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John William Trevan's concept of Median Lethal Dose (LD_{50}/LC_{50}) – more misused than used

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Abstract

Introduction. Median lethal dose (MLD) has been a controversial subject among biologists and animal ethicists since its inception in 1927 by Trevan. Toxicologists use MLD (LD_{50}) as the first step to assess the toxicity of a substance. Animal ethicists criticize LD_{50} tests because animals suffer pain, and LD_{50} is irreproducible. The disadvantage of classifying chemicals based on LD_{50}, the importance of the 'characteristics' proposed by Trevan, and the ideal mortality range for determining the best estimate of LD_{50} are also discussed.

Objective. The aim of this review was to understand Trevan's concept of LD_{50} and the method of Litchfield and Wilcoxon (L and W), and Finney's probit analysis for LD_{50} determination.

Materials and method. A literature survey was conducted using Google search and Pubmed. Simulated data set was used for identifying the ideal mortality range for calculating the 'best estimate' of LD_{50}.

Brief description of the state of knowledge. After Trevan, the extensively used classical methods for LD_{50} determination are Finney's probit analysis and the L and W method. Animal ethicists questioned LD_{50}, because of its irreproducibility. Presently used methods for LD_{50} tests do not provide information on the dose-response, hence assessing the complete spectrum of toxicity is not possible. However, LD_{50} is used to classify chemicals.

Conclusions. 'The 'characteristic' is more specific than the slope or LD_{50} of a dose-response curve. LD_{50} does not manifest the exact nature of the toxicity of a substance; hence, classifying chemicals based on LD_{50}s may have little relevance.

Key words

LD_{50}, JW Trevan, acute toxicity, median lethal dose, MLD, Litchfield and Wicoxon

INTRODUCTION

The 'minimal lethal dose' was widely used terminology in the early 1900s to express quantitatively the toxicity of a substance. The terminology was interpreted differently among toxicologists – some interpreted 'minimal lethal dose' as the dose just sufficient to kill only an occasional animal or the dose that kills 50% of animals of the dosed group, or the dose that kills all the dosed animals. Trevan [1] suggested abolishing the terminology 'minimal lethal dose', and introduced new terminology to express the toxicity of a substance quantitatively, the 'Median Lethal Dose' (MLD) or LD_{50}. The LD_{50} proposed by Trevan was for the biological standardization of digitalis extract, insulin, and diphtheria toxin. But, after Trevan, acute toxicity studies were conducted to evaluate the effect of a substance [2], not for the biological standardization of drugs. Nowadays, most of the LD_{50} tests are conducted to determine the acute toxicity of pesticides [3l] and drugs [4, 5]. Since Trevan, several methods have been proposed to calculate LD_{50}, to name only a few, Karber's method, the arithmetic method of Reed and Muench, Litchfield and Wilcoxon's method, the method of Miller and Tainter, the moving average method, Lorke's method, fixed dose procedure and the up-and-down procedure [6]. The method introduced by Litchfield and Wilcoxon [7] has been widely used for determining LD_{50} and confidence intervals due to its easy-to-perform calculation steps. But the method of probit analysis became more popular as it calculates 'accurate' LD_{50} and confidence intervals (fiducial limits) [Finney, 1971 8]. Probit analysis by Finney is difficult to perform if one does not have some skill in mathematics. Nowadays, several types of commercial software are available for performing probit analysis. The question is: do we need to calculate LD_{50} so 'accurately,' when LD_{50} itself is irreproducible? Therefore, using examples, the LD_{50}s calculated using Finney's probit analysis and Litchfield and Wilcoxon's method are discussed in this review paper. Trevan's method for calculating LD_{50} and the terminology, 'characteristic' of the dose-response curve proposed by him are also discussed in detail.

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OBJECTIVES

The aim of the review is to present an insight into the concept of median lethal dose (LD_{50}) invented by J.W. Trevan. A comparison was made between the methods of Litchfield and Wilcoxon and Finney's probit analysis in determining LD_{50} to know which method gives the best estimate of LD_{50}. An attempt was also made to determine the minimum number of dosed groups required, and to understand the distribution of the mortality on the dose-response curve to obtain the best estimate of the LD_{50}.

BRIEF DESCRIPTION OF THE STATE OF KNOWLEDGE

To begin with, a brief understanding of Finney's probit analysis and the Litchfield and Wicoxon method for determining LD_{50} and confidence intervals is necessary.

