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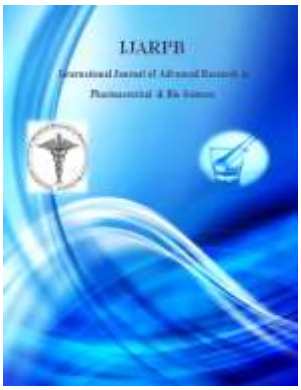
Study of acute, Sub acute and chronic toxicity test

Bhardwaj S, Deepika Gupta*[1],

Seth G. L. Bihani S.D. College of Technical Education, Institute of Pharmaceutical Sciences and Drug Research, Sriganganagar,



Corresponding Author

Deepika Gupta

M.pharm Q.A.

Seth G. L. Bihani S.D. College of Technical Education, Institute of Pharmaceutical Sciences and Drug Research, Sriganganagar, India

Email-deepika.sgnr@gmail.com

ABSTRACT

The types of toxicity tests which are routinely performed by pharmaceutical manufacturers in the investigation of a new drug which are routinely performed by pharmaceutical manufacturers in the investigation of a new drug involve Acute, Sub acute and chronic toxicity. The acute toxicity test in which a single dose is used in each animal on one occasion only for the determination of gross behavior and LD50 or median lethal dose. The chronic tests in which two species, one rodent and one non rodent are dosed daily for six months. The sub acute tests in which animals (usually rats and dogs) are dosed daily, starting at around expected therapeutic level and increasing stepwise every two to three days until toxic signs are observed.

KEYWORDS - Dose-related response, lethal dose, median lethal concentration, toxicity tests.

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INTRODUCTION

It is essential to use at least two species (usually a rodent and a non- rodent) in the evaluation of the potential toxicity of a drug because species differ in their responses to toxic agents. It is also unwise to use a homogenous strain (inbred strain) in toxicity tests, and the aim should be to discover new and unexpected effects of a drug in animals of wider variability like random bred animals.

A drug effect that is seen both in the rat and in the dog probably involves a common physiology mechanism that is likely to be present in the human, whereas an effect seen only in one of the two species indicates that the same is peculiar to that species, and is less likely to be present in the third species. For instances, a toxic effect observed only in rats or dogs would indicate its probability of occurring in about 25 percent in cases of man; while an effect observed in both rats and dogs would indicates a probability of 80 percent. 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. It is the detail of these that forms the basis of the experimental toxicology that is applied to new drug development. Unpredictable idiosyncratic adverse effects, not related to time or dose, are considerably more difficult to identify in preclinical drug evaluation[1].

Most chemicals are now subject to stringent government requirements for safety testing before they can be marketed. This is especially true for pharmaceuticals, food additives, pesticides, and industrial chemicals. Exposure of the public to inadequately tested drugs or environmental agents has resulted in several notable disasters. Examples include:

Severe toxicity from the use of arsenic to treat syphilis Deaths from a solvent (ethylene glycol) used in sulfanilamide preparations (one of the first antibiotics)

Thousands of children born with severe birth defects resulting from pregnant women using thalidomide, an anti-nausea medicine By the mid-twentieth century, disasters were becoming commonplace with the increasing rate of development of new synthetic chemicals. Knowledge of potential toxicity was absent prior to exposures of the general public [9]. The following tests are performed on laboratory animals for detection of toxicity of a compound.

Acute toxicity test (single dose)

Sub-acute toxicity test (daily dose)

Chronic toxicity test (daily dose) [2]

ACUTE TOXICITY TEST

Acute toxicity tests are generally the first tests conducted. They provide data on the relative toxicity likely to arise from a single or brief exposure. Standardized tests are available for oral, dermal, and inhalation exposures.

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Table 1: Basic parameters of Acute toxicity tests

Species	Rats preferred for oral and inhalation tests; rabbits preferred for dermal tests
Age	Young adults
Number of animal	5 of each sex per dose level
Dosage	Three dose levels recommended; exposures are single doses or fractionated doses up to 24 hours for oral and dermal studies; and 4 hour exposure for inhalation studies
Observation period	14 days

Test in which single dose of the drug is used in each animal on one occasion only for the determination of gross behavior and LD50 (the dose which has proved to be lethal (causing death to 50% of the tested group of animals) is usually an initial step in the assessment and evaluation of the toxic characteristics of a substance. It is an initial assessment of toxic manifestations (provides information on health hazards likely to arise from short-term exposure to drugs) and is one of the initial screening experiments performed with all compounds [3].

Data from the acute study may

(a) Serve as the basis for classification and labeling.

(b) Provide initial information on the mode of toxic action of a substance.

(c) Help arrive at a dose of a new compound.

(d) Help in dose determination in animal studies.

(e) Help determine LD50 values that provide many indices of potential types of drug activity [3].

Each LD50 test performed for a chemical must include at least two routes of exposure, usually oral and parenteral routes. Depending on the nature of a substance, the routes can be modified to include testing for inhalation, dermal, or other selective exposure. For inhalation or aquatic studies, toxicology is presented as the median lethal concentration (LC50) the estimated concentration of environmental exposure resulting in 50% mortality of the population of experimental animals.

LD50 data represent lethality and do not reflect the acute toxic properties of a compound, nor does the value suggest enough information to adequately categorize a compound. The LD50 also does not correlate well with information on different mechanisms of action of toxic agents, especially when the agents are in different toxicological categories. Comparisons are significant only when agents are homologous and have the same or similar mechanisms of action. In fact, analysis of LD50data simply translates a value into a comparative indicator of immediate

toxicology for a species agent in a particular strain, age group, and sex of a species of animal[1].

AIM OF ACUTE TOXICITY TEST

To determine the therapeutic index, i.e. ratio between the lethal dose and the pharmacologically effective dose in the same strain and species (LD50/ED50).

The greater the index, safer is the compound. LD50 with confidence limits is to be established on one common laboratory species such as mouse/rat using the standard method. The LD50 dose thus found was administered to guinea pigs, rabbits, cats or dogs on weight basis (on basis of relative surface area gives better results).

To determine the absolute dose for a species in the column, the absolute dose given to the species in a row was multiplied by the factor given at intersection of the relevant row and column (Table 1). Because of species variation, several species of animals (one rodent and one non-rodent) were used to determine LD50.

When a clearly different response was observed in any of these species, a larger number of that species needs to be tested to establish the approximate LD50 value [3].

