Acute toxicity studies and determination of median lethal dose

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Whenever an investigator 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 LD_{50} (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. This article reviews the methods so far utilized for the determination of median lethal dose (LD_{50}) and the new changes which could be made. This has to go through the entire process of validation with different categories of substances before its final acceptance by regulatory bodies.

Keywords: Dose-related response, lethal dose, toxicity tests.

In screening drugs, determination of LD_{50} (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.

Data from the acute study may: (a) Serve as the basis for classification and labelling; (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 LD_{50} values that provide many indices of potential types of drug activity.

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 (LD_{50}/ED_{50}).
- The greater the index, safer is the compound. LD_{50} with confidence limits is to be established on one common laboratory species such as mouse/rat using the standard method. The LD_{50} 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 LD_{50}.
- 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 LD_{50} value[1].

Test procedure

The test substance was administered orally/intraperitoneally 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 anaesthesia.

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[2].

(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

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Table 1. Surface area ratios of some common laboratory species and man[2]

	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

group of five animals of the other sex was dosed[1,2]. The females were nulliparous and non-pregnant[1]. 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[1].

(iii) Age – If a compound is to be administered in infants under six months of age, the LD_{50} 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[1].

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[1], 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[3].
- 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[1,2] an acceptable estimation of LD_{50}.
- 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[3].
- 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[1,4], 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[1].

Route of administration: The LD_{50} 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[3]. The intravenous route is preferable to the intraperitoneal route (because many drugs get detoxified by the liver if the intraperitoneal route is employed)[1].

Signs recorded during acute toxicity studies: These are increased motor activity, anaesthesia, 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[1].

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[3].

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[1,2].

Before the actual LD_{50} 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 LD_{50}.

In another method each dose was given to one animal only, and LD_{50} estimated from the mean of the logarithms of the smallest effective dose and the largest ineffective dose[1].

A simple method is the 'up and down' or the 'staircase' method. Two mice were injected with a particular dose and observed for a period of 24 h for any mortality. The subsequent doses were then increased by a factor 1.5 if the dose was tolerated, or decreased by a factor of 0.7 if it was

lethal. The maximum nonlethal and the minimum lethal doses were thus determined using only about ten mice[1].

Once the approximate LD_{50} or the range between the maximum nonlethal and minimum lethal doses was found, a final and more reliable LD_{50} assay was planned using at least three or four dose levels within this range, with a larger number of animals in each group[1].

Calculation of LD_{50} for zinc sulfate

Five animals in each group (inbred mice, 10–12 weeks old) obtained from the Institutional Animal House, Kasturba Medical College, Mangalore were used.

Arithmetical method of Karber[4]

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 LD_{50} value.

LD_{50} = The apparent least dose lethal to all in a group $- \sum \frac{(a \times b)}{N}$,

where N is the number of animals in each group, a the dose difference and b the mean mortality (Table 2).

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

Table 2. Arithmetic method of Karber[4]

Group	Dose (mg/ml)	No. of animals dead	
1	Vehicle	0	
2	600	0	
3	700	1	
4	800	3	
5	900	3	
6	1000	4	
7	1100	5	
Group	Dose difference (a)	Mean mortality (b)	Probit
2	0	Mortality in 2nd + 1st 2	($a \times b$)
3	100 (700–600)	0.5	50
4	100	2	200
5	100	3	300
6	100	3.5	350
7	100	4.5	450

Sum of the product = 1350

LD_{50} = Least lethal dose $- \sum \frac{(a \times b)}{N}$,

$LD_{50} = 700 - \frac{1350}{5} = 700 - 270 = 430$ mg/kg.

Graphical method of Miller and Tainter[4]

The observed percentage mortality was converted into probit referring to the probit table (Table 3). The values thus obtained were plotted against log dose. The LD_{50} value and its standard error were determined from the graph, if the line was straight enough (Table 4).

Transformation of percentages to probits was done based on the table of probits (Table 3). 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 LD_{50}.

Disadvantage – Too many animals had been utilized.

Determination of median lethal dose (Lorke[5])

Phase 1: Three-groups of three mice/group. One dose was given to each group intraperitoneally. The treated mice were monitored for 24 h for mortality and general behaviour.

Phase 2: After 24 h 3–4 groups of one mouse were given doses based on the findings of phase 1, intraperitoneally. The mice were again monitored for 24 h. The geographic mean of the least dose that killed mice and the highest dose that did not kill mice was taken as the median lethal dose[6,7].

Advantage – Fewer animals were sacrificed.

Disadvantage – Accuracy, reproducibility and reliability are questionable.

Recommended methodology

(i) There could be only two animals per group. A wide range of doses can be tested, starting from the lowest dose, with increments of two (so that the minimum dose which is lethal to one rodent is not missed).

(ii) At a time there should be not more than five groups (so that there are not many groups showing mortality). Mice were monitored for 24 h for mortality.

(iii) If there is no mortality noted, the experiments was continued with another five groups. At no point of time there should be more than two groups showing >1 mortality.

The lowest dose which had killed one animal and the highest dose which had not killed any animal was noted. The geographic mean of these two doses gave LD_{50}.

This is a simple and reliable method which uses lesser number of animals and hence unnecessary exploitation of animals is avoided. The research and development sector will have to spend less on animal maintenance and will

Table 3. Transformation of percentages to probits[1]

%	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

Abridged from table IX of Fisher and Yates: *Statistical Tables for Biological, Agricultural and Medical Resarch*, Oliver and Boyd, Edinburgh, and by permission of the authors and publishers.

Table 4. Graphical method of Miller and Tainter[4] for zinc sulfate

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%)

$$0\% = 100 \times \frac{0.25}{N} = 100 \times \frac{0.25}{5} = 5, \quad 100\%\ \text{dead} = \left(\frac{n - 0.25}{N}\right),$$

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[2].

Table 5. Comparison of different methods

	Method Karber[4]	Method of Miller and Tainter[4]	Method of Lorke[5]	Recommended methodology
No. of rodents used	More than necessary	More than necessary	Appropriate	Appropriate
Expenditure	High	High	Average	Average
Accuracy of results	Inaccurate	Inaccurate	Doubtful (since dose ranges are inadequately explored)	Appears to be accurate and reliable

also get reliable results in a shorter span of time. The disadvantages of the earlier methods which follow complex procedures using a large number of animals are avoided (Table 5).

Thus we suggest a method of acute toxicity testing and calculation of LD_{50} that has to go through the entire process of validation with different categories of substances before its final acceptance by the regulatory bodies.

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