COMPARISON OF FIVE METHODS FOR THE DETERMINATION OF LETHAL DOSE IN ACUTE TOXICITY STUDIES

F.J.R. PAUMGARTTEN, O.A.F. PRESGRAVE, M.A.C. MENEZES, F.F. FINGOLA, J.C.B.R. FREITAS, R.R. CARVALHO and F.Q. CUNHA

Setor de Farmacologia e Toxicologia, Instituto Nacional de Controle de Qualidade em Saúde, FIOCRUZ, 21040 Rio de Janeiro, RJ, Brasil

The aim of the present study was to compare the reliability of LD_{50} determination using the traditional Litchfield and Wilcoxon method with that obtained by four alternative tests requiring smaller numbers of animals, for the purpose of classifying chemicals according to their acute toxicity. Acute lethal dose determinations were carried out in mice for oral and intraperitoneal administration of hexachlorophene, lidocaine, methanol, phenobarbital and physostigmine. The Molinengo method proved not to be as reliable as suggested by its author. Determination of LD_{50} using the Thompson and Weil method or, alternatively, the maximal non-lethal dose and the approximate lethal dose permitted the classification of the chemicals in essentially the same order. The approximate lethal dose method, in particular, seems to be a very suitable alternative method to the classical LD_{50} test since it requires only about 6 animals, provides enough information to order chemicals according to their toxicities, and provides useful information for planning subsequent repeated-dose studies.

Key words: LD_{50}, acute toxicity, lethality indices, alternative toxicity tests.

During the past few years, for ethical, scientific and economic reasons, toxicologists have been more and more compelled to reduce the number of animals used for safety evaluation of chemicals and to use alternative *in vitro* methods whenever possible. Tests for determination of the median lethal dose (LD_{50}) have been particularly questioned due to the large number of animals that must die in order to provide statistically valid LD_{50} data.

The LD_{50} method was originally developed by Trevan (1) in 1927 for the biological standardization of digitalis extracts and other highly active pharmacological agents. Since then, the test has been used widely as a toxicological test to assess the toxicity of drugs, food additives and environmental chemicals. Although high statistical precision is really required for biological standardization, such as in bioassays where a test compound is compared with a reference substance, it seems to have no practical importance when LD_{50} is used for toxicological evaluation. Zbinden and Flury-Roversi (2) have pointed out that the

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The present address of F.Q.C. is Departamento de Farmacologia, Faculdade de Medicina de Ribeirão Preto, USP, 14049 Ribeirão Preto, SP, Brasil.

Correspondence: Dr. F.J.R. Paumgartten, Setor de Farmacologia e Toxicologia, INCQS, FIOCRUZ, Av. Brasil 4365, Manguinhos, 21040 Rio de Janeiro, RJ, Brasil.

LD_{50} method depends on so many variables, such as animal species and strain, sex and age, diet, food deprivation prior to dosing, environmental temperature and humidity, circadian and seasonal rhythms, experimental procedures and so on, that the variability of the LD_{50} test can only be kept at an adequately low level when it is repeated in the same laboratory under strictly controlled conditions. Moreover, a European Community Multicentre study has demonstrated that the interlaboratory reproducibility of LD_{50}s is very poor even when the test is carried out under carefully standardized experimental conditions (3,4). This lack of reproducibility indicates that, far from being a biological constant, the LD_{50} value should be regarded as a unique result of one particular experiment.

In support of the LD_{50} test (5), it has been claimed that it provides a valuable method to learn about acute toxicity and to evaluate the hazards to human beings accidentally exposed to large amounts of a chemical substance, to obtain some information useful in selecting doses for repeated-dose and chronic toxicity studies, and to classify chemicals in order to assign them to a toxicity class in official lists of poisonous substances (5). Nevertheless, test procedures requiring smaller numbers of animals can suitably replace the conventional LD_{50} test for all of these purposes. In spite of this controversy, evaluation by the LD_{50} method is still required by regulatory agencies in many countries, in most of them, only for the labelling of chemicals.

