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The Toxicity and Trypanocidal Activity of Commercial Neoarsphenamine

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The toxicity of neoarsphenamine, as well as of arsphenamine and sulpharsphenamine, offered for sale in Canada is subject to regulations under the Food and Drugs Act of Canada (1). According to the method of assay used in the control of these products in Canada the toxicity is expressed as per cent of the International Standard preparation (2), and in accordance with a recommendation contained in a memorandum from the National Institute for Medical Research (3), batches having a toxicity greater than 120 per cent of the International Standard are not sold in Canada.

Although a test for the therapeutic potency of these drugs is not required under the Canadian regulations, examination of a number of commercial samples by a method recently developed in this laboratory (4) has been carried out. By this test the trypanocidal activity of the samples is also determined as per cent of the International Standard. In the memorandum referred to above (3) it is recommended that the trypanocidal activity of commercial neoarsphenamine should not be less than 80 per cent of the International Standard.

The results of approximately 200 routine assays of neoarsphenamine for toxicity and 76 tests for trypanocidal activity are reported here, and they show, as well as can be done with present methods, the safety and effectiveness of the different preparations offered for sale in Canada.

In addition, a number of observations have been made on the use of dosage-response curves in the assay of arsenicals. Single-dose and multiple-dose methods (4, 5) have been employed, and the regression lines for toxicity and trypanocidal activity have been redetermined and compared with those previously published (2, 4).

A report on the secondary standards used in Canada in routine assays of neoarsphenamine is also included.

MATERIALS AND METHODS

The methods used for the biological tests for toxicity and trypanocidal activity have already been described (2, 4). Both one-dose and multiple-dose methods were employed in this