RESEARCH PAPERS

ASSAY OF THE CURATIVE ACTION OF NEOARSPHENAMINE BY TIME-MORTALITY DATA

BY ÅKE LILJESTRAND

From the State Pharmaceutical Laboratory and the Medical Clinic of the Caroline Hospital, Stockholm

Received November 6, 1948

THE commonest current methods employed for testing the action of neoarsphenamine are those in which the drug is injected into mice or rats already lightly or heavily infected with *Trypanosoma equiperdum* on a previous day. Blood specimens removed from every animal are examined daily, involving the counting of many squares of the counting-chamber, before deciding whether or not the animals are cured. These methods are laborious and time-consuming, and for this reason, and because of other disadvantages, Bülbring and Burn[1] proposed that the activity of a preparation should be estimated from the survival times of mice infected and treated on the same day. In the present work, this method has been extended and examined statistically.

METHOD

Blood taken from rats which had been infected 2 days earlier was diluted with 1 per cent. sodium citrate solution till it contained 7,000 trypanosomes in 1 microlitre. Mice weighing about 16 to 18 g. were infected by intraperitoneal injection with 0·5 ml. of this trypanosome suspension. Neoarsphenamine was injected intravenously in 0·2 per cent. solution within 2 hours from the time of infection. The doses were calculated in proportion to the body weight. The usual precautions to prevent the oxidation of the neoarsphenamine were taken.

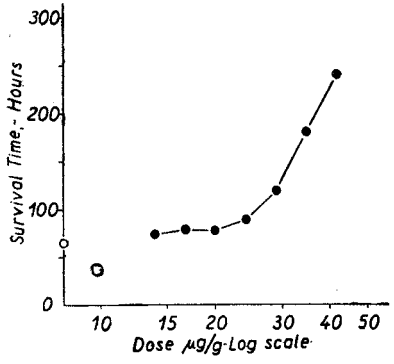

FIG. 1. Survival in hours from the time of infection for the median in every group.

Untreated. ● Treated.

RESULTS

The results of a preliminary pilot-experiment provided a curve relating dosage with survival time. Seven groups each of 15 infected mice were injected with graded doses of neoarsphenamine ranging from 13·9 µg./g. of body weight upwards by steps of 20 per cent. From 2 days after the infection the mortality was noted every hour for 36 hours. After that, observations at night were discontinued. For the two highest doses the mortality was noted only once a day. A control group of 40 mice was followed at the same time. The survival time-

dosage curve was drawn from the medians of the survival times in every group (see Fig. 1). All animals, except those receiving the highest dose, died, the last mouse surviving until the 15th day. In the highest dose group, i.e., those receiving 41·5 μg./g., 3 animals survived, the last one dying on the 18th day. Since we rarely found any animals dying after this time it was decided that any mouse living beyond the 18th day should be counted as definitely cured.