The aim of the present study was to compare LD_{50} values estimated by the conventional Litchfield and Wilcoxon method (6) with those obtained by alternative tests for classifying chemicals according to their acute toxicity.

Albino Swiss mice of either sex (25-30g) from our colony were kept in plastic cages on wood shavings in air-conditioned rooms (21-23°C). All animals were fasted overnight before and approximately 3 h after receiving the test substance. Drugs were administered in the morning and the mice were observed for 14 days. Lethality indices were determined following oral (to males by gavage) and intraperitoneal (females) administration of either methanol PA (Merck, Brazil), lidocaine HCl (Far-Manguinhos, Brazil), phenobarbital (Alkaloida Chemical Factory, Hungary), hexachlorophene (Merck) or physostigmine (Merck). All chemicals were dissolved in distilled water except hexachlorophene which was suspended in sesame oil. LD_{50} values were determined as described by Litchfield and Wilcoxon (6), Thompson and Weil (7,8) and Molinengo (9). The maximal non-lethal dose (MNLD) was determined according to Malmfors and Teiling (10) and the approximate lethal dose (ALD) as described both by Deichman and Le Blanc (11) and by Kennedy et al. (12).

Determinations of the LD_{50} and corresponding confidence limits (CL) by the moving average or Thompson and Weil method (T & W) and the Molinengo method require fewer animals than the classical Litchfield and Wilcoxon method (L & W). However, they do not estimate the slope of the dose-mortality curve and the confidence intervals tend to increase as the number of animals used decreases (Table 1). With the T & W method, the LD_{50} can be determined starting from 2 animals per dose and at least 4 dose levels, provided that the logarithms of successive dose levels differ by a constant value. Thus, in theory, it would be possible to determine an LD_{50} value and CL starting from 8 animals. In practice, however, a very reduced number of animals may not be the best choice since the probability of obtaining a set of mortality data which does not match any of those found in the Weil tables (8) increases. We used 5 mice per dose but sometimes had to repeat the test and, in two

Table 1 - *Comparison of acute lethal doses and 95% confidence limits determined by five methods.*

The lethality indices include: LD_{50} determined by the methods of Litchfield and Wilcoxon (L & W), Thompson and Weil (T & W) and Molinengo, the maximal non-lethal dose method (MNLD) and the approximate lethal dose method (ALD).

Chemical	Route of administration	LD_{50}			MNLD	ALD
		L & W	T & W	Molinengo		
Physostigmine (mg/kg)	*ip*	1.0 (0.9-1.1)	1.0 (0.9-1.1)	1.3 (0.1-2.6)	0.8	1.0
	po	2.2 (2.1-2.4)	2.1 (1.5-2.8)	2.0 (1.4-2.5)	1.4	4.4
Phenobarbital (mg/kg)	*ip*	215 (185-249)	180	237 (174-299)	151.2	200.0
	po	250 (219-286)	252 (211-301)	389 (330-448)	116.6	213.0
Methanol (g/kg)	*ip*	4.6 (4.3-4.8)	4.2 (3.9-4.6)	6.3 (5.7-6.8)	4.1	5.0
	po	10.4 (9.9-10.9)	9.5	15.3 (11.8-18.9)	7.5	11.3
Lidocaine (mg/kg)	*ip*	134 (126-142)	136 (127-147)	175 (69.1-281)	133.0	130.0
	po	400 (337-474)	400 (317-503)	393 (240-546)	230.0	450.0
Hexachlorophene (mg/kg)	*ip*	14.0 (8.6-22.6)	35.7 (35.1-36.3)	109 (42-176)	15.9	40.0

instances, although we were able to determine the LD_{50}, it was not possible to estimate the confidence interval from the mortality data obtained. Despite this drawback, a smaller number of animals was used and the LD_{50} values were quite close to those determined by the L & W method (Tables 1 and 2).