Table 2: Surface area ratios of some common laboratory species and man

	20 g Mouse	200 g Rat	400 g Guinea-pig	1.5 kg Rabbit	2 kg Cat	4 kg Monkey	12 kg Dog	70 kg Man
20 g Mouse	1.0	7.0	12.25	27.8	29.7	64.1	124.2	387.9
200 g Rat	0.14	1.0	1.74	3.9	4.2	9.2	17.8	56.0
400 g Guinea-pig	0.08	0.57	1.0	2.25	2.4	5.2	10.2	31.5
1.5 kg Rabbit	0.04	0.25	0.44	1.0	1.08	2.4	4.5	14.2
2 kg Cat	0.03	0.23	0.41	0.92	1.0	2.2	4.1	13.0
4 kg Monkey	0.016	0.11	0.19	0.42	0.45	1.0	1.9	6.1
12 kg Dog	0.008	0.06	0.10	0.22	0.24	0.52	1.0	3.1
70 kg Man	0.0026	0.018	0.031	0.07	0.076	0.16	0.32	1.0

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CLASSIC ACUTE TOXICITY TESTING

A variety of factors should be considered when planning and organizing a study for the determination of LD50, not all of which are associated with the calculation of 95% confidence limits. Most preparations among the different routes of administration are similar although some changes are required as shown below.

Oral LD50: Table summarizes some of the parameters and factors that are monitored as a classic oral LD50 study commences. Such Factors include but are not limited to (1) randomization of animals, (2) maintenance of a narrow range of body weights, (3) appropriate number of animal per group, (4) identification of individual test subjects, (5) fasting, and (6) availability of water. Preliminary range-finding experiments are initiated to minimize the extent of no lethality or 100% lethality, thus reducing the number of groups in the total study. This method also improves the precision of the LD50 determination.

Table 3: Consideration during preparation for oral LD50 study

Parameter	Factor for consideration
Randomization of animals	Unbiased distribution into groups
Narrow range of body weights	Uniform distribution of similar sized animals
Number of animals	For classic LD50 average of 10 per treatment group
Identification of individual animals	Ensure individual observation and monitoring: allows for group housing
Fasting (16 to 24 hr)	Optimal GI absorption
Water ad libitum	Prevention of dehydration

Dermal LD50

Dermal LD50 studies are conducted on toxicants if the probable exposure route is through skin absorption. As with the oral LD50, lethality is generally assessed in two species, one of which is non-rodent. Also, the test substance is applied to shaved skin in increasing doses to several groups of experimental animal, one dose per group. The parameters involved with the dermal LD50 determination are the same as those described for the oral LD50. Some of the factors that are unique to dermal studies are summarized in table [3].

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Most of the variability in dermal LD50 studies arises from these parameters. Incomplete absorption of a toxicant due to poor vehicle solubility, inability to penetrate intact skin, and lack of uniformity in the application method are largely responsible for the inconsistency seen with dermal LD50 studies. Because of known or suspected inadequate absorption, an LC50 (median lethal concentration 50%) is determined after absorption and may substitute for the lack of a reliable dosage determination. Dermal toxicity testing is required to determine this [1].

Skin

In order to evaluate the degree of skin irritation that may be exerted by a potentially toxic substance, it is necessary to examine the effect in human subjects. Due to enormous variability in the response of the skin of different animal species to toxic chemicals, there is little value in skin irritancy testing that requires extrapolation of finding from one species to another.

Eyes

Any chemical with irritant properties when applied to the skin is also likely to be irritant to the cornea and conjunctiva, and ocular irritancy tests need not be carried out. The most widely used predicting test for ophthalmological irritancy is still the Draize test in rabbits.

Mucosal surface

Irritancy testing of mucosal surfaces is necessary when substances are designed for application to particular surfaces such as the vagina, where local factors such as pH have to be considered. There is little difference between species, and between individuals, in mucosal responses to toxic injury [4].

Table 4: Consideration during preparation for Dermal LD50 study

Parameter	Factor for consideration
Formulation	Solids dissolved in water or inert oil-based vehicle
Application to skin	Shave for 24 hr prior to test, uniform application
Absorption of toxicant	Depend on water- soluble or lipid- soluble properties
Variability of results	High degree of variability; determine LC50

Inhalation LD50

Air-borne toxic materials that are transported via gases, aerosol, smoke, or ventilation necessitate the determination of acute inhalation LD50. for the classic LD50 , rodents and non-rodents are exposed for 4 to 24 hr to a test substance in increasing concentrations (one concentration per

group, at least four doses plus a control group). Well controlled inhalation studies incorporate a

negative pressure dynamic inhalation system with programmable airflow settings. Currently used systems are capable of delivering precise test material concentration, continuously monitoring toxicant in the exposure chamber. The range of doses is capable of producing a corresponding series of toxic effects and mortality rates to facilitate assessment of acute toxicity for LD50 or LC50 determination. Table 4 represents some of the difficulties unique to inhalation studies [4].

Table 5: Consideration during preparation for Inhalation LD50 study

Parameter	Factor for consideration
Concentration of delivered test agent	Air flow rate into chamber; Air temperature and humidity; monitoring concentration in chamber; check integrity of exposure chamber
Particle size	Determines distribution to target organ
Control group	Influence of co-solvents, additives, air pressure.

DESIGN OF ACUTE TOXICITY TEST

The test substance was administered orally/intraperitoneal in graduated doses to several groups of experimental animals, one dose being used per group.

Dose selection: This is based on the results of a range finding test. Animals showing severe and enduring signs of distress and pain were killed after anesthesia.

Animal selection: (i) Species and strain – Two species were selected, one rodent and other non-rodent, because species differ in their response to toxic agents. Animals were obtained from random breeding in a closed colony, because the aim was to discover new and unexpected effects of a drug in groups of animals of wider variability or F/1 hybrids of two inbred strains.

(ii) Number and sex of animals – At least five rodents were used at each dose level. They were all of the same sex. After completion of the study in one sex, at least one group of five animals of the other sex was dosed. The females were nulliparous and non-pregnant. In acute toxicity tests with animals of a higher order than rodents, the use of smaller numbers may be considered. A drug effect that is seen in say, both a rat and a Dog, probably involves a common physiological mechanism that is likely to be present in humans. Whereas an effect seen only in one of the two species indicates that it is peculiar to that species and is less likely to be present in the third species.

(iii) Age – If a compound is to be administered in infants under six months of age, the LD50 values

in newborn rats under 24 h of age, were compared with those of mature rats in order to assess any difference in sensitivity due to age.

