Chapter 15. Determination of drug toxicity in animals

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Introduction

Toxicity is the degree to which a substance (a toxin or poison) can harm humans or animals. Toxicity can refer to the effect on a cell (cytotoxicity), an organ (e.g. renal or liver toxicity), or the whole organism thus explaining why scientists use different procedures to assess toxicity and to provide an estimate of how much of a substance causes a kind of harm. All substances are potentially toxic depending on the quantity. Many therapeutic medications can be acutely toxic, but are beneficial when used at the appropriate level: vitamin D, oxygen or water being the simplest examples. However, a larger quantity of a substance does not automatically imply harm. This is why toxicity determination is primarily focused on determining the type and degree of harm, caused by different amounts of a substance or drug.

Many serious toxic reactions caused by new chemical entities may be detected by routine toxicological testing. Experience has shown that predictable “dose time-dependent” reactions are likely to be revealed in animal experiments. These tests form the basis of the experimental toxicology that is applied to every new drug development or unknown substance. Unpredictable idiosyncratic adverse effects, not related to time
or dose, are considerably more difficult to identify in preclinical drug evaluation and do not constitute the purpose of this chapter.

There is no measure of toxicity, and its effects may occur in short term (acute effects), or after repeated exposure over a long period (sub-acute or chronic effects). The following tests are performed in the Centre for Study and Therapy of Pain and Central Drug Testing Laboratory, “Gr.T.Popa” University of Medicine and Pharmacy Iasi, using rodent models, for detection:

- Acute toxicity tests (single dose)
- Sub-acute toxicity test (daily dose - 14 to 28 days)
- Sub-chronic toxicity test (daily dose – up to 90 days)
- Chronic toxicity test (daily dose – up to 12 months)

**ACUTE TOXICITY TEST**

1. **Definition:**

The Globally Harmonized System (GHS) defines Acute Toxicity as “*those adverse effects occurring following oral or dermal administration of a single dose of a substance, or multiple doses given within 24 hours, or an inhalation exposure of 4 hours*”.

Acute toxicity tests are generally the first tests conducted and they provide critical data on the relative toxicity likely to arise from a single or brief exposure. Standardized tests are available for oral, dermal and inhalation exposures and the preferred species for oral and inhalation testing is the rat, and for dermal
testing the rat or the rabbit. Oral administration is the most common form of acute systemic toxicity testing. The Organization for Economic Cooperation and Development (OECD) elaborate five Test Guidelines for describing acute systemic testing:

- Fixed Dose Procedure (OECD TG 420)
- Acute toxic Class method (OECD TG 423)
- Up-and-Down Procedure (OECD TG 425)
- Acute Dermal Toxicity (OECD TG 402)
- Acute Inhalation Toxicity (OECD TG 403)

2. Aims

The aim of the acute toxicity test is to determine the therapeutic index, which is the ratio between the lethal dose and the pharmacologically effective dose, in the same species and strains (LD50/ED50). The greater the index, the safer the compound is. The acute test may also provide initial information on the mode of toxic action of a substance and to determine the LD50 value that provide indices of potential types of drug activity.

The median lethal dose (LD50) is a statistically derived single dose of a substance that can be expected to cause death in 50% of test animals at the end of the experiment (up to 14 days), when administrated via oral, dermal or inhalation way. Its value is expressed in terms of weight of test substance per unit weight of test animal (usually mg/kg). LD50 is the endpoint for oral or dermal administration, where as the lethal concentration or LC50 is the end point for inhalation administration. The LD50 does not reflect the acute toxic properties of a substance, nor suggest enough information to
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categorize a compound. It is just a data that represent lethality and also does not correlate well with information on different mechanism of action of toxic agents, especially compounds from different toxicological categories. The lower the LD₅₀ - the lower the lethal dose - the more toxic is the substance. LD₅₀ values are unknown for humans, but animal LD₅₀ value can be used to estimate lethal amounts for humans.

Interpretation example: A reported “rat oral LD₅₀ of 35 mg/kg” means that half of the rats that ingested 35 milligrams of investigated substance per kilogram of body weight died in 14 days.