Probit analysis by Finney [8]. The first edition of Probit analysis was published in 1947, and the second edition in 1952. Finney's probit analysis is considered a robust method to determine the LD_{50}. In the second edition [9], an example of LD_{50} determination was given for chrysanthemum aphid (*Macrosiphoniellas anborni*) exposed to rotenone. Six groups, each with about 50 aphids were exposed to various concentrations of rotenone. All steps for calculation of LD_{50}, and standard error and fiducial limits of LD_{50} were meticulously explained in this example in such a way that a biologist with a basic knowledge of algebra could understand the calculation procedure. Since Finney used about 50 aphids for each dose group in this example, the biologists took it for granted that about 50 animals, irrespective of species, are required to be exposed to each dose group to obtain a statistically reliable LD_{50}. Trevan [1] indicated that once the 'characteristic' is determined, the number of animals necessary depends on the 'accuracy' desired [1]..

The mortality, 0 and 100% are not included in the Finney's probit analysis as there are no probit values corresponding to 0 and 100% mortality. However, commercially available software accepts the input of 0 and 100% mortality. The software assigns a value close to 0 (e.g. 0.1) for 0% mortality, and a value close to 100 (e.g. 99.9) for 100% mortality. The corresponding probit values for 0.1 and 100% mortality will be included in the calculation procedure. However, 0.1 and 99.9% mortality may not contribute much to the LD_{50} value if the dose-response curve covers 16% and 84% mortality.

Litchfield and Wilcoxon [7]. The Litchfield and Wilcoxon [7] method is widely used for determining LD_{50} and its 95% confidence limits. This method [7] has provided a procedure for correcting 0 and 100 % effects [10]. However, an LD_{50} value determined using dose-mortality data with a range of 16–84% mortality, may not show a marked change in the value by performing the 0 and 100% correction. Determination of LD_{50} by Litchfield and Wilcoxon [7] is performed by a graphical method, and for calculating 95% confidence intervals, 16% and 84% mortality are needed.

Comparison of LD_{50} calculated by Trevan [1] and LD_{50} calculated using Litchfield and Wilcoxon [7] method and probit analysis [8]. LD_{50} is calculated using the Litchfield and Wilcoxon method and the probit analysis of Finney of the same data (Tab. 1) published by Trevan in 1927, to discover which calculation method results in a more 'accurate' LD_{50}. The dose-mortality relationship of the data (Tab. 1) is given in Figure 1.

Table 1. Mortality in mice intravenously administered with different doses of cocaine hydrochloride [1]

Dose of cocaine hydrochloride (mg for 20 g mouse)	Mortality (%)
0.8	100
0.7	84
0.6	78
0.5	55
0.4	16
0.3	0

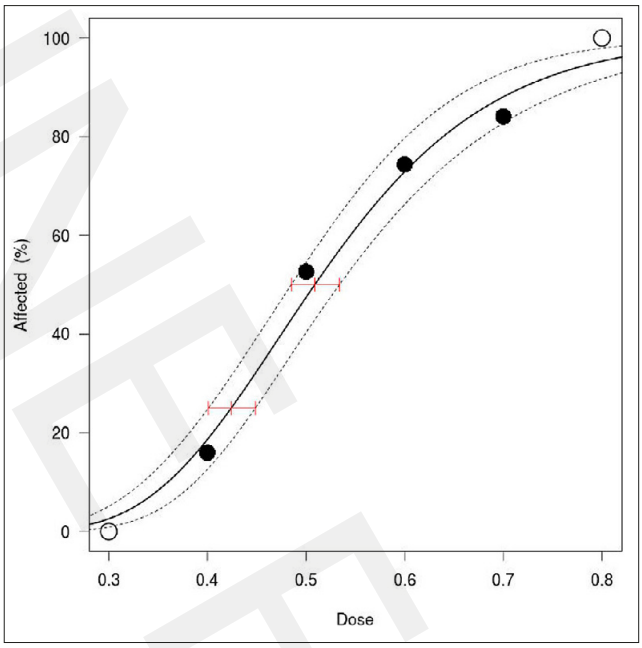

Figure 1. Dose-mortality relationship of the data given in Table 1.
Note: Dark circles are observed mortality. White circles are 0 and 100% mortality. The LD_{50}s calculated using the method of Litchfield and Wilcoxon [5] and Finney's probit analysis (Finney, [8] are given in Table 2