Assignment of animals – Each animal was assigned a unique identification number. A system to assign animals to test groups and control groups randomly is required.

Housing – Animals were group-caged by sex, but the, number of animals per cage must not interfere with clear observation of each animal. The biological properties of the test substance or toxic effects (e.g. morbidity, excitability, etc.) may indicate the need for individual caging.

Administration – The compound was administered once, orally or parenterally, to rats that have been fasted for 18 h.

Dose levels and dose selection

The substance used in the toxicity tests should be as pure as the material eventually to be given to humans.

At least three to four dose levels were used, 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 permit an acceptable estimation of LD50.If the lethality of the groups is such that only one group has a lethality falling between 4 and 6 probits, more groups may be required.

Solvent: Where necessary, the test substance was dissolved or suspended in a suitable solvent.

Volume: This depends on size of the test animal. In rodents it should not exceed 1 ml/100 g body weight maximum of 50 ml/kg. Injection was given slowly and uniformly. This will avoid undue killing by a drug having predominant action on the CNS/heart.

Route of administration

The LD50 value depends on the route of administration. 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 intraperitoneal route (because many drugs get detoxified by the liver if the intraperitoneal route is employed).

Signs recorded during acute toxicity studies: These are 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.

Observation period

After the test the animal is the sole occupant of the cage, with free access to food and water during the observation period of 1–2 h, and thereafter at intervals. At the end of the test surviving animals were weighed and sacrificed. A gross necropsy was performed; all gross pathology changes were recounted. 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.

Before the actual LD50 determination, a pilot study was conducted on a small group of mice

mainly to select the dose ranges for the subsequent study. The compound was administered intravenously to pairs of mice in ascending and widely spaced doses. The injected mice were observed continuously for 2 h and then occasionally for further 4 h, and finally overnight mortality was recorded. The dose killing one out of two mice in such experiments gives an approximate estimate of LD50.

In another method each dose was given to one animal only, and LD50 estimated from the mean of the logarithms of the smallest effective dose and the largest ineffective dose [4].

A variety of established techniques have been traditionally used for LD50 determinations with the intention of minimizing the number of animals. These include the Up-and-down procedure (UDP) or staircase method:- In this type of procedure a single animal (or sometimes two to four animals) is exposed with subsequent does adjusted up or down by a constant factor depending on the outcome of the previous dose. If an animal dies during the initial step of the test, another animal is given a dose reduced by a factor. If this exposure does not result in toxicity, the dose is elevated by an equivalent constant factor until five animals have been dosed or the limit dose is reached. Although time consuming, the up-and-down procedure can give good results with the use of as few as six to nine animals. Proposals are being circulated for acceptance of the up-and-down method into OECD guidelines

Fixed-dose approach (FDP, British Toxicology Society)

The fixed- dose procedure was first proposed by the British Toxicology Society in 1984. After an international validation study involving 20 reference chemicals tested in 31 laboratories from 11 different countries, the procedure was incorporated into the OECD guidelines (guideline 420) in 1992. The result of the validation study showed a remarkable consistency between laboratories and it was concluded that the data generated could be used both for risk assessment and ranking chemicals for classification. Further evaluation of the method has proven its usefulness the test substance is given at one of the four fixed-dose levels (5, 50, 500, and 2000 mg/kg) to five male and five female rats. The objectives is to identify a dose that produces clear signs of toxicity but no mortality. Depending on the results of the first test, either no further testing is needed or a higher or lower dose s tested: mortality occurs, retesting at a lower dose level is necessary (except if the original dose chosen is 5mg/kg). if no signs of toxicity occur at the initial dose, it is necessary to retest at a higher dose level. The results are thus interpreted in relation to animal survival and evident toxicity and it becomes possible to assign the chemical to one of the OECD classification categories.

Acute toxic class method (ATCM)

The toxic class method has been validated both nationally and internationally. The latter study included 20 test substances and nine laboratories in five

Countries

The method is described in OECD guideline 423 and is based on the assessment of lethality. In principle three levels corresponding to the oral LD50 classification limits. The purpose of the procedure is to identify the lowest dose level that causes two or three animals to die. Three animals of one sex are dosed at the middle level.

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If two or three animals die, retesting is done at the lower level. When fewer than two die, the test is repeated at same level, but with the other sex. If two or three die in this step, the test is repeated at the lower level, and if fewer than two die, the test is repeated at the higher level. Several studies have evaluated the method and found it a valuable alternative test for classification of chemicals [5].

Determination of acute lethality

Five animals in each group (inbred mice, 10-12 weeks old) obtained from the Institutional Animal House, Kasturba Medical College, Mangalore were used. for calculation of LD50 value of any given

Chemical there are two following methods

Graphical method (Miller and Tainter):- This method is simple and accurate enough in most of the cases and should always be tried first. The observed percentage mortality was converted into probit referring to the probit table (Table 5). The values thus obtained were plotted against log dose. The LD50 value and its standard error were determined from the graph, if the line was straight enough (Table 3). Transformation of percentages to probits was done based on the table of probits (Table 5). For 96%, the value that is present at the intersection of 90 on the vertical line on the left and 6 in the horizontal line on the top was taken. If decimal was present, e.g. 97.5, then the value against 90 and 7 + 90 and 8 was taken, and average of the two considered as the probit. The probit value was plotted against the logarithm of dose. The dose corresponding to 50% or probit 5 was taken as LD50.