3. Initial Considerations

Test substances, at doses that are known to cause marked pain and distress due to corrosive or severely irritant actions, need not be administered. It is a principle of the method that in the main study only moderately toxic doses are used, and that administration of doses that are expected to be lethal should be avoided. Moribund animals, or animals obviously in pain, or showing signs of severe and enduring distress shall be sacrificed according the ethical principles, and are considered in the interpretation of the test results in the same way as animals that died on test.

Our laboratory considers all available information on the test substance prior to conducting the study. Such information includes:

- The identity and chemical structure of the test substance;
- Its physical chemical properties;
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- The results of any other *in vitro* or *in vivo* toxicity tests on the substance;
- Toxicological data on structurally related substances or similar mixtures;
- The anticipated use(s) of the substance.

This information is useful to determine the relevance of the test for the protection of human health and the environment, and helps in the selection of an appropriate starting dose.

4. Principle of the test

**Acute Toxic Class Method**

The substance is administered orally to a group of experimental animals at one of the defined doses. The substance is then tested using a stepwise procedure, each step using three animals of single sex (normally females). 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

This method is the most used in our laboratory.

**Fixed Dose Procedure**

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. The initial dose level is selected on the basis of a sighting study
as the dose expected to produce some signs of toxicity, without causing severe toxic effects or mortality. Further groups of animals may be dosed at higher or lower fixed doses, depending on the presence or absence of signs of toxicity or mortality. This procedure continues until the dose causing evident toxicity, or no more than one death is identified, or when no effects are seen at the highest dose, or when deaths occur at the lowest dose.

**Up-and-Down-Procedure (UDP)**

The Limit Test is a sequential test that uses a maximum of 5 animals. A maximum test dose of 2000 mg/kg may be used. The selection of a sequential test plan increases the statistical power and also has been made to intentionally bias the procedure towards rejection of the limit test for compounds with LD50s near the limit dose; i.e., to error on the side of safety. As with any limit test protocol, the probability of correctly classifying a compound will decrease, as the actual LD50 better resembles the limit dose.

**Acute Dermal Toxicity**

The test substance is applied to the skin, in graduated doses, to several groups of experimental animals, one dose being used per group. Subsequently, observations of effects and deaths are made. Animals that die during the test are forensically investigated, and at the conclusion of the test the surviving animals are also sacrificed and forensic analysis performed. Animals showing severe and enduring signs of distress and pain may need to be sacrificed. Administration of test substances in a way known to cause marked pain and distress
due to corrosive or irritating properties is not carried out. This test is particularly useful in testing novel analgesic combinations with dermal administration.

5. Description of the method

- Selection of animal species and sex

The preferred rodent species is the rat, although other rodent species may be used and females are preferred because literature survey of conventional LD50 tests showed that females are slightly more sensitive. However if knowledge of the toxicological or toxicokinetic properties of structurally related chemicals indicates that males are likely to be more sensitive, then this sex should be used. When the test is conducted in males, adequate justification should be provided. At least five rodents are used at each dose level. They are all of the same sex. After completion of the study in one sex, at least one group of five animals of the other sex will also be tested.

Healthy young adult animals of commonly used laboratory strains should be employed. Females should be nulliparous and non-pregnant. Each animal, at the start of administration, should be between 8 and 12 weeks old and its weight should fall in an interval within ± 20 % of the mean weight of any previously dosed animals.

- Housing and feeding conditions

The temperature in the experimental animal room should be 22°C (± 3°C), with a relative humidity between 30%-70%, with artificial/natural light in sequence 12 hours light/12hour dark.
The conventional laboratory diets may be used for feeding and water ad libitum. Animals may be group-caged by dose or by sex (where appropriate), or in single cages with the mention that the number of animal per cage must not interfere with clear observational of each animal.

• Preparation of animals

The animals are randomly selected, marked to permit identification and caged for at least 5 days prior to dosing, to allow for acclimatization to the laboratory condition and should also be acclimatized to the test apparatus for a short period prior to testing, as this will lessen the stress caused by introduction to the new environment.