The LD_{50} calculated by Trevan [1] is very close to the LD_{50}s calculated using the method of Litchfield and Wilcoxon [7] and probit analysis [8]. The data of Trevan shown in Figure 1 is a typical sigmoid dose-response curve, covering the mortality range of 16% and 84%. If such data with a mortality range between 16 – 84% are plotted on a log-probit graph, the mortality data will be distributed linearly (Fig. 2). In an acute toxicity study, if the lowest mortality recorded is around 16% and the highest mortality is around 84%, the LD_{50} calculated using Finney's probit analysis [8] or Litchfield-Wilcoxon method [7] will be more or less the same. It is expected that a well-designed acute toxicity study should provide mortality data covering the range of 16 – 84%. Trevan suggested obtaining information on the 'characteristics' of the dose-response curve, initially for selecting the doses to obtain a mortality range, as explained above. However, this view was not considered by biologists after Trevan in designing their studies, although in earlier years a pilot study

Table 2. Comparison of LD_{50} calculated by Trevan [1] with the LD_{50} calculated using the method of Litchfield and Wilcoxon [5] and probit analysis [8]

Method used for calculation of LD_{50}	LD_{50} (mg/20 g mouse)
Trevan (1927)[1]	0.49
Litchfield and Wilcoxon (1949)[2]	0.51
Probit analysis (Finney, 1972)[3]	0.51

[1] Taken from Trevan's paper [1]
[2] Calculated using LW1949 software [12]
[3] Calculated manually

or range-finding study were used with smaller number of animals to determine the dose range for the main study. This would provide information on the highest dose that would result in 0% mortality and the lowest dose that would result in 100% mortality.

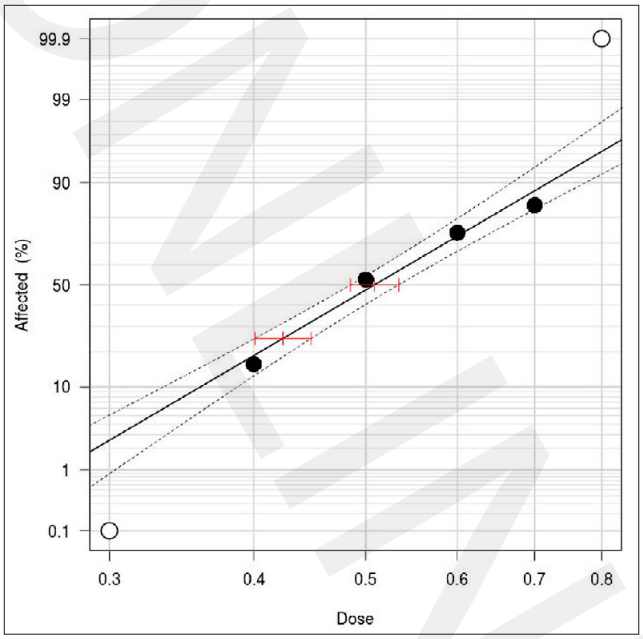

Figure 2. Dose (logarithmic scale)-mortality (probit scale) relationship of data shown in Table 1.
Note: The dark line is dose-response curve. Dark circles are observed mortality. 0 and 100% mortality (light circles) are not used for plotting the curve (there is no probit values for 0 and 100% mortality). The two dotted lines on either side of the dose-response curve are 95% confidence levels of doses. The plot was made using LW1949software [12, 13]

The 'accuracy' of LD_{50}. Several authors speak about determining the MLD accurately [13]. The definition of accuracy in analytical chemistry is the closeness of the analytical value to the true value [14]. The median lethal dose cannot be determined accurately since its true value is not known. With the mortality data obtained from a study, the MLD is calculated which is assumed to be the true value or close to the true value. Let us make a comparison between the Litchfield and Wilcoxon method [7] and Finney's probit analysis [8] to determine which of these methods calculates the 'best estimate' of MLD. The terminology, the 'best estimate' is used with some reservation since it is not known which MLD is the 'best' estimate. The LD_{50}s determined using Litchfield and Wilcoxon method [7] and Finney's probit analysis [8] in different situations are shown in Table 3.