Disadvantage – Too many animals had been utilized

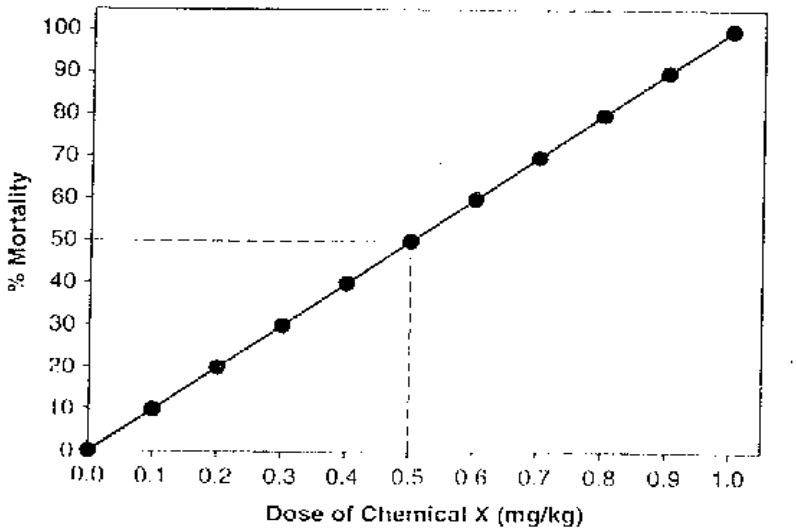

Fig 1: Graph between % mortality and Dose of Chemical

Table 6: Transformation of percentages to probits

%	0	1	2	3	4	5	6	7	8	9
0	–	2.67	2.95	3.12	3.25	3.36	3.45	3.52	3.59	3.66
10	3.75	3.77	3.82	3.87	3.92	3.96	4.01	4.05	4.08	4.12
20	4.16	4.19	4.23	4.26	4.29	4.33	4.36	4.39	4.42	4.45
30	4.48	4.50	4.53	4.56	4.59	4.61	4.64	4.67	4.69	4.72
40	4.75	4.77	4.80	4.82	4.85	4.87	4.90	4.92	4.95	4.97
50	5.00	5.03	5.05	5.08	5.10	5.13	5.15	5.18	5.20	5.23
60	5.25	5.28	5.31	5.33	5.36	5.39	5.41	5.44	5.47	5.50
70	5.52	5.55	5.58	5.61	5.64	5.67	5.71	5.74	5.77	5.81
80	5.84	5.88	5.92	5.95	5.99	6.04	6.08	6.13	6.18	6.23
90	6.28	6.34	6.41	6.48	6.55	6.64	6.75	6.88	7.05	7.33

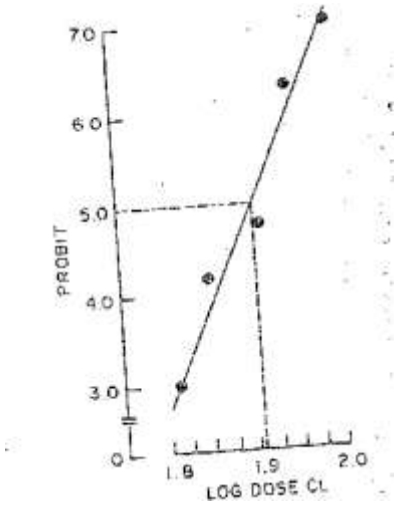

Fig 2: Graph between probit and Log dose

Table 7: Graphical method of Miller and Tainter

Group	Dose (mg/ml)	Log dose	Dead	Dead (%)	Corrected (%)	Probit
1	Vehicle		Total	0		
2	600	2.7782	0	0	5	
3	700	2.8451	1	20	20	4.16
4	800	2.9031	3	60	60	5.25
5	900	2.9542	3	60	60	5.25
6	1000	3.0000	4	80	80	5.84
7	1100	3.0414	5	100	100	

Corrected % (if there is 0 and 100%)

0%= 1000.25/N

1000.25/5= 5

100% dead = (n-0.25/N)

N= number of animal in each group

To determine the absolute dose for a species in the columns, multiply the absolute dose given to the species in a row by the factor given at the intersection of the relevant row and column. Thus an effect is produced in a 12 kg dog by a dose of 10 mg/kg; the absolute dose to the dog is 120 mg. Extrapolated to man by surface area, the effect might be expected at a dose of 120 mg 3.1 = 372 mg, as opposed to 700 mg, given by the ratio of weights.

Determination of the LD50 further entails that the response to a chemical is normally distributed – the highest number of respondents are gathered in the middle dosage range. Fig 3 represents a normal frequency distribution achieved with increasing doses of a chemical versus the cumulative percent mortality. The bars represent the percentage of animal that died at each dose minus the percentage that died at the immediately lower dose. As shown by the normal (Gaussian) distribution, to lowest percentage of animals died at the lowest and highest doses, accounted for by biological variation [6].

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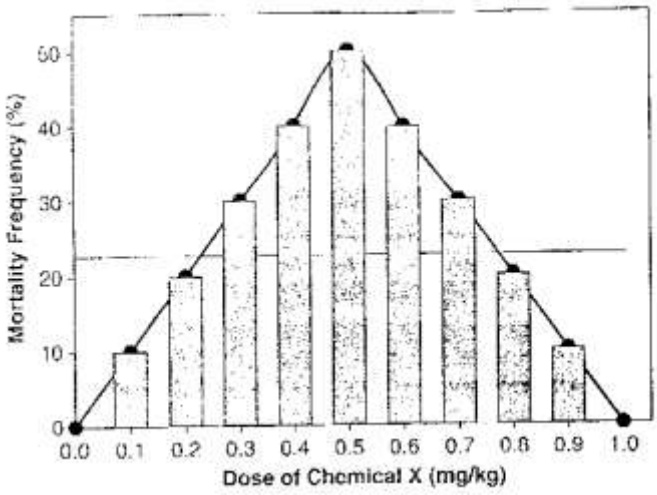

Fig 3: Graph between mortality Fequency and Dose of chemical

Consequently, the calculation of the LD50 considers the slope of the line with respect to response and the 95% confidence limits. The slope is an indication of the range of intensity of toxicity of the chemical in question. For instance, a steep slope represents proportionately greater responses to increase in concentration, whereas a shallow slope suggests less toxic response in proportion to increase in dose. The confidence interval is a relative measure of the degree of error present in the population sample and allows for intra and inters laboratory comparisons [6].

Arithmetical method of Karber:- The LD50 may be calculated by Karber's method that not involve any plotting of dose-response curve. It is the simplest and rapid though crud method of deriving LD50 value particularly when the number of animal is small. Te interval mean of the number dead in each group the interval mean of the number dead in each group of animals was used as well as the difference between doses for the same interval. The product of interval mean and dose difference was obtained. The sum of the product was divided by the number of animals in a group and the resulting quotient was subtracted from the least lethal dose in order to obtain LD50 value.

LD50 = The apparent least dose lethal to all in a group

$$- (ab)/N$$

Where N is the number of animals in each group, a dose difference and b the mean mortality (Table 5).

Disadvantage – When we look back this was the dose which had not killed a single mouse; hence too many animals were unnecessarily sacrificed.