• Preparation of doses

The substance used in the toxicity tests should be as pure as the one intended to be given to humans. In general test, substances should be administered in a constant volume over the range of doses to be tested, by varying the concentration of the dosing preparation. Where a liquid end product or mixture is to be tested however, the use of the undiluted test substance, i.e. at a constant concentration, may be more relevant to the subsequent risk assessment of that substance, and is a requirement of some regulatory authorities. In either case, the maximum dose volume (50 ml/kg) for administration must not be exceeded. The maximum volume of liquid that can be administered at one time depends on the size of the test animal. In rodents, the volume should not normally exceed 1mL/100g of body weight: however in the case of aqueous solutions 2 mL/100g body weight can be considered. With respect to the
formulation of the dosing preparation, the use of an aqueous solution/suspension/emulsion is recommended whenever possible, followed in order of preference by a solution/suspension/emulsion in oil (e.g. corn oil) and then in other vehicles. For vehicles other than water the toxicological characteristics of the vehicle should be known. Injection must be given slowly and uniformly to avoid undue killing by a drug having predominant action on the CNS or heart. Doses must be prepared shortly prior to administration unless the stability of the preparation over the period during which it will be used is known and shown to be acceptable.

• Procedure: Administration of doses

The LD$_{50}$ value depends on the route of administration and usually the values are found to increase with the following sequences of routes: intravenous, intraperitoneal, subcutaneous and oral. The intravenous route is preferable to the peritoneal route because the liver detoxifies many drugs if the peritoneal route is employed, but the oral and the peritoneal administration are most commonly used. Administration is made in a single dose by gavage using a stomach tube or an intubation cannula, and when this is not possible, the dose can be given in smaller fraction within 24 hour. Animal should be fastened prior to dosing - in rat food withheld over-night; in mice 3-4 hours with unrestricted water. Following the fasting period the animals should be weighted and the test substance administered, after which the food may be withheld for further 3-4 hours in rats or 1-2 hours in mice, with the exception of drugs administered in fractions.
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- **Limit test**

The limit test is primary used in situation where the experimenter has information, gained from knowledge about similar tested compounds or similar tested mixtures or products, indicating that the test is likely to be nontoxic. In those situations where there is little or no information about compound toxicity, the main test should be performed.

- **Limit Test at 2000mg/kg**

Dose one animal at the test dose, if the animal dies, conduct the main test to determine the LD50. If the animal survives, dose four additional animals so a total of five animals are tested. However, if three animals die, the limit test is terminated and the main test is performed. If three or more animals survive then the LD50 is greater than 2000 mg/kg.

- **Maximum tolerated dose**

If there is sufficient information that the compound is likely not toxic and a limit test is to be performed, there is the possibility of a physical limit for administration. Specifically, each administration way has a physical limit (e.g. volume, size, weight) of solution, compound, capsule, etc. that can be administered without harming the animal. The limit of volume for a solution for example, means that the maximum substance quantity that can be administered is the maximum substance that can be diluted, but still possible to inject in that limit volume. The dose administered this way is called Maximum Tolerated Dose (MTD). MTD can be larger or smaller than the 2000 limits.
• Observation

After the administration, the animal is the sole occupant of the cage, with free access to food and water, and is observed at least once during the first 30 minutes, periodically during the first 24 hours (with special attention during the first 4 hours), and daily thereafter, for a total of up to 14 days, except where they need to be removed from the study and sacrificed for animal welfare reasons or are found dead.

Signs recorded during acute toxicity studies: increased motor activity, anesthesia, tremors, arching and rolling, clonic convulsions, ptosis, tonic extension, lacrimation, Straub reaction, exophthalmos, pilo-erection, salivation, muscle spasm, opisthotonus, writhing, hyperesthesia, loss of righting reflex, depression, ataxia, stimulation, sedation, blanching, hypnosis, cyanosis and analgesia. The times at which signs of toxicity appear and disappear are important, especially if there is a tendency for toxic signs to be delayed. All observations are systematically recorded, with individual records being maintained for each animal. Additional observations will be necessary if the animals continue to display signs of toxicity.