The LD_{50}s calculated using Litchfield and Wilcoxon method [7] and Finney's probit analysis [8] are more or less the same in situations 1, 2, 3, and 7, where the mortality range 15–85% was included in the calculation procedure. The confidence interval (Litchfield and Wilcoxon method) and SE (Finney's probit analysis) of the LD_{50}s were similar. When mortality of 85% and above were excluded from the analysis (situation 4), LD_{50} and confidence interval (Litchfield and Wilcoxon method) and LD_{50}±SE (Finney's probit analysis) increased, and the increase was much higher when the mortality of 55% and abov, were excluded from the analysis (Situation 5). In Situation 6, where only the mortality range 15–55% was used for the analysis, both Litchfield and Wilcoxon method and Finney's probit analysis resulted in similar LD_{50}s with higher confidence intervals than Situations 1, 2, 3 and 7, but lower than that of Situations 4 and 5. From Table 3 it can be stated that both the Litchfield and Wilcoxon method [7] and Finney's probit analysis [8] provide the 'best estimate' of LD_{50}, for a four-group dose-response data (doses are logarithmically spaced), covering the mortality of about 20–80%.

DISCUSSION

John William Trevan (1887–1956). Trevan studied mathematics, chemistry, and biology before taking up medicine. He obtained the B.Sc. with honours in physiology in 1908 and medical qualifications M.B.B.S., and M.R.C.S., L.R.C.P. in 1911. Trevan was fond of drawing curves on graph

Table 3. LD_{50}s determined using Litchfield-Wilcoxon method [5] and Finney's probit analysis [8] in different situations

Situation	Dose (Mortality)							LD_{50}	
								L-F[1]	Finney[2]
1	10 (5)	20 (10)	40 (15)	80 (30)	160 (55)	320 (85)	640 (95)	112(76-165)	116±18
2	-	-	40 (15)	80 (30)	160 (55)	320 (85)	640 (95)	125(87-179)	126±18
3	-	-	-	80 (30)	160 (55)	320 (85)	640 (95)	132(95-185)	133±22
4	10 (5)	20 (10)	40 (15)	80 (30)	160 (55)	-	-	162(92-285)	156±55
5	10 (5)	20 (10)	40 (15)	80 (30)	-	-	-	235 (103-534)	229±210
6	-	-	40 (15)	80 (30)	160 (55)	-	-	141 (84-239)	141±40
7	-		40 (15)	80 (30)	160 (55)	320 (85)	-	126(88-181)	126±20

Note: The LD_{50}s were calculated in different situations. For example in the Situation 1, 7 dose-mortality data were used for the LD_{50} calculation, in the Situation 2, the first 2 dose-mortality data of Situation 1 were excluded for the calculation and so on.
[1]LD_{50} and confidence intervals are given; [2]LD_{50} and standard error (SE) are given.
L-F- Litchfield and Wicoxon method [5]. The LD_{50} and confidence intervals were calculated using LW1949 software [12, 13].
Finney-LD_{50} and SE were calculated manually as given by Finney [8]

paper and was skilled at making apparatus, contributing to the designing of the microbalance and microsyringe. His main interest, however, was the standardization of drugs, such as digitalis and insulin [15]. Being a medical professional he felt the need to provide standardized medicines for patients. He was aware that the safety margin of digitalis extract, insulin, and diphtheria toxin between therapeutic and toxic doses was small, hence the standardization of these drugs had to be performed precisely. His knowledge of mathematics and biology, and particularly physiology, were beneficial in his animal experiments which led to the invention of the 'MLD' (LD_{50}).

Trevan's calculation procedure of LD_{50} was simple and easy to perform. Briefly, the difference in percent mortality (*f*) is multiplied with the corresponding dose interval (*d*) to obtain (*fd);* the mean, mode, and median of the (*fd*) are then calculated. The median is the LD_{50} value. This may be why the LD_{50} is called as 'median lethal dose', although since Trevan, the commonly used methods like Litchfield and Wilcoxon [7] and Finney's probit analysis [8] do not use the median value in the calculation procedures of the LD_{50}. However, even today, biologists acknowledge Trevan's contribution to the determination of MLD (LD_{50}), knowingly or unknowingly, by retaining the word 'median' in the terminology and 'median lethal dose' for LD_{50}. Trevan also explained how to calculate the standard error of the LD_{50}, and the mode is calculated from mean and median, which he named 'characteristic'. Trevan defined the 'characteristic' as the curve expressing the percentage of effect, produced by varying doses of a drug on animals of a specific species, for that particular drug, effect, and species. Perhaps, Trevan could have borrowed the word 'characteristic' from Gregor Johann Mendel, the father of modern genetics, who used this word to define a specific property of an organism. According to Mendel, individuals belong to a species display precisely the same characteristics. In genetics, character [16] is a synonym for characteristic [17]. The 'characteristic' is different from the slope of the dose-response curve. Trevan [1] mentioned in his paper that by determining LD_{25} and LD_{75}, a slope can be obtained with sufficient accuracy for most drugs. The method of obtaining a straight line using this method is similar to that suggested by Litchfield and Wilcoxon [7]. According to Litchfield and Wilcoxon [7], mortality between 16 and 84% fall in a linear fashion and according to Trevan the precision of the LD_{50} test can be improved by increasing the number of animals in the test groups. Trevan, however, never claimed that the LD_{50} determined using his method is always reproducible.