Table 8: Arithmetic method of Karber

Group	Dose (mg/ml)	No. of animals dead	Dose difference (a)	Mean mortality (b)	Probit (a b)
1	Vehicle	0	-	-	-
2	600	0	0	mortality in 2nd + 1st 2	0
3	700	1	100 (700-600)	0.5	50
4	800	3	100	2	13
5	900	3	100	3	300
6	1000	4	100	3.5	350
7	1100	5	100	4.5	450

Sum of the product = 1350

LD50 = Least lethal dose -- (ab)/N

LD50 = 700 – (1350/5) = 700-270 = 430 mg/kg.

APPLICATION OF LD50 STUDIES

The LD50 value is a statistical estimate of the acute lethality of a chemical administered under specific circumstances; it provides a measure of relative toxicities of chemical under similar or identical conditions. Thus the major application of the LD50 is comparative, allowing for semi-quantitative toxic evaluation of compounds. Especially with in the vast database of acute toxicology recorded for laboratory rodents. In addition, the test provides a screening method for toxic evaluation, particularly useful for new unclassified substances. The determination however is not without limitations. By current scientific methodologies, the LD50 is antiquated, require large numbers of animals, does not provide significant information regarding mechanistic effects or selective target organs, and does not suggest complementary or discriminating pathways of toxicology. It is also limited by the route and duration of exposure. Consequently, its routine use in toxicology testing

has become the subject of continue debate and regulatory review.

CHRONIC TOXICITY TEST

Chronic toxicity tests determine toxicity from exposure for a substantial portion of a subject's life. They are similar to the subchronic tests except that they extend over a longer period of time and involve larger groups of animals.

Table 9: Basic parameters of Acute toxicity tests

Species	Two species recommended; rodent and non rodent (rat and dog)
Age	Young adults
Number of animal	20 of each sex for rodents, 4 of each sex for non rodents per dose level
Dosage	Three dose levels recommended; include a toxic dose level and NOAEL; exposures generally for 12 months; FDA requests 24 months for food chemicals
Observation period	12-24 months

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. These studies range in duration from 1 to 2 weeks to 1 to 2 years. The length of studies required for a drug depends mainly upon the duration of treatment and the 118 Animals in Drug Development intended clinical dosing regimen. (This is discussed further under Governmental Guidelines). 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. Parallel sub chronic and chronic studies are almost always conducted in two species, usually the rat and dog, because there is a large historical data base for these species and they are easy to work with, relatively economical to house, and readily available from commercial vendors. However, special circumstances may dictate that other species be used. For instance, if a drug causes excessive vomiting in dogs, which are known to be particularly sensitive to such effects, then some other non-rodent species such as the monkey may have to be used. Also, if the absorption or metabolic handling of a drug in the rat or dog is found to be markedly different from that in humans such that one of these species

would not be a reasonable predictor of toxicity in humans, then another species would be used.

The usual protocol for subchronic and chronic studies includes groups of animals containing equal numbers of both sexes receiving at least three dosage levels of drug plus vehicle or other control groups. These animals are observed daily for clinical signs of toxicity. Their body weights and food consumption are measured frequently.

These three parameters: clinical signs, body weight, and food consumption— can be very sensitive indicators of toxicity. Complete hematology and serum chemistry profiles are determined at least at the end of the administration period and in some cases at intervals during the period of administration. Thorough physical examinations by a veterinarian or a trained technician are conducted at regular intervals. Periodic electrocardiograms are commonly recorded in studies with dogs. At the end of the period of drug administration, all the animals are subjected to a complete necropsy under the supervision of a veterinary pathologist. The tissues are then subjected to complete microscopic examination by a veterinary pathologist to detect morphologic alterations in the tissue that may have resulted from drug administration. In some cases it may be desirable to allow some animals from the drug-treated groups to live for a period of time after the end of drug administration to determine if any drug-related changes will disappear upon withdrawal of the drug.

As mentioned, the results of the subchronic and chronic studies are used to help determine the dosage this, the lowest dosage causing no toxicity (the nontoxic- effect dosage) is determined for each study, and a safety factor is applied depending on the species. For instance, for rats the no-toxic-effect dosage is divided by 10 and for dogs by 6 to arrive at an estimate for the initial human dosage. These factors are derived from the observations that laboratory animals can usually tolerate higher dosages of drugs and other chemicals without exhibiting toxicity than can humans and that the differences in tolerance vary with differences min basal metabolic rate, which in turn varies with body surface area to weight ratio. This ratio varies by a factor of approximately 10 in rats and 6 in dogs relative to humans. The descriptions of toxic effects elicited in the subchronic and chronic studies allow clinicians conducting clinical trials to know which side effects to anticipate so as to protect the patient volunteers [6].

The standardized methods for the chronic toxicity test, the test of impact on reproductive toxicity and future generations, the teratogenicity test, the mutagenicity test, the carcinogenicity test, are as follows

GENERAL RULES

Test animals

As a rule, mammals are selected to be test animals, and animals with a clearly known origin, species and breed are to be used. With the exception of certain special tests, it is necessary to use animals that are not much affected by age (affected by youth or old age). When the metabolic pattern of the test substance in people is known, it is desirable to use an animal with a

metabolic pattern similar to that of people. As a rule, test animals of the same type, species and breed are used across all tests. Furthermore, it is desirable to use species for which the types and frequency of naturally occurring pathologies under appropriate breeding conditions are known.

Management of breeding

When breeding animals over a long period, be careful to maintain appropriate management conditions in particular (the breeding environment: temperature, humidity, ventilation, lighting, and food for the animals, etc.) and to avoid the outbreak of infectious diseases.

Test substances

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.

Control group

When administering a test substance by adding it to the food for the animals, establish a control group and give it food, which does not contain the test substance. When administering a test substance using a solvent, suspension agent, emulsion agent, it is desirable to establish a control group which is raised by being given feed, containing solvents, suspension agents or emulsion agents only. If the added test substance is highly concentrated, it is necessary to take into account nutritional balance.

Test Animals

Animal type and sex

Use male and female animals of two or more types (mice, rats, etc.) of the same kind as are used in 1 to 3-month short-term preliminary tests. It is desirable for one of these types to be a non-rodent.

Age

For types of animals with a short life span, such as mice, rats, etc., use 5 to 6-week old animals with uniform weight. For types of animals with a relatively long life span, use animals of an age that in general corresponds to that of mice, rats, etc.

Number of animals

In the case of mice, rats, etc., use 20 or more males and 20 or more females in each group. In the case of non-rodents, use 4 or more males and 4 or more females in each group. If mice, rats, etc. are to be put down during the testing period in order to conduct tests on them, add the number of animals that will be required for that purpose in advance.