Observations should include changes in skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous systems, as well as somatomotor activity and behavior pattern. Attention should be directed to observations of tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma. Animals found in a moribund condition and animals showing severe pain or enduring signs of severe distress should be sacrificed. When
animals are sacrificed for ethical reasons or found dead, the time of death should be recorded as precisely as possible.

- **Body weight**

Individual weights of animals should be determined shortly before the test substance is applied, weekly thereafter, and at death; changes in weight should be calculated and recorded when survival exceeds one day. At the end of the test, surviving animals are weighed and then sacrificed.

- **Pathology**

All test animals (including those that die during the test or are removed from the study for animal welfare reasons) should be subjected to gross necropsy. All gross pathological changes should be recorded for each animal. Microscopic examination of organs showing evidence of gross pathology in animals surviving 24 or more hours may also be considered because it may yield useful information. If necropsy cannot be performed immediately after the death of the animal it should be refrigerated to minimize autolysis. Necropsies must be performed no later than 16 h after death.

6. **Data and reporting**

**Data**

Individual data of all animals are provided and all data summarized showing for each test group the number of animals used, the number displaying signs of toxicity, found dead during the test or sacrificed, time of death of individual
animals, a description and the time course of toxic effects, reversibility, and necropsy findings.

Calculation of LD50

The LD50 is calculated by graphical method (Miller and Tainter) or arithmetical method of Karber, depending on the preferences of the experimenters and the number of animals used.

Test report

Must include the following information as appropriate:

- Test substance—physical nature, purity, physicochemical properties and identification data
- Vehicle — justification for choice of vehicle, if other than water.
- Test animals: -species/strain used, microbiological status of the animals, number, age and sex of the animals, source, housing condition, diet etc.
- Test conditions:
  - Details of test substance formulation including details of the physical form of the material administered;
  - Details of the administration of the test substance including dosing volumes and time of dosing;
  - Details of food and water quality (including diet type/source, water source);
  - The rationale for the selection of the starting dose.
- Results:
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- Tabulation of response data and dose level for each animal (i.e. animals showing signs of toxicity including mortality; nature, severity, and duration of effects);
- Tabulation of body weight and body weight changes;
- Individual weights of animals at the day of dosing, in weekly intervals thereafter, and at the time of death or sacrifice;
- Date and time of death if prior to scheduled sacrifice;
- Time course of onset of signs of toxicity, and whether these were reversible for each animal;
- Necropsy findings and pathological findings for each animal, if available;

- Discussion and interpretation of results
- Conclusions

SUB-ACUTE AND CHRONIC TOXICITY TEST

1. Introduction

Sub-acute and chronic toxicity test determine toxicity from exposure for a substantial portion of a subject's life and the Globally Harmonized System (GHS) defines it as "specific target organ/systemic toxicity arising from a repeated exposure". In rats these studies range in duration from 28-days (sub-acute studies) to 90-day (sub-chronic studies), and even 12-months (chronic studies), and consist of repeated doses in oral, inhalation and dermal administration.

These studies are conducted in stages so that the results of one study can be used to design the subsequent study of longer duration. The first are usually 2 weeks in length followed by 1-month, 3-month, 6-month, and then 1-year studies. The
endpoints for repeat dose testing consist of an evaluation of clinical observations, blood analysis, whole body gross necropsy, and microscopic examination of all organs and tissues (histopathology). The data from these studies provide valuable information on the cumulative exposure of target organs, and on general health hazards likely to occur as a consequence of repeated low-dose exposure to a chemical.

There are six OECD (Organization for Economic Cooperation and Development) Test Guidelines describing short-term repeat-dose toxicity testing:

- Repeated Dose 28-day Oral Toxicity Study in Rodents (OECD TG 407)
- Repeated Dose 90-Day Oral Toxicity Study in Rodents (OECD TG 408)
- Repeated Dose Dermal Toxicity: 21/28-day Study (OECD TG 410)
- Sub-chronic Dermal Toxicity: 90-day Study (OECD TG 411)
- Repeated Dose Inhalation Toxicity: 28-day or 14-day Study (OECD TG 412)
- Sub-chronic Inhalation Toxicity: 90-day Study (OECD TG 413).

