Scientific Edition

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

A. G. DuMez, Editor, Baltimore, Maryland

Volume XXX | *JULY, 1941* | Number 7 Consecutive No. 13

A Simple Statistical Method for the Calculation of Mortality Percentages in Digitalis Assays*

By Harold N. Wright†

The percentage mortalities found by actual experiment in all procedures involving biological material are likely to show discordant results because of the variability in resistance of individuals, unless very large groups of animals are employed at each dosage. The latter is often not easily practicable, particularly where a relatively wide dosage range must be covered in the assay.

In digitalis assays by the official one-hour frog method, for example, it is usually most satisfactory to employ a relatively large number of small groups of frogs at different dosages, supplementing this procedure by the use of appreciably larger groups at the dosages in the critical range. Even so, discordant results frequently appear and the determination of clear-cut percentage mortality comparisons by purely experimental means without the use of an inordinately large number of animals is often difficult.

The calculation of the comparative strengths of (*a*) the standard tincture prepared from the U. S. Reference Standard Powder and (*b*) the preparation being assayed, according to the requirements of the United States Pharmacopœia (1), requires the injection of the two series of frogs to proceed "until the amounts of the percolate of digitalis and the standard preparation of digitalis, respectively, required to give equivalent percentages of positive reactions in approximately equal groups of frogs, are determined." The Pharmacopœia also states in the same paragraph that the injections must be repeated "if the largest doses of the respective preparations are not enough to give systolic standstill in at least 25 per cent of their respective groups or if more than 75 per cent of either group receiving the smallest doses are in systolic standstill." It would appear, therefore, that the "equivalent percentages of positive reactions" employed to "calculate the relationship of the activity of the preparation being assayed to the reference preparation" may lie anywhere within the range of 25 to 75 per cent mortality.

* Presented at the Group Meeting on Statistical Methods as Applied to Biological Assays of the Committee of Revision of the United States Pharmacopœia, Washington, D. C., May 13, 1940. A part of the funds used in this research was supplied by a grant from the Medical Research Fund of the University of Minnesota.

† From the Department of Pharmacology, University of Minnesota Medical School, Minneapolis, Minnesota.

Statistical methods of treatment of bioassay are not new. Trevan (2) showed that a sigmoid curve is obtained when the percentage of frogs showing a positive response to digitalis is plotted against the dosage. Later workers, notably Gaddum (3), Bliss (4) and Miller, Bliss and Braun (5) have demonstrated that this curve can be transformed into a straight line by plotting the logarithm of the dose against "an expression of the percentage effect in units derived from the normal frequency curve," termed "probits" by Bliss. The complexities of the theoretical arguments justifying such transformations deter many workers from employing these methods.

The method here presented is not intended to supplant these more elaborate methods, but simply to draw attention to a simple statistical method of increasing the accuracy of conclusions drawn from the experimental data without transformation of the data into artificial units which may tend to obscure the interpretation of the experimental results and prevent their comparison with the results of other workers.

The simple statistical method here presented for the more accurate calculation of percentage mortality statistics in digitalis assays than is given by the actual experimental data appears to have been first stated by Dragstedt and Lang (6). The method employs two assumptions which are logically quite sound and generally acceptable. These assumptions are: (1) that an animal which dies from a given dose would, under the same conditions, have died had it received any higher dose, and (2) that an animal which survives a given dose would also have survived had it received a smaller dose.

These assumptions are applied statistically by (1) integrating all survivals at higher dosages with those at the dosage under consideration and (2) simultaneously integrating all deaths at lower dosages with those at the dosage under consideration. Dragstedt and Lang clearly enunciated these assumptions and applied them in the above manner to the calculation of percentage mortality statistics of the effects of various respiratory stimulants in acute cocaine poisoning in rabbits.

Behrens (7) in 1929, apparently unaware of Dragstedt's publication, stated and applied the same fundamental assumptions to the calculation of a percentage mortality curve for the intravenous infusion of a strophanthin solution to frogs. He found that when his experimental determinations of the individual intravenous fatal dose on 140 frogs were analyzed as grouped data on the basis of the above assumptions, the percentage mortality curve agreed quite closely with the sigmoid dose-mortality curve previously determined experimentally on the same species of frogs (*Rana temporaria*) by Trevan.

Apparently unaware of the publications of either of these previous workers, Reed (8) and Reed and Muench (9) have more recently applied this method of calculating LD50 to problems of percentage mortality in irradiation and the biological titration of sera and viruses. Holck (10) has also recently discussed the applicability of the method to laboratory bioassay results.

The usefulness of the "Dragstedt method" of calculating percentage mortalities lies in the fact that the statistical procedure of simultaneous integration of survivals and deaths gives an accuracy to the percentage mortalities equivalent to that which would be obtained without integration only by the use of several times the number of animals employed with integration.