(Research Article)

Test Substance

Method of administration

As a rule, the test substance is administered orally. It is desirable to administer the test substance by adding it to food for the animal or the animal's drinking water. The concentration of the test substance added to the food for the animal must be 5W/W% or less. However, if due to the properties of the test substance it cannot be administered orally, administer it parenterally. In the case of forced administration, administer the test substance at a set time each day.

Dosage

Administer the dosage in three stages or more in order to determine the relationship between dosage and effect.
Carry out 1 to 3-month short-term preliminary tests in advance and determine the maximum dosage for which some toxic effect caused by the test substance has been established without causing a large number of deaths.
Make the minimum dosage the amount which produces no effect on the animals during the period of the test. Establish a separate control group.
Calculate the actual amount of the test substance assimilated from the food intake of the animals or from the water intake and the concentration of the test substance.

Administration period

Make the administration period 12 months or more.

Observation and Measurement Items
As a rule, conduct observations on the following items.

General condition and death rate

Weight, food intake and water intake, food intake efficiency

Blood tests

Hematologictest
Blood biochemistry test

Urine test

Pathology tests

Observation with the naked eye and internal organ-weight
Observation with a microscope (carry out a test using an electronic microscope or a histochemical test as necessary)

Other-necessary-items
Study the cause of death of animals that died during the testing. Also, promptly put down and do an autopsy on animals whose general condition is very bad and are about to die.

When using mice or rats, it is desirable to use at least five males and at least five females to study at least once during the administration period the same test items that are in the tests that will be carried out at the end of the test period.

It is desirable to measure water intake only when administering the test substance by mixing it into the animals' drinking water and calculate food intake efficiency during the period of growth of the animals.

The hematologic tests that are usually carried out are as follows. For the measurements of each item the test methods and measurement units

widely used internationally are adopted. In addition, it is desirable to perform tests on any other items for which a relationship to toxicity is indicated.

Red blood cell count, reticulocyte count, hemoglobin, hematocrit, white blood cell count, white blood cell percentage, platelet count, etc.

The blood biochemistry tests that are usually carried out are as follows. For the measurements of each item, the test methods and measurement units widely used internationally are adopted. In addition, it is desirable to perform tests on any other items for which a relationship to toxicity is indicated.

Total protein, A/G ratio, blood sugar, triglyceride, phospholipid, total cholesterol, urea nitrogen, creatinine, uric acid, sodium, potassium, chlorine, calcium, phosphorus, glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), lipotropic hormone (LPH), alkaline phosphatase, creatine phosphokinase, γ-guanosine triphosphate (GTP), ornithine decarboxylase, etc.

Carry out semiquantitative tests of urine volume, photogrammetry, occult blood, total protein, sugar, ketone body, urobilinogen and bilirubin and carry out tests of sediment with a microscope as necessary.Conduct autopsies on all the animals used in the testing (including those that died or were put down during the testing period) and carry out sufficient observations with the naked eye of all internal organs and tissues. Store all of the organs and tissues described in an appropriate storage liquid for each group.

The organs and tissues for which a histopathological test is necessary are as follows. Implement this test for a maximum dosage group and a control group, and implement a test of other dosage groups for organs and tissues that showed changes in the maximum dosage group. Brain, spinal cord, peripheral nerve, pituitary gland, eyeballs, nasal cavity, lungs(including the bronchial tubes), tongue, esophagus, stomach, small intestine, large intestine, skin, salivary gland, lymph node, thyroid gland (including the parathyroid gland), thymus gland, heart, liver, pancreas, spleen, kidney, adrenal gland, bladder, testicles, seminal vesicle, prostate gland, mammary gland (females), ovaries, uterus, sternum (including the bone marrow), vertebrae and femur (including the joint), and organs and tissues for which changes have been confirmed with the naked eye.

In the case of an inhalation test, the nasal cavity, pharynx, larynx, and trachea.

TEST OF IMPACT ON REPRODUCTIVE POTENTIAL AND FUTURE GENERATIONS

Objective

The objective of this test is to determine the damage to reproductive potential and to the emergence of future generations caused by the test substance by administering the test substance to male and female animals over many generations.

Test Animals

Animal type

Use at least one type of animal (mice or rats, etc.) and choose the test animals from among the animals used in the V. Teratogenicity test.

(Research Article)

When selecting the type, species and breed of the test animals, take into account knowledge concerning reproduction, such as knowledge about fertility, etc., the frequency of occurrence of naturally occurring abnormalities, sensitivity to substances known to have reproductive or birth toxicity. Also, it is desirable to select animals for which the frequency of occurrence of naturally occurring-deformities-is-low.

When using the same type of animal as for the chronic toxicity tests, it is desirable to select animals-of-the-same-species.

When using animal types other than rats or mice, it is necessary to carry out appropriate modifications to these guidelines in order to achieve the objective of this test.

Number of animals

With rats or mice, prepare the number of females that are expected to be necessary to produce 20 pregnant animals, and the equivalent number of males, in the control group to which the test substance is not administered.

Test substance

Method of administration

As a rule, the test substance is administered orally. It is desirable to administer the test substance by adding it to food for the animal or the animal's drinking water. The concentration of

the test substance added to the food for the animal must be 5W/W% or less. However, if due to the properties of the test substance it can not be administered orally, administer it parenterally.

Dosage

In order to determine the dosage-response relationship and estimate the maximum dosage with no effect, establish dosage test groups with at least three stages. Make the maximum dosage the amount that causes the parental generation of animals (F_0) to show slight indications of toxicity, such as declining food intake or suppression of growth in weight, but does not cause a death rate of more than 10%. Make the minimum dosage the amount that does not have any toxic effects on reproductive potential or the emergence of future generations. Set up a control group separately.

Crossbreeding and administration of the test substance

For F_0, commence administration of the test substance by the time the animals are 5-8 weeks old, as a rule, and after administering it every day for more than 10 weeks (for mice, 8 weeks), begin-crossbreeding.

Make the cohabitation period of the same males and females 2 or 3 weeks and during that period verify every day whether copulation has occurred.

Separate out the females that have mated, and create the first generation (F_1) through natural birth.When adjusting the number of progeny in the litter, at a relatively early time after birth,

randomly leave a fixed number of animals composed of approximately the same number of males and females for one mother. Leave the progeny to be raised by the mother animal. Continue to administer the test substance to the father animals until crossbreeding to produce the

F_1 generation is completed and to the mother animal until weaning of the F_1 generation.

