant Lethal Test, Adopted in 29th July, 2016.
51. OECD Draft Test guideline 417, Toxicokinetics, Adopted in 22 July, 2010.
52. OECD Test guideline 503, Metabolism in Livestock, Adopted in 8th January, 2007.
53. US EPA, Biological Test Guidelines, OPPTS 880.3550 Immunotoxicity, February 1996.
54. OECD Test guidelines 223, Avian Acute Oral Toxicity test, adopted on 22nd July, 2010
55. OECD Test Guideline 205, Avian Dietary Toxicity Test, Adopted on 4th April, 1984
56. OECD Test Guideline 206, Avian Reproduction Test, Adopted on 4th April, 1984
57. OECD Test guidelines 203, Fish Acute Toxicity Test, adopted on 17th July, 1992
58. US EPA, Ecological Effects Test Guidelines, OPPTS 850.1075 Fish Acute Toxicity Test, Freshwater and Marine adopted on April, 1996.
59. OECD Test guidelines 213, Acute Oral toxicity to Honey Bee, Adopted on 21st September, 1998
60. OECD Test guidelines 214, Acute Contact Toxicity to Honey Bee, Adopted on 21st September, 1998

61. OECD Test guidelines 207, Acute Toxicity to Earthworm, Adopted on 4th April, 1984.
62. Section 5.2.3, Commission Regulation (EU) No: 283/2013 of March 1, 2013 setting out data requirements for active substances, in accordance with Regulation (EC)No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market
63. Section 7.1, Commission Regulation (EU) No: 284/2013 of March 1, 2013 setting out data requirements for plant protection products, in accordance with Regulation (EC)No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market
64. OECD Guidance Document on Considerations for Waiving or Bridging of Mammalian Acute Toxicity Tests Series on Testing & Assessment No. 237
65. Section 5.2.4, Commission Regulation (EU) No: 283/2013 of March 1, 2013 setting out data requirements for active substances, in accordance with Regulation (EC)No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market
66. OECD Guideline for the Testing of Chemicals no: 431; *In vitro* skin corrosion: Reconstructed human epidermis (RHE) test method
67. OECD Guideline for the Testing of Chemicals no: 435; *In vitro* Membrane Barrier Test Method for Skin Corrosion
68. OECD Guideline for the Testing of Chemicals no: 439; ; *In vitro* skin irritation: Reconstructed human epidermis (RHE) test method
69. Page 12 – 13; OECD Guidance Document on Considerations for Waiving or Bridging of Mammalian Acute Toxicity Tests Series on Testing & Assessment No. 237
70. Section 5.2.5, Commission Regulation (EU) No: 283/2013 of March 1, 2013 setting out data requirements for active substances, in accordance with Regulation (EC)No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market
71. OECD Guideline for the Testing of Chemicals no: 437; Bovine Corneal Opacity and Permeability Test Method for Identifying i) Chemicals inducing serious eye damage and ii) Chemicals not requiring classification for eye irritation or serious eye damage
72. OECD Guideline for the Testing of Chemicals no: 438; Isolated Chicken Eye Test Method for Identifying i) Chemicals inducing serious eye damage and ii) Chemicals not requiring classification for eye irritation or serious eye damage
73. OECD Guideline for the Testing of Chemicals no: 460; Fluorescein Leakage Test Method for Identifying Ocular Corrosives and Severe Irritants

74. OECD Guideline for the Testing of Chemicals no: 491; Short Time Exposure *In vitro* Test Method for Identifying i) Chemicals inducing serious eye damage and ii) Chemicals not requiring classification for eye irritation or serious eye damage
75. OECD Guideline for the Testing of Chemicals no: 492; Reconstructed human Cornea-like Epithelium (RhCE) test method for identifying chemicals not requiring classification and labelling for eye irritation or serious eye damage
76. Commission Regulation (EU) No: 283/2013 of March 1, 2013 setting out data requirements for active substances, in accordance with Regulation (EC)No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market
77. Section 8.9.2, Regulation (EU) No 528/2012 of the European Parliament and of the council of 22 may 2012 concerning the making available on the market and use of biocidal products
78. OECD Environment, Health and Safety Publications Series on Testing and Assessment No. 20 :- Guidance Document For Neurotoxicity Testing
79. Part 158 Toxicology Data Requirements: Guidance for Neurotoxicity Battery, Subchronic Inhalation, Subchronic Dermal and Immunotoxicity Studies
80. USEPA toxicity data requirement
81. OPPTS 870.4300: Combined Chronic Toxicity/Carcinogenicity
82. OPPTS 870.4200: Carcinogenicity
83. OPPTS 870.4100: Chronic Toxicity
84. Guidance Document on Revisions to OECD Genetic Toxicology Test Guidelines
85. Scientific opinion on genotoxicity testing strategies applicable to food and feed safety assessment
86. Combining Genotoxicity Testing with Standard Repeated Dose Toxicology Testing: <https://www.epa.gov/pesticide-science-and-assessing-pesticide-risks/advances-genetic-toxicology-and-integration-vivo>
87. OPPTS 870.7485: Metabolism and Pharmacokinetics
88. OPPTS 870.7800 Immunotoxicity (August 1998) Guideline Studies serve a limited role in existing pesticide risk assessment methodology—A retrospective
89. Retrospective analysis of the immunotoxic effects of plant protection products as reported in the Draft Assessment Reports for their peer review at EU level
90. A Retrospective Analysis of the Immunotoxicity Study (OCSPP Test Guideline No. 870. 7800)
91. Part 158 Toxicology Data Requirements: Guidance for Neurotoxicity Battery, Subchronic Inhalation, Subchronic Dermal and Immunotoxicity Studies

92. S. K. MAYNARD, P. EDWARDS, and J. R. WHEELER. Saving Two Birds With One Stone: Using Active Substance Avian Acute Toxicity Data To Predict Formulated Plant Protection Product Toxicity. *Environmental Toxicology and Chemistry*, Vol. 33, No. 7, pp. 1578–1583, 2014
93. Risk Assessment for Birds and Mammals *EFSA Journal* 2009; 7(12):1438
94. Setting out the data requirements for active substances, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market. <http://eurlex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2013:093:0001:0084:EN:PDF>
95. EURL ECVAM Strategy to replace, reduce and refine the use of fish in aquatic toxicity and bioaccumulation testing. <https://eurl-ecvam.jrc.ec.europa.eu/eurl-ecvam-strategy-papers/strategy-fish>
96. OCED Series on Testing and Assessment No. 126: Short Guidance On The Threshold Approach For Acute Fish Toxicity