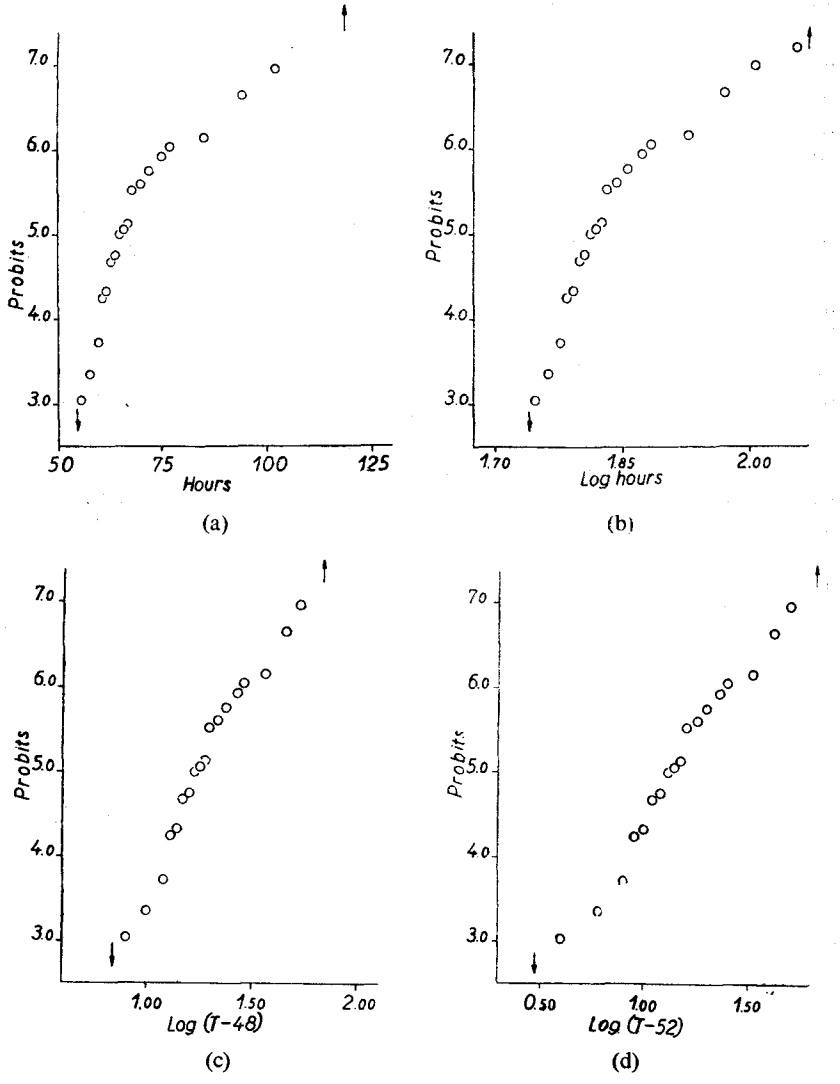

FIG. 2. Probit transformations for the survival times from the time of infection of the 40 infected control mice. (a) Survival time in hours; (b) Log survival time in hours; (c) Log (survival time in hours − 48); (d) Log (survival time in hours −52). The times of the last observation with all living animals and those of the first observation with all dead animals are marked with arrows.

Using this basis we observed which function of survival time had a normal distribution. In toxicity tests in which the lethal time is delayed, some function of the time elapsing between the time of injecting the animal and its death can be found to be normally distributed, and the standard deviation of this function becomes a suitable measure of the varying sensitivity of the animals to the drug. Since the untreated infected controls show differences in their survival times, these were also determined and their limits of variation ascertained.

Of the 40 controls mentioned above, 4 animals died during periods when observations were more infrequent. For the other 36, the time of death could be stated to within an hour. The percentage of animals that had died up to the various times was transformed into probits according to Bliss[2]. These values were then plotted on graph paper with the probits along one axis and the time or the logarithm of the time along the other, the time being measured from the moment when the animals were infected (*see* Fig. 2 a and b). If the plotted function of time had been normally distributed, the points should have fallen mainly along a straight line. In neither case did this happen, however, but the points fell along curved lines. That this was not accidental could be seen from similarly curved lines with corresponding probit transformations for the lower neoarsphenamine doses, in spite of the small number of animals in each one of these groups.

Every mouse was inoculated with about 3·5 million trypanosomes. In this way it received such a large number of trypanosomes that one hardly needs to take into account any differences in virulence between the infecting material of the different mice due to random variation. The dispersion of survival times will thus be mainly due to the host animals, i.e., the possibilities of growth for the trypanosomes in the different mice and the varying resistance of these to the fully developed infection. With intraperitoneal infection the conditions of growth may be regarded as nearly optimal, and so no great differences should exist between the different mice on this ground. This line of reasoning is confirmed by the results from the trypanosome counting method. In this the animals are used when the infection is very strong in the blood, that is, 2 days after being infected. Relatively few animals, however, need to be rejected on account of badly developed infection. In other words, full development of the infection is reached at approximately the same time by the majority of the infected animals. Consequently the differences in survival times would chiefly be due to the varying resistance of the mice against the fully developed infection. If such is the case some function of the time between the point when full infection is reached and the time of death might have a normal distribution.

The first control mouse died 55 hours after being infected. Evidently full infection must have been reached some time earlier, after which the remaining time of survival was influenced only by the resistance of the mouse. Times of 48 and 52 hours after infection were, therefore, chosen, as it was considered that full infection might have been attained

in the control group at one of these periods. New probit diagrams were made with the logarithms of the times from these new starting points along one axis, that is, the logarithm of the survival time in hours minus 48 and hours minus 52 respectively. In both cases good agreement with a normal distribution was obtained (*see* Fig. 2 c and d). In view of the small amount of material the points on both figures appear to lie reasonably near a straight line.