Randomly select the animals to produce the next generation at the time of weaning of the F_1generation and perform an autopsy examination on the remaining animals. After administering the test substance to the animals used to produce the next generation over a period of at least 10 weeks (for mice, 8 weeks) in the same way as for F_0 after weaning, as a rule, take at least 20 pairs of males and females from different litters, and crossbreed them in the same way as for F_0 to produce the second generation (F_2). As a rule, raise the F_2 generation by administering the test substance from after weaning until sexual maturation.

Observation Items

Observe general condition and whether there are deaths, measure weight and food intake (as necessary water intake), and calculate the intake of the test substance.

Calculate the copulation rate and conception rate for the parent animal. Search for birth abnormalities and calculate the birthrate for the mother-animals.Do an autopsy on the mother animal at the time of weaning of the F_1 generation and observe the internal-organs.

At an appropriate time, put down the males and those females that did not copulate, conceive or give birth, and observe their internal organs.

F_1

Examine the number born, survival rate, sex, weight, and changes in the external appearance of the new progeny. After birth, observe general condition, whether there are deaths, growth, and the development of form and function. Measure weight at least once a week. Calculate survival rate at appropriate intervals and calculate the weaning rate at the time-of-weaning.

Conduct the same kind of examination of F_1 animals used in crossbreeding to produce the F_2 generation as was done for the F_0 generation. Do an autopsy on the remaining F_1generation at the time of weaning.

F_2

Carry out the same observations as for the F_1 generation. As a rule, do an autopsy when the animals reach sexual maturity. As necessary, conduct a detailed examination using histological or biochemical methods.

How to draw conclusions from the observations Study the observed abnormalities and the relationship between the symptoms of toxicity and amount of the test substance administered using appropriate statistical methods, and state an opinion concerning the maximum dosage with no effect. When doing so, it is desirable to make the progeny of one litter the sample unit until weaning.

TERATOGENICITY TEST

Objective

The objective of this test is to administer the test substance to pregnant animals during the period that the internal organs of the fetus are forming, and determine what damage to the birth of the fetus is caused by the test substance, in particular the teratogenicity of the test substance.

(Research Article)

Test Animals

Animal type

Use one or more types of rodents, such as rats or mice, and non-rodents such as rabbits. When selecting the animal type, species and breed, take into consideration knowledge concerning reproduction, such as knowledge about fertility, the frequency of occurrence of naturally-occurring abnormalities, sensitivity of the animal to substances known to have reproductive or birth toxicity, etc. Also, it is desirable to select animals with a low frequency of occurrence of naturally-occurring abnormalities.

When using the same animal type as for the chronic toxicity test, it is desirable for the species of the animals to be the same.

When using animal types other than rats, mice or rabbits, it is necessary to carry out appropriate modifications to these guidelines in order to achieve the objective of this test.

Number of animals

With rats and mice, use 20 or more animals for each dosage group as the number of individuals that conceived. With rabbits, use 12 or more animals.

Test substance

Method of administration

As a rule, the test substance is administered forcibly and orally.

Dosage

In order to determine the dosage-response relationship and estimate the maximum dosage with no effect, establish dosage test groups with, as a rule, at least three stages. Make the maximum dosage the amount that causes the mother animals to show slight indications of toxicity, such as declining food intake or suppression of increase in weight, but does not cause a death rate of over 10%. In the case that the mother animal shows no indications of toxicity even from the maximum amount of the test substance that can be administered (make the limit 1000mg/kg), make that amount the maximum dosage. Make the minimum dosage the amount for which toxic impact on the birth of the fetus is not shown. Set up a control group separately to administer the solvent only.

Administration period

Administer the test substance every day during the period that the internal organs of the fetus are forming. Normally, when the date of verification of copulation is defined to be day 0 of pregnancy, the administration period is from the 6th day to the 15th day of pregnancy for mice, from the 7th day to the 17th day of pregnancy for rats, and from the 6th day to the 18th day of pregnancy for rabbits. However, for rats it is also acceptable to make the administration period from the 6th day to the 15thday of pregnancy.

Observation Items

Mother animal

Observe general condition throughout the test period and measure weight and food intake. Do an autopsy on all of the animals approximately one day before the expected date of birth, study the establishment of pregnancy, count the number of corpus lutea and implantations, and observe the internal organs with the naked eye.

(Research Article)

Fetus

Determine whether the fetus has survived and estimate the time of death of the dead progeny. Measure the weight of the living progeny and determine their sex. In addition, conduct an examination with the naked eye of the external appearance and internal organs, and an examination of the shape and ossification of bones through skeletal staining transparent sample.

How to draw conclusions from the observations Study the observed abnormalities and the relationship between the symptoms of toxicity and amount of the test substance administered using appropriate statistical methods, and state an opinion concerning the maximum dosage with no effect. When doing so, it is desirable to make the progeny of one litter the sample unit [10].

CARCINOGENICITY TEST

Objective

The objective of this test is to determine whether the test substance is carcinogenic when it is administered to the animal continuously over its lifetime.

Test Animals

Animal-type-and-sex
Use two or more types of male and female animals (mice, rats, etc.).
In general, use types of animals whose natural rate of incidence for tumors under normal breeding conditions as well as sensitivity to known carcinogenic substances are well-known, animals with inbred genealogies or their first filial generation. In this case, selection of animals with a low rate of incidence for tumors is desirable.

Age

Use animals 5 to 6 weeks old with uniform weight.

Number of animals
Use 50 or more males and 50 or more females in each group.

Test Substance

Method of administration

As a rule, the test substance is administered orally. It is desirable to administer the test substance by adding it to food or drinking water. The concentration of the test substance added to the food for the animal must be 5W/W% or less. However, if due to the properties of the test substance it can not be administered orally, administer it parenterally. In the case of forced administration, administer the test substance at a set time each day.

Dosage

Administer the dosage in three stages or more in order to know the relationship between dosage and-response.
Carry out 1 to 3-month short-term tests in advance and take as the maximum dosage the greatest dosage amount that will maintain weight loss to around 10% compared to the control group that does not result in poisoning-related death, and does not generate marked changes to the animal's general condition. As a rule, median dosage and minimum dosage are established from maximum dosage using common ratio 2,3. Actual intake is calculated using the animal's

(Research Article)

food intake or water intake and the concentration of the test substance.