2. Aims

Repeat-dosing toxicity studies are conducted to determine what side effects will arise from repeated administration of a drug at lower dosages than those used in acute toxicity studies, and to determine safe dosages to be used in the initial human clinical trials. The study will provide information on the major toxic effects, indicate target organs and the possibility of
accumulation, and can provide an estimate of no-observable-adverse-effects levels (NOAEL) or point of departure for establishment of a Benchmark Dose (BMD), of exposure which can be used in selecting dose levels for chronic studies and for establishing safety criteria for human exposure. Also the identification of the hazardous properties of a chemical, characterization of the dose-response relationship or the provision of data to test hypotheses regarding the mode of action is some of the aims of chronic toxicity tests.

As these studies are aimed at detecting any systemic toxic effect, a wide variety of parameters is monitored, such as clinical appearance including ophthalmological examination, body weight, body weight gain, and food consumption; and sometimes water intake, hematology, clinical biochemistry, and pathology including histopathology. To conduct the dose-response studies the scientists are looking in these tests for so called Lowest Observable Effect Level (LOEL), which is the smallest dose that causes any detectable effect. In addition to LOEL, the term LOAEL (Lowest Observable Adverse Effect Level) is sometimes used. The term LOAEL implies a judgment that the effect is adverse. A LOEL refers to any effect and may or may not be judged to be adverse. These parameters are measured in milligrams (mg) of substance per kilogram (kg) of body weight, or in parts per million (ppm) of substance in food.

For determining safe levels of a substance the usual method is to determine the highest dose at which no effects occur - the No Observable Effect Level (NOEL) and it is considered the “safe level” for that substance, in the studied species, but not necessarily the “safe level” for humans.
3. Initial Consideration

Information that assists in the selection of appropriate test concentrations can include:

- The identity, chemical structure, and physicochemical properties of the test article;
- Results of any in vitro or in vivo toxicity tests; anticipated use(s) and potential for human exposure;
- Available (Q) SAR data and toxicological data on structurally related substances;
- Data derived from acute inhalation toxicity testing.

If neurotoxicity is expected or is observed in the course of the study, the study Principal Investigator may choose to include appropriate evaluations such as a functional observational battery (FOB) and measurement of motor activity. Dilutions of corrosive or irritating test articles may be tested at concentrations that will yield the desired degree of toxicity. When exposing animals to these materials, the targeted concentrations should be low enough to not cause marked pain and distress, yet sufficient to extend the concentration-response curve to levels that reach the regulatory and scientific objective of the test. These concentrations should be selected on a case-by-case basis, preferably based upon an adequately designed range-finding study that provides information regarding the critical endpoint, any irritation threshold, and the time of onset. The justification for concentration selection should be provided.
4. **Principles of the test:**

- **Repeated Dose 28-Day Oral Toxicity:** “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 that die or are euthanized during the test are necropsied and at the conclusion of the test surviving animals are euthanized and necropsied. 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. The data derived from using the TG should allow for the characterization of the test substance toxicity, for an indication of the dose response relationship and the determination of the No-Observed Adverse Effect Level (NOAEL).”

- **Repeated Dose 90-day Oral Toxicity:** ”The test substance is orally administered daily in graduated doses to several groups of experimental animals, one dose level per group for a period of 90 days. During the period of administration the animals are observed closely for signs of toxicity. Animals which die or are killed during the test are necropsied and, at the conclusion of the test, surviving animals are also killed and necropsied”.

- **Sub-chronic Dermal Toxicity 90-day Study:** “The test substance is applied daily to the skin in graduated doses to several groups of experimental animals, one dose per group, for a period of 90 days. During the period of application the
animals are observed daily to detect signs of toxicity. Animals which die during the test are necropsied, and at the conclusion of the test the surviving animals are sacrificed and necropsied”.