Also for the mice treated with neoarsphenamine the logarithms of (survival time in hours − 48) seemed to be normally distributed for every dose. As the groups were rather small, on a later occasion 2 groups with 40 infected mice in each were treated with doses of 20 and 31 g./g. of body weight respectively. In these groups, too, no certain deviation from the formula just mentioned could be found, the points lying fairly well along a straight line in the probit diagram. On this occasion a certain change in sensitivity to the neoarsphenamine was observed as compared to that shown in Figure 1 since half of the animals in these two groups died after 108 and 218 hours.

If this transformation of the primary values, i.e., the logarithm of (survival time in hours—48) proves to be generally useful for experiments of this kind in different laboratories, the mice of a group in different tests or at different times should as a rule be approximately normally distributed when their times of survival are transformed in this manner. The values given by Bülbring and Burn and also those found in earlier experiments from this laboratory, lend support to this contention. These figures confirm, among other things, that the maximum mortality of the controls as well as that of the animals treated with small neoarsphenamine doses occurs during the night between the second and third day after the infection.

TABLE I

Reading Time: Day after Infection	Reading Time: Time of Day	Time in Hours After the Infection (T)	Class Limit Log (T-48)	Class Width	Class Middle
2	22 o'clock	61	1·114	0·248	1·238
3	8 ,, ...	71	1·362	0·104	1·414
3	14^{15} ,, ...	77·25	1·466	0·102	1·517
3	22 ,, ...	85	1·568	0·104	1·620
4	8 ,, ...	95	1·672	0·104	1·724
4	20^{45} ,, ...	107·75	1·776	0·105	1·828
5	13 ,, ...	124	1·881	0·104	1·933
6	9^{30} ,, ...	144·5	1·985	0·098	2·034
7	10 ,, ...	169	2·083	0·099	2·132
8	17 ,, ...	200	2·182	0·104	2·234
10	10 ,, ...	241	2·286	0·103	2·337
12	14 ,, ...	293	2·389	0·105	2·442
15	9 ,, ...	360	2·494	0·099	2·544
18	17 ,, ...	440	2·593	0·103	*2·645

*Calculated with the class width taken as 0·103, which is the mean of all class widths except the first

As it thus seems that the logarithm of (survival time in hours − 48) in practice may be taken as normally distributed in the different dosage groups, due regard should be given to this fact in the spacing of the reading times. The following example shows how this may be performed so that the intervals between the observations are as far as possible equally large when expressed in the normally distributed function, i.e., log. (time in hours between the time of infection and the observation −48). Even allowing for this, the readings can be so arranged that most of them are made during the normal working day.

EXAMPLE

Groups of 20 newly infected mice, which had been kept in the laboratory for over a week before this experiment were injected intravenously with doses of 24 and 30 μg./g. of body weight of the International Standard and of a commercial preparation respectively, both in 0·3 per cent. solution. The mice were infected at 9 o'clock in the morning and the injections of neoarsphenamine were made within the following 2 hours. The readings were performed according to Table I. The times when the mice were found dead are given in Table IIa and the corresponding class middle for each animal in Table IIb. Two methods of analysis of the data are now available.

TABLE IIA

RESULTS. NUMBER OF DEAD MICE

Reading Time		Standard		Test Preparation	
Day	Time of day	24 μg./g.(S_L)	30 μg./g.(S_H)	24 μg./g.(U_L)	30 μg./g.(U_H)
2	22 o'clock			4	
3	8 „			7	2
3	14^{15} „			2	4
3	22 „	1		5	5
4	8 „	4		2	6
4	20^{45} „	8	3		2
5	13 „	4	6		
6	9^{30} „	2	1		
7	10 „		2		
8	17 „	1	5		1
10	10 „		2		
12	14 „				
15	9 „				
18	17 „				
Surviving after the 18th day			1		

The simplest method is to calculate the means, standard deviations, and standard errors of the means in the usual manner for each group as in Table IIB t-analysis for the differences between the means of S_H and S_L and between U_H and U_L shows in the first case $P < 0.001$ and in the second case $P = 0.001$. The differences are therefore not attributable to random variation alone. This shows that the test really is sensitive to a difference in dosage of 20 per cent. For the difference between the means of S_L and U_H $P = 0{\cdot}05$, i.e. the larger test dose has smaller effect than the smaller standard dose.