The LD_{50} determination procedure proposed by Trevan was 'used', or rather 'misused', extensively by biologists. In order to calculate $LD_{50,}$ a huge number of animals were killed by biologists across the world; for example, in the 1960s, experiments were conducted to determine the LD_{50} of reduced iron and distilled water in rats [18]. In the experiments to determine LD_{50}, reduced iron was administered intragastrically as a thick suspension in 11 doses ranging between 60 – 200 g/kg b.w., at a dose volume of 75–100 ml/kg b.w. to 10–24 male rats. The doses were spaced by 5 – 10 g. There could have been need to conduct such a study at that time, but the number of dose groups used in the study and the narrow spacing between the doses is scientifically and ethically questionable. The volume of the doses administered was too high, considering the volume of the rat stomach (approximately 3.4 ml). According to the present regulatory guidelines, in toxicology studies with rodents the volume of the dose administered should not exceed 1 – 2 ml/100g body weight [19]. In the above study, the dose-response curve obtained was a straight line and the LD_{50} (98.6 g/kg body weight) was calculated from the linear regression equation:

Y=35.0+1.272X, where Y=Dose and X = Mortality.

In the study to determine the LD_{50} of distilled water, the volume of distilled water administered was 70 ml/kg b.w. and the LD_{50} was determined as 500 g/kg. Interestingly the authors of the above studies were well aware that a large volume of water used as a vehicle itself could produce toxicity. The authors also knew that the LD_{50} cannot be determined accurately as it is affected by several factors, such as the selection of species, body weight, age, gender, day and seasons. Ethics in using animals in acute toxicity tests and irreproducibility of LD_{50} have been extensively discussed during the past thirty years [20] and several methods have been proposed to determine LD_{50} using the minimum possible number of animals [21, 22].

It is inappropriate to state that Trevan sacrificed animals only to determine LD_{50}. If we study in depth his articles on similar subjects, we will realize that he has given much importance to interpret the data in the light of the dose-response relationship. It is generally accepted by biologists that the dose-response relationship provides a better understanding of causality between a toxicant and its effects, especially the rate of manifestation of the adverse effect [23]. Several chemicals may have the same LD_{50}, but their rate of manifestation of the adverse effect may differ. Similarly, there are chemicals with different LD_{50}s, but their rate of manifestation of the adverse effect is similar. Therefore, classifying chemicals into various groups based on LD_{50} has little relevance; here comes the relevance of the term, 'characteristic' mentioned by Trevan. According to him, 'characteristic' is species and test substance-specific. Classifying the chemicals using both LD_{50} and 'characteristic' would have been more relevant fom the toxicology point of view. The currently used procedures to determine LD_{50} by the regulatory agencies are up-and-down, and the fixed dose procedures [24] have scientific deficiencies and cannot be extrapolated to humans [25]. Determination of 'characteristic' in the up-and-down and the fixed dose procedures is not possible. For toxicological classification of chemicals, regulatory agencies use LD_{50} [26]. $LD_{50,}$ is not considered as a useful measure of toxicity in new drug development [27; however, it is considered useful for estimating the potential hazard of a chemical in humans [8]

CONCLUSIONS

Trevan calculated the LD_{50} to ensure the required potency of certain drugs before they reached a patient. He never promoted sacrificing more animals to determine LD_{50}. He was aware of the fact that the determination of LD_{50} is affected by several factors. The 'characteristic' of a dose-response curve proposed by Trevan is species and test substance-specific than the slope and LD_{50} determined of a dose-response curve. The manifestation of the toxicity of a drug or a chemical cannot be assessed by an LD_{50} value, as drugs or chemicals

having similar LD_{50} values manifest toxicity differently. Similarly, drugs or chemicals with different LD_{50} values may manifest similar toxicity effects; hence, classification of chemicals into various groups based on LD_{50} values may not have much relevance.

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