- **Repeated Dose Dermal Toxicity 21/28-day Study:** "The test substance is applied daily to the skin in graduated doses to several groups of experimental animals, one dose per group, for a period of 21/28 days. During the period of application the animals are observed daily to detect signs of toxicity. Animals which die during the test are necropsied, and at the conclusion of the test the surviving animals are sacrificed and necropsied”.

5. **Description of the method**

- **Selection of animal species and sex**

As a rule, mammals are selected to be test animals, and animals with a clearly known origin, species and breed are to be used. The preferred rodent species is the rat, although other rodent species may be used and if so, justification should be given. Although it is biologically plausible that other species should respond to toxicants in a similar manner to the rat, the use of smaller species may result in increased variability due to technical challenges of dissecting smaller organs. In the international validation program for the detection of endocrine disrupters, the rat was the only species used. Young healthy adult animals of commonly used laboratory strains should be employed. Females should be nulliparous and non-pregnant. Dosing should begin as soon as feasible after weaning, and, in any case, before the animals are nine weeks
old. At the commencement of the study the weight variation of animals used should be minimal and not exceed ± 20% of the mean weight of each sex. When the test is conducted as a preliminary to a longer-term study, it is preferable that animals from the same strain and source should be used in both studies.

- Housing and feeding conditions

Animals should be individually identified, if possible with subcutaneous transponders, to facilitate observations and avoid confusion. All procedures should conform to local standards of laboratory animal care. 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 to exceed 70% (other than during room cleaning), the aim should be 50-60%. Lighting should be artificial, the photoperiod 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 orally. Animals should be group housed in small groups of the same sex; animals may be housed individually if scientifically justified. For group caging, no more than five animals should be housed per cage.

- Preparation of animals

Healthy animals, which have been acclimated to laboratory conditions for at least 5 days and have not been subjected to previous experimental procedures, should be used. The test animals should be characterized as to species, strain, source, sex, weight and/or age. Animals should be randomly assigned
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to the control and treatment groups. Cages should be arranged in such a way that possible effects due to cage placement are minimized.

- **Preparation of doses**

The test compound is administered different depending on the aims of the study. The method of oral administration is dependent on the purpose of the study, and the physical/chemical properties of the test material. Where necessary, the test substance is dissolved or suspended in a suitable vehicle. It is recommended that, whenever possible, the use of an aqueous solution/suspension be considered first, followed by consideration of a solution/emulsion in oil (e.g., corn oil) and then by possible solution in other vehicles. For vehicles other than water the toxic characteristics of the vehicle must be known. The stability of the test substance under the conditions of administration should be determined.

6. **Procedure**

- **Administration of doses**

*In oral toxicity studies*

The animals are dosed with the test substance daily seven days each week for a period of 28/90 days. Any other dosing regime, e.g., five days per week, needs to be justified. When the test substance is administered by gavage, this is done in a single dose to the animals using a stomach tube or a suitable intubation cannula at similar times each day, and adjusted as necessary to maintain a constant dose level in terms of animal body weight. The maximum volume of liquid that can be
administered at one time depends on the size of the test animal. The volume should not exceed 1 ml/100g body weight, except in the case of aqueous solutions where 2-ml/100g body weights may be used. Except for irritating or corrosive substances, which will normally reveal exacerbated effects with higher concentrations, adjusting the concentration to ensure a constant volume at all dose levels should minimize variability in test volume.

When administering a test substance by adding it to the food for the animals, closely monitor the homogeneity, additive concentration and safety of the test substance after it has been added, and confirm these factors at fixed intervals. When dissolving the test substance in a solvent, to form a suspension or emulsion, clearly determine the concentration and safety of the test substance. Where a repeated dose or a 90-day study is used as a preliminary to a long-term chronic toxicity study, a similar diet should be used in both studies.

**In dermal toxicity studies**

The animals are treated with the test substance, ideally for at least 6 hours per day on a 7-day per week basis, for a period of 90 days. However, based primarily on practical considerations, application on a 5-day per week basis is considered to be acceptable. Animals in a satellite group scheduled for follow-up observations should be kept for at least a further 28 days without treatment to detect recovery from, or persistence of, toxic effects.