TABLE IIB

RESULTS TRANSFORMED
CLASS MEANS FROM TABLE I FOR THE DEATH OF EACH MOUSE

Mouse	Standard		Test Preparation	
Number	24 μg./g.(S_L)	30 μg./g.(S_H)	24 μg./g.(S_L)	30 μg./g.(U_H)
1	1·517	1·724	1·238	1·414
2	1·620	1·724	1·238	1·414
3	1·620	1·724	1·238	1·517
4	1·620	1·828	1·238	1·517
5	1·620	1·828	1·414	1·517
6	1·724	1·828	1·414	1·517
7	1·724	1·828	1·414	1·620
8	1·724	1·828	1·414	1·620
9	1·724	1·828	1·414	1·620
10	1·724	1·933	1·414	1·620
11	1·724	2·034	1·414	1·620
12	1·724	2·034	1·517	1·724
13	1·724	2·132	1·517	1·724
14	1·828	2·132	1·620	1·724
15	1·828	2·132	1·620	1·724
16	1·828	2·132	1·620	1·724
17	1·828	2·132	1·620	1·724
18	1·933	2·234	1·620	1·828
19	1·933	2·234	1·724	1·828
20	2·132	2·645	1·724	2·132
Total	35·099	39·914	29·432	33·128
Mean	1·75495	1·9957	1·4716	1·6564
Standard deviation	0·138	0·232	0·159	0·164
Standard error of the mean	0·031	0·052	0·036	0·037
Total S	75·013		Total U	62·560

More information, however, may be extracted from the material if it is subjected to variance analysis (Fisher[3]), the results of which are shown in Table III.

TABLE III

ANALYSIS OF VARIANCE. DATA OF TABLE IIB

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F	P
Between samples	1	1·9385	1·93850	61·74	<0·001
Between doses of same substance	2	0·9211	0·46055	14·67	<0·001
Random sampling	76	2·3867	0·03140	—	—
Total	79	5·2463	—	—	—

Slope, 2·18 Potency, 0·72 Fiducial limits, 0·580 to 0·809 ($P = 0·05$).
Percentage limits, 80·5—112·0

The slopes of the dosage-response lines were calculated and found to be homogeneous. It was therefore legitimate to calculate a combined slope and to use this value in order to obtain the potency of the unknown in terms of the standard from the equation provided by Gaddum[4], and the fiducial limits of error of the estimated potency from the equation provided by Fieller[5].

Summarising the results, the unknown preparation had a potency of 0·72 of the standard with fiducial limits of error of 0·580 to 0·809 for $P = 0·05$.

When material is grouped it is desirable to group it within narrow class limits, i.e. to group it in many classes, in order to extract as much

information as possible. In this case the number of classes directly depends on the frequency of the readings; many times of observations allow many classes with narrow class limits, whereas few readings allow only a few classes and widely spaced class limits. The number of the observations, however, is limited by the fact that in practice it is hardly feasible to make observations during the night. An increase of the number of readings during the day but none during the night implies alternating small and large intervals between the observations, with varying class widths, and this naturally gives no increased precision.

This could also be verified in the material from our example in which more readings were made during the days than are recorded in the tables. The number of readings in the example is near the maximum possible in practice with approximately equally spaced intervals. The fact that the first class has a much larger width than the rest has no great importance as in any case the doses of neoarsphenamine must be so large that only single animals die here.

One objection that has been raised against using survival times for estimating the curative action is that one does not know if the death of the animal is due to the infection. A certain control of this, however, exists. No deaths are caused by the infection on the day when the animals are infected or on the first or even on the greater part of the second day after infection. If the animals die during this time, it is due either to technical faults, which should be few with proper technique, or to non-specific deaths. Mice dying during this time must therefore be excluded from the analysis. When the groups are not too small and the times of survival not too long, the death of one or two mice during this period does not appreciably alter the accuracy of the test. If more mice die, it shows that they were in bad condition and the assay must be rejected. It has been found convenient to keep the mice in the laboratory for some days before they are used in order to make sure that they are in good condition.

When it is desired accurately to determine the curative potency of a preparation, the calculations will be performed as indicated in the example quoted above. Often, however, it is only necessary to ascertain that the potency of the test preparation is not less than that of the standard or of a certain proportion of it. The doses to be used should give a clearly prolonged survival time without making the test unwieldly. In our experiments a dose of 24 to 30 μg/g. of body weight fulfilled these conditions. As a control of the sensitivity of the method a weaker dose of the standard was given as well, by way of example say 20 per cent. weaker. In a successful experiment a difference between the two should be evident.