APPLICATION OF THE METHOD

The application of the Dragstedt double integration method to digitalis assays is shown in the following figures and tables. Table I shows the actual experimental data and the double integrated data for a single assay of the standard tincture prepared from the U. S. Reference Digitalis Powder. Forty-eight frogs were employed at nine dosage levels between 200 and 400 mg./Kg. In this particular assay (chosen to illustrate the utility of the statistical method rather than the perfection of the experimental results) the experiment shows a 50 per cent mortality at four dosage levels, namely 200, 250, 300 and 325 mg./Kg. Determination of mortalities from the integrated totals, however, smooths out these irregularities and places the LD50 at 322 mg./Kg. The graphic representation of these results is shown in Fig. 1. The effect of integrating survivals only, and of deaths only, is also shown in this

Table I.—Experimental and Integrated Mortality Statistics for a Single Assay Employing 48 Frogs of a Standard Tincture Prepared from the U. S. Reference Digitalis Powder by the U. S. P. One-Hour Frog Method (See Also Fig. 1)

Dose mg./Kg.	Experimental Data Alive	Dead	% Mort.	Integrated Data Alive	Dead	% Mort.
200	1	1	50	21	1	4.5
225	2	0	0	20	1	4.8
250	1	1	50	18	2	10
275	3	1	25	17	3	15
300	4	4	50	14	7	33.3
325	4	4	50	10	11	52.3
350	5	7	58	6	18	75
375	1	3	75	1	21	95.5
400	0	6	100	0	27	100

figure, from which it is apparent that this practice, which has been widely followed in bioassay statistical methods since its employment by Behrens, especially that of integrating survivals only, is not nearly as satisfactory as the Dragstedt double integration method. In the experiment here discussed use of survivals only has the effect of moving the LD50 too far to the right, thereby producing a fictitious lowering of the apparent toxicity of the drug, while use of deaths only assigns too great a toxicity to the drug.

The application of the method to the evaluation of the potency of an unknown tincture is illustrated in Fig. 2 and Table II. Figure 2 shows the experimental and double integrated curves for the simultaneous assays of the standard tincture prepared from the U. S. P. Reference Standard powder and the unknown tincture. The figures given in Table II have been read from this graph (plotted on coordinate paper). Since the U. S. P. one-hour frog method permits the calculation of corresponding mortality percentages throughout the 25–75 per cent range the comparative ratio strength of the unknown tincture to the reference standard tincture has been calculated for both curves at mortalities of 25, 40, 50, 60 and 75 per cent (Table II).

Table II.—Potency of Unknown Tincture of Digitalis in Terms of U. S. Reference Standard at Various Percentage Mortalities (See Also Fig. 2)

% Mortality	U. S. Ref., mg./Kg.	Unknown, mg./Kg.	Ratio Strength
	Experimental Curves		
25	225.0	275.0	75.0; 81.8
	265.5	300.0	88.3; 96.4
	312.0		104.0; 113.4
40	240.0	317.5	75.6
	255.0		80.3
	315.0		99.2
50	250.0	325.0	76.9
	322.5		97.0
60	327.5	350.0	93.6
75	350.0	361.0	97.0

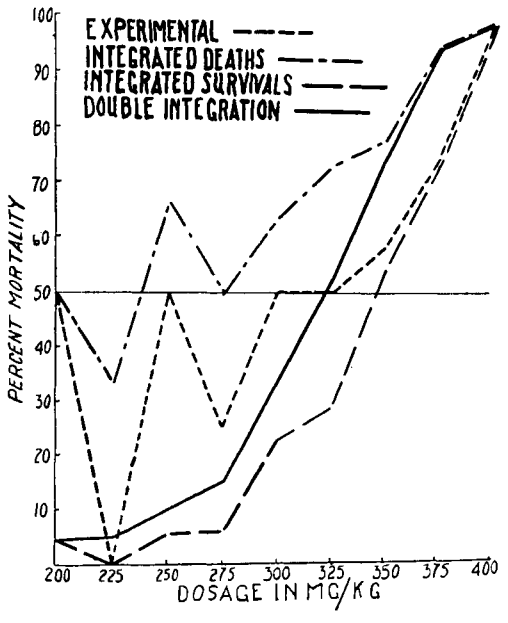

Fig. 1.—Graphic Representation of Various Methods of Integrating Survivals and Deaths in Digitalis Assays in Comparison with the Actual Experimental Data.