The test substance should be applied uniformly over an area that is approximately 10 per cent of the total body surface area. With highly toxic substances the surface area covered may be
less, but the area should be covered with as thin and uniform a film as possible. Between applications the test substance is held in contact with the skin with a porous gauze dressing and non-irritating tape. The test site should be further covered in a suitable manner to retain the gauze dressing and test substance and ensure that the animals cannot ingest the test substance. Restrainers may be used to prevent ingestion of the test substance, but complete immobilization is not a recommended method.

- **Limit test**

If a test at one dose level of at least 1000 mg/kg body weight/day or, for dietary or drinking water administration, an equivalent percentage in the diet, or drinking water (based upon body weight determinations), using the procedures described for this studies, produces no observable toxic effects and if toxicity would not be expected based upon data from structurally related compounds, then a full study using three dose levels may not be considered necessary. The limit test applies, except when human exposure indicates the need for a higher dose level to be used.

- **Observation**

The observation period should be 28 days in sub-acute toxicity and at least 90 days in sub-chronic and chronic toxicity tests. Animals in a satellite group scheduled for follow-up observations should be kept for an appropriate period without treatment to detect delayed occurrence, or persistence of, or recovery from toxic effects. As a rule, observations are conducted on the following items:
a) General condition and death rate - should be made at least once a day, preferably at the same time(s) each day, and considering the peak period of anticipated effects after dosing. The health condition of the animals should be recorded. At least twice daily, all animals are observed for morbidity and mortality.

b) Weight, food intake and water intake, food intake efficiency- all animals should be weighed at least once a week. Measurements of food consumption should be made at least weekly. If the test substance is administered via the drinking water, water consumption should also be measured at least weekly. Water consumption may also be considered for dietary or gavage studies during which drinking activity may be altered.

c) Hematologic test - blood samples should be taken from a named site and stored, if applicable, under appropriate conditions. At the end of the test period, samples are collected just prior to or as part of the procedure for killing the animals. The following hematological examinations should be made at the end of the test period and when any interim blood samples may have been collected: hematocrit, hemoglobin concentration, erythrocyte count, total and differential leukocyte count, platelet count and a measure of blood clotting time/potential.

d) Blood biochemistry test - are used to investigate major toxic effects in tissues and, specifically, effects on kidney and liver, should be performed on blood samples obtained from each animal just prior to or as part of the procedure for killing the animals (apart from those found moribund and/or intercurrently killed). In a similar manner to hematological investigations, interim sampling for clinical
biochemical tests may be performed. Determinations in plasma or serum should include sodium, potassium, glucose, total cholesterol, urea, blood urea nitrogen, creatinine, total protein and albumin, and more than two enzymes indicative of hepatocellular effects (such as alanine-aminotransferase, aspartate-aminotransferase, alkaline phosphatase, gamma glutamyl-transpeptidase, and sorbitol-dehydrogenase). Measurements of additional enzymes (of hepatic or other origin) and bile acids, which may provide useful information under certain circumstances, may also be included.

e) Urine test - using timed urine volume collection: appearance, volume, osmolality or specific gravity, pH, protein, glucose and blood/blood cells.

f) Pathology test- observation with the naked eye and internal organ-weight and with a microscope.

All animals in the study shall be subjected to a full, detailed gross necropsy that includes careful examination of the external surface of the body, all orifices, the cranial, thoracic and abdominal cavities and their contents. The liver, kidneys, adrenals, testes, epididymides, uterus, ovaries, thymus, spleen, brain and heart of all animals (apart from those found moribund and/or intercurrently killed) should be trimmed of any adherent tissue, as appropriate, and their wet weight taken as soon as possible after dissection to avoid drying. 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.
7. Data and reporting

Data

Individual data should be provided. Additionally, all data should be summarized in tabular form showing for each test group the number of animals at the start of the test, the number of animals found dead during the test or euthanized for ethical reasons and the time of any death or euthanasia, the number showing signs of toxicity, a description of the signs of toxicity observed, including time of onset, duration, and severity of any toxic effects, the number of animals showing lesions, the type of lesions, their severity and the percentage of animals displaying each type of lesion. When applicable, numerical results should be evaluated by an appropriate and generally acceptable statistical method. The statistical methods and the data to be analyzed should be selected during the design of the study.