The Molinengo method permitted the estimation of LD_{50} values and CL from a very reduced number of mice (ranging from 7 to 10) but the method proved not to be as reliable as previously suggested (9). In 4 out of 9 determinations, the LD_{50} values obtained by the Molinengo method were so different from those obtained by the L & W method, that, in spite of the large confidence intervals of the Molinengo LD_{50}, there was no confidence interval overlap between the two sets of data (Table 1). Also, the Molinengo-derived LD_{50} tended to underestimate toxicity and three chemicals ranked lower than they did when other methods were used (Table 2).

A disadvantage of the LD_{50}-based system of ranking the toxic potencies of chemicals is that, even assuming a lognormal distribution of lethalities, it does not consider the slope of the probit line. Depending on whether the dose-mortality curve is flatter or

Table 2 - *Acute toxicity classification of chemicals using different lethality indices.*

The lethality indices presented include: LD_{50}s determined by the methods of Litchfield and Wilcoxon (L & W), Thompson and Weil (T & W) and Molinengo; the maximal non-lethal dose method (MNLD) and the approximate lethal dose method (ALD). Chemicals were classified according to a toxicity rating chart adapted from Klaassen (13). Practically nontoxic (No) > 15 g/kg, slightly toxic (Sl) 5-15 g/kg, moderately toxic (Mo) 0.5-5 g/kg, very toxic (Ve) 50-500 mg/kg, extremely toxic (Ex) 5-50 mg/kg and supertoxic (Su) < 5 mg/kg. The numbers in parentheses indicate the number of animals used for each determination. *Different from L & W-derived toxicity rating.

Chemical	Route of administration	LD_{50}			MNLD	ALD
		L & W	T & W	Molinengo		
Physostigmine	*ip*	Su (85)	Su (20)	Su (8)	Su (58)	Su (6)
	po	Su (67)	Su (20)	Su (7)	Su (52)	Su (6)
Phenobarbital	*ip*	Ve (65)	Ve (20)	Ve (8)	Ve (52)	Ve (6)
	po	Ve (65)	Ve (20)	Ve (9)	Ve (46)	Ve (6)
Methanol	*ip*	Mo (126)	Mo (20)	Sl* (10)	Mo (48)	Mo (5)
	po	Sl (90)	Sl (40)	No* (7)	Sl (28)	Sl (7)
Lidocaine	*ip*	Ve (72)	Ve (20)	Ve (9)	Ve (50)	Ve (7)
	po	Ve (90)	Ve (40)	Ve (8)	Ve (38)	Ve (6)
Hexachlorophene	*ip*	Ex (40)	Ex (20)	Ve*(10)	Ex (48)	Ex (6)
Average No. of mice used, Mean ± SD		77.7 ± 23.9	24.4 ± 8.8	8.4 ± 1.1	46.4 ± 8.8	6.1 ± 0.6

steeper, the toxic effects will start with lower or higher doses, respectively. Therefore, systems based on the detection of threshold toxic doses would be more suitable and safer than LD_{50}-based systems. The MNLD method, a threshold lethality index, provided values which were lower than LD_{50} values in every case except for hexachlorophene. However, the rank of toxic potency of the chemical using MNLD was identical to that derived from the LD_{50}s determined by the L & W method (Table 2). The MNLD method requires fewer animals than the L & W method but is very time-consuming as each of its 4 successive steps takes at least one week to complete.

The ALD method required fewer animals than any of the other methods and in all but two instances it provided values close to the L & W-derived LD_{50}s (Table 1). In all instances, the ALD method ranked the chemicals just as the L & W method did (Table 2).

In conclusion, with the exception of the Molinengo method, all other tests allowed the chemicals to be classified in the same way, even though they required quite different numbers of animals. The approximate lethal dose, in particular, seems to be a suitable alternative method to the classical LD_{50} test, since it requires only a minimum number of

animals and gives enough information to enable the classification of chemicals according to their toxicities and to plan further repeated-dose studies.

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