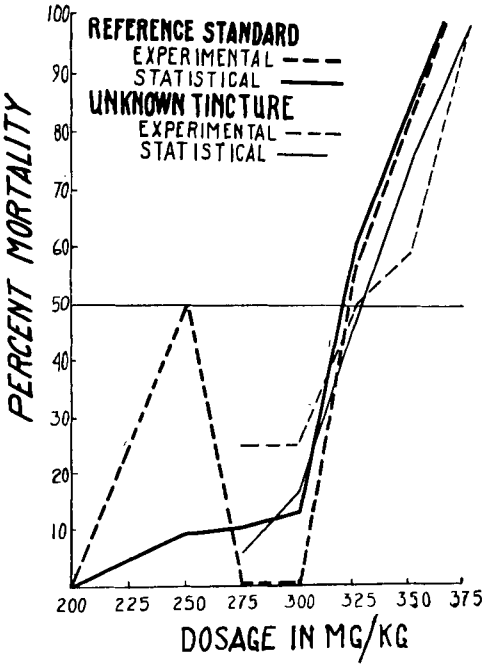

Fig. 2.—Experimental and Double Integrated Curves for Assay of U. S. Reference Standard Digitalis Tincture and Unknown Tincture.

Table II.—(*Continued*)

Dragstedt Curves

25	307.5	306.0	100.5
40	315.0	319.0	98.8
50	320.0	327.0	97.9
60	325.0	337.0	96.4
75	337.5	344.0	98.1

It will be noted that in the experimental curves a mortality of 25 per cent is shown at three dosage levels for the U. S. P. Reference Standard tincture and at two dosage levels for the unknown tincture, thus giving six possible ratio strengths for the unknown tincture at this percentage mortality, ranging from 75.0 to 113.4 per cent of the U. S. P. potency. The application of the double integration procedure to the two experimental curves, however, completely smooths out these irregularities and gives curves for the corresponding mortality percentages of the two preparations to be composed, which run almost parallel throughout the 25–75 per cent mortality range.

Taking the figures from the Dragstedt curves the potency of the unknown tincture varies through the corresponding percentage mortality values only between 96.4 and 100.5 per cent of U. S. P. potency, with a mean value of 98.3 per cent and a probable error of the mean ±0.403 per cent.

SUMMARY

1. A simple statistical method is presented for the rapid and accurate calculation of comparative potencies in digitalis assays.

2. The method employs the simultaneous integration of survivals and deaths at all dosage levels.

3. The method does not require any transposition of units and the results obtained are directly comparable with those obtained by other workers.

4. The integrated results have an accuracy of approximately ±1 per cent throughout the 25–75 per cent mortality range.

REFERENCES

(1) Pharmacopœia of the United States, 11th Edition, Mack Printing Co., Easton, 1936.

(2) Trevan, I. W., *Proc. Roy. Soc.* (London), Ser. B., 101 (1927), 483.

(3) Gaddum, J. H., *Med. Res. Council (Brit.), Special Rept. Series No. 183*, 1933.

(4) Bliss, C. I., *Science*, 79 (1934), 409; *Quart. J. Pharm. and Pharmacol.*, 11 (1938), 192.

(5) Miller, L. C., Bliss, C. I., and Braun, H. A., JOUR. A. PH. A., 28 (1939), 644.

(6) Dragstedt, C. A., and Lang, V. F., *J. Pharmacol., 32* (Dec. 1927), 215.

(7) Behrens, B., *Arch. Exp. Path. Pharmakol.*, 140 (1929), 237.

(8) Reed, L. J., "Statistical Treatment of Biological Problems in Irradiation," Chap. 2, in Duggar, B. J., "Biological Effects of Radiation," McGraw-Hill, New York, 1936.

(9) Reed, L. J., and Muench, H., *Am. J. Hyg.*, 27 (May 1938), 493.

(10) Holck, H. G. O., *Am. J. Pharmaceut. Ed.*, 4 (1940), 602.

A Pharmacological Study of Some New Synthetic Hypnotics*

By John W. Nelson, Stanley C. Lyster and George F. Cartland

The studies here reported were undertaken with the purpose of evaluating in a preliminary way, the hypnotic activity of a large group of synthetic compounds, most of which have not previously been described in the literature. All of the compounds here studied, with the exceptions noted in Table I, were prepared by Blicke and Centolella (1, 2). The rat has generally been regarded as the most suitable animal for such studies, and the intraperitoneal route has been adopted by us as the most sensitive method of estimating hypnotic activity in a group comprising compounds of widely different solubilities. Fitch and Tatum (3) have suggested the intraperitoneal route as closely approximating slow intravenous injection. For references to the literature and a discussion of methods we refer to this paper.

Further studies on the more effective members of the series, involving measurement of induction time, and duration of sleep by oral administration in rabbits and intraperitoneal injection in rats are included in this report.

EXPERIMENTAL

Rat Experiments.—Healthy young adult male Wistar strain albino rats raised in our animal colony and weighing 175 to 300 Gm. were used in all experiments included in this report. No animal received more than one injection in order to avoid any tolerance for the material injected. The rats were not starved previous to injection since preliminary ex-

* From the Research Laboratories, The Upjohn Company, Kalamazoo.