Test report

The test report must include the following information:

- Test substance;
- Vehicle (if appropriate);
- Test animals;
- Test conditions:
  - Rationale for dose level selection;
  - Details of test substance formulation/diet preparation, achieved concentration, stability and homogeneity of the preparation;
  - Details of the administration of the test substance;
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- Actual doses (mg/kg body weight/day), and conversion factor from diet/drinking water;
- Test substance concentration (ppm) to the actual dose, if applicable;
- Details of food and water quality.

• Results:
  - Body weight and body weight changes;
  - Food consumption, and water consumption, if applicable;
  - Toxic response data by sex and dose level, including signs of toxicity;
  - Nature, severity and duration of clinical observations (whether reversible or not);
  - Results of ophthalmological examination;
  - Sensory activity, grip strength and motor activity assessments (when available);
  - Hematological tests with relevant base-line values;
  - Clinical biochemistry tests with relevant base-line values;
  - Terminal body weight, organ weights and organ/body weight ratios;
  - Necropsy findings;
  - A detailed description of all pathological findings;
  - Absorption data if available;
  - Statistical treatment of results, where appropriate.

• Discussion of results
• Conclusions

When a chemical substance is administrated to a subject, different types of interactions can occur and a series of dose-related responses result which in most cases are desired and
useful but they came with a number of other effects which are not so useful and can even harm the patient.

The types of toxicity tests that are routinely performed by pharmaceutical manufactures in the investigation of a new drug involve acute, sub-acute and chronic toxicity. Acute toxicity is involved in estimation of LD50 (the dose which has proved to be lethal (causing death) to 50% of the tested group of animals). Determination of acute oral toxicity is usually an initial screening step in the assessment and evaluation of the toxic characteristics of all compounds. The methods so far utilized for the determination of median lethal dose (LD50) and the new changes which could be made. Sub acute and chronic toxicity studies are designed to characterize the toxic effects of drugs upon repeated daily administration for periods of time ranging from 2 weeks to 1 year and to determine no-toxic-effect dosage levels for short to long-term repeated dosing.

References

3. Horn H.J., Simplified LD50 (or ED50) Calculations; Biometrics Vol. 12, No. 3,1956; 311-322. Published by: International Biometric Society
5. Occupational Safety and Health Standards Toxic and Hazardous Substances-“Health Hazard Criteria (Mandatory)” App A
6. OECD GUIDELINE FOR TESTING OF CHEMICALS “Repeated Dose Dermal Toxicity: 21/28-day Study”-TG 410
7. OECD GUIDELINE FOR TESTING OF CHEMICALS “Subchronic Dermal Toxicity: 90-day Study”-TG 411
8. OECD GUIDELINE FOR TESTING OF CHEMICALS “Acute Dermal Toxicity” -TG 402
9. OECD GUIDELINE FOR TESTING OF CHEMICALS “Acute Inhalation Toxicity” - TG 403
10. OECD GUIDELINE FOR TESTING OF CHEMICALS “Acute Oral Toxicity – Acute Toxic Class Method”- TG 423
11. OECD GUIDELINE FOR TESTING OF CHEMICALS “Acute Oral Toxicity – Up-and-Down-Procedure (UDP)”- TG 425
12. OECD GUIDELINE FOR TESTING OF CHEMICALS “Acute Oral Toxicity – Fixed Dose Procedure“-TG 420
13. OECD GUIDELINE FOR THE TESTING OF CHEMICALS “Repeated Dose 90-day Oral Toxicity Study in Rodents”-TG 408
14. OECD GUIDELINE FOR THE TESTING OF CHEMICALS “Subacute Inhalation Toxicity: 28-Day Study”-TG 412
15. OECD GUIDELINE FOR THE TESTING OF CHEMICALS “Subchronic Inhalation Toxicity: 90-Day Study”-TG 413
17. Prieto et al.- “Subacute and subchronic toxicity”