Practical Performance of a Test

Guided by these principles and by the aforementioned results, the test is performed as follows: 60 mice which have been kept in the laboratory for a week are infected intraperitoneally with 0·5 ml. per mouse of a trypanosome suspension containing 7,000 trypanosomes per

microlitre. This is obtained from the blood of rats that have been infected 2 days earlier. The trypanosomes are counted in a counting chamber and the blood is diluted with 1 per cent. sodium citrate solution to the desired concentration. The infection is performed at 9 o'clock in the morning. Within the next hour the mice are injected intravenously with the different doses of the 0·3 per cent. neoarsphenamine solutions. During the preparation of the solutions the usual precautions against oxidation are observed (*see* Burn[6]). 20 mice receive 24 μg. and 20 mice 30 μg./g. of body weight of the standard and 20 mice receive the dose of the test preparation that is to be compared with the higher standard dose, and which must not be less potent than this standard dose if the preparation is to pass the test. The mice are observed at 16 o'clock on the second day after the infection. Those which have died are rejected and excluded from the calculations. After that the readings of the mortality are spaced according to the times in Table 1. The mean and the standard error of the mean are calculated for every group, the value for every animal being that of the corresponding class middle seen in the table. Thus, if an animal has died only on the fourth day at 8 o'clock its value is 1·620. The preparation passes the test if t-analysis shows that it is stronger or not weaker than the larger standard dose. In the latter case, however, a significant difference between the standard doses must exist, otherwise the test must be repeated. If t-analysis shows that the test preparation is significantly weaker than the larger standard dose it is rejected.

Discussion

When testing substances on animals, it can be shown in many cases that the logarithm of the duration of the effect or the logarithm of the time till the effect appears is approximately normally distributed (Bliss[2], Goodwin and Marshall[7], Goldberg[8] and others). A close study of this question, however, may reveal that a more complicated function of the time has a normal distribution (Ipsen[9]). On the other hand, it is sufficient to have an approximate knowledge of the kind of function that is normally distributed when grouping the observations as the laws for calculating the means, standard errors and t-values are also applicable to a number of different distributions, more or less deviating from the normal. The grouping of the observations advocated in this paper seems to be more rational than making one or two readings every day for a limited number of days as proposed by Bülbring and Burn[1] and Goodwin[10] or for a longer times according to the method of Chen, Geiling and MacHatton[11]. A further advantage is that an estimate of the error may be obtained for every dose, so extracting all information inherent in the material. A comparison with the results of Hawking[12] shows that, whereas in the trypanosome counting method every animal gives only a qualitative expression for the strength of the preparation in this method, the survival times are quantitative estimates of the strength of the drug, and thus furnish more detailed information.

SUMMARY

The basis for using time-mortality data in estimating the curative effect of neoarsphenamine is examined. It is shown how the survival times may be transformed so that they become approximately normally distributed. A routine test has been designed on these lines.

The author is indebted to Dr. W. L. M. Perry, of the National Institute for Medical Research, London, for suggesting the treatment of the material with variance analysis and also for its application to the data.

REFERENCES

1. Bülbring and Burn, *Quart. J. Pharm. Pharmacol.*, 1938, **11**, 67.
2. Bliss, *Ann. appl. Biol.*, 1938, **22**, 134.
3. Fisher, *Statistical Methods for Research Workers*, Oliver and Boyd, Edinburgh, 1946.
4. Gaddum, *Med. Res. Coun. Spec. Rep.*, 1933, No. 183.
5. Fieller, *Quart. J. Pharm. Pharmacol.*, 1944, **17**, 117.
6. Burn, *Biological Standardisation*, Oxford University Press, 1937.
7. Goodwin and Marshall, *J. Pharmacol.*, 1945, **84**, 12.
8. Goldberg, *Acta physiol. Scand.*, 1948, **15**, 173.
9. Ipsen, *Contribution to the Theory of Biological Standardisation*, Copenhagen, 1941.
10. Goodwin, *J. Pharmacol.*, 1944, **81**, 224.
11. Chen, Geiling and MacHatton, *J. infect. Dis.*, 1945, **76**, 144.
12. Hawking, *Quart. J. Pharm. Pharmacol.*, 1943, **16**, 13.