Administration period

For most of the animal's lifetime (over 18 months for mice and hamsters, over 24 months for rats). The death rate due to causes other than neoplastic lesions that stem from administering the test substance for 18 months for mice and hamsters and 24 months for rats must be, however, under 50%.

Observation and Measurement Items

General-observation

Weight, food intake and water intake, food intake efficiency Pathology tests Observation with the naked eye Observation with a microscope (carry out a test using an electronic microscope or a histochemical test as necessary)

Blood tests

Other necessary items

Study the cause of death of animals that died during the testing. Promptly put down and do an autopsy on animals whose general condition is very bad and are about to die.

Calculate the survival rate by observing the animal's general condition and incidence of death.

It is desirable to measure water intake only when administering the test substance by mixing it into the animals' drinking water and calculate food intake efficiency during the period of growth of the animals.

Do autopsies on all the animals used in the testing (including those that died or were put down during the testing period) and carry out sufficient observations with the naked eye of all internal organs and tissues. Store all of the organs and tissues described in *4 in an appropriate storage liquid for each group.

In addition to all neoplastic lesions that are visible to the eye, conduct microscopic studies for the following organs and tissues for all groups including the control group and maximum dosage group.

For organs and tissues affected by maximum dosage, implement studies for other dosage groups as well

Brain, spinal cord, peripheral nerve, pituitary gland, eyeballs, nasal cavity (#), lungs (including bronchial tubes), oral cavity and tongue, esophagus, stomach, duodenum, jejunum, ileum, appendix, colon, rectum, external auditory canal, skin, salivary gland, lymph node, thyroid gland (including parathyroid gland), thymus gland, heart, liver, pancreas, spleen, kidney, adrenal gland, bladder, testicles, seminal vesicle, prostate gland, mammary gland (females), ovaries, uterus, vagina, sternum (including bone marrow), vertebrae, and femur (including the joints).

In the case of inhalation test, nasal cavity, pharynx, larynx, and trachea. If the number of animals alive in the maximum dosage group is extremely small compared to the control group, conduct studies for the following dosage groups as well.

If the maximum dosage group corresponds with the above assume the organs and tissues are those which are affected in the maximum dosage group and the following dosage groups. Effects that are recognized for the maximum

dosage group and the following dosage groups include not just neoplastic lesions but also general toxic changes.

During the autopsy, create a blood smear sample for all groups and measure the blood cell count and conduct a blood biochemistry test as necessary [7].

PHOTOTOXICITY

Phototoxicity testing is not often included in the safety assessment of new pharmaceuticals; however, such studies may be conducted if phototoxicity is expected on the basis of reports on chemicals of similar structure or pharmacological class. Information on UV absorption spectrum of a compound may prompt phototoxicity testing. Sometimes reports of phototoxicity after a drug has reached the marketplace prompt laboratory studies of phototoxicity to determine if the drug is causing phototoxicity and, if so, by what mechanism. Phototoxicity is to be distinguished from photosensitization (or photoallergy). Phototoxicity results from interaction of the drug or drug metabolites in the skin with light, usually in the UV region, to produce reactive molecular species that cause cell injury or death in the skin.

Phototoxicity is most commonly produced under sunlight where the skin is exposed to the full range of UV light. The phototoxic response generally shows a dose response to both the amount of drug and the intensity or duration of light exposure. Photosensitization, on the other hand, results from an immunological response to by-products formed in the skin when the drug or its metabolites interact with light. The initial exposure to the drug and light may not result in an adverse reaction, but subsequent exposures to drug and light will result in an allergic reaction in sensitized individuals. The models described in this section are confined to the assessment of phototoxicity. Modifications of the models for dermal sensitization referred to in the section on immunological sensitization, incorporating light (i.e., UV light) as well as drug exposure, can be used to test for photosensitization.

Several sensitive and convenient models can be used to test for phototoxicity. Guinea pigs, rabbits, and mice are the most commonly used species. Normal, albino guinea pigs and rabbits must be shaved or their hair removed with a depilatory before light exposure.Hairless mice have been used because of the obvious advantage of not having to remove the hair prior to testing. The euthymic, hairless guinea pig has also been used.

These animals provide a convenient and sensitive model for phototoxicity testing. When conducting a phototoxicity test with normal animals, one finds that the hair can never be completely removed from the test site, and that this interferes to varying degrees with light exposure and scoring the resulting skin reaction. In addition, the ears, a site containing relatively smallskin area, are sensitive indicators of phototoxicity. In some cases, the ears may be the only part of the animal where a skin reaction is obtained. With hairless animals, a much larger skin area is available for accurate observation of the skin reaction. The larger size of the hairless guinea pig compared with that of hairless mice maximizes this advantage. With hairless animals, accurate scoring of skin reactions is facilitated because inevitably there will be an area where exposed and unexposed skin are juxtaposed along the sides of the animal. (This assumes the animal was exposed to light coming from above with the animal in the normal resting position on all four feet.) The comparison of the appearance

of exposed and unexposed skin side by side in the same animal makes it easier to discern slight changes in skin appearance due to light exposure. A test using the mouse tail has also been recommended for determining phototoxicity. In this test mice of the same strain, age, sex, and weight are used. Groups of mice are exposed to the drug and UV light; after a period of 24–48 hours, depending on the drug, the tails are removed and the wet weight of the tails is determined. An increase in tail wet weight indicates an inflammatory response due to phototoxicity. Obviously, an untreated group as well as groups treated with only the drug or the light are included as controls [7].

SUB ACUTE TOXICITY TEST

Subacute toxicity tests are employed to determine toxicity likely to arise from repeated exposures of several weeks to several months. Standardized tests are available for oral, dermal, and inhalation exposures. Detailed clinical observations and pathology examinations are conducted.

Table 10: Basic parameters of acute toxicity tests

Species	Rodents (usually rats) preferred for oral and inhalation studies; rabbits for dermal studies; non rodents (Usually dogs) recommended as a second species for oral tests.
Age	Young adults
Number of animal	10 of each sex for rodents, 4 of each sex for non rodents per dose level
Dosage	Three dose levels plus a control group; include a toxic dose level plus NOAEL; exposure are 90 days
Observation period	90 days (same as treatment period)

CONCLUSION

When administers a chemical substance to a biological system, different types of interactions can occur and a series of dose-related responses result. In most cases these responses are desired and useful, but there are a number of other effects which are not advantageous. These may or may not be harmful to the patients. The types of toxicity tests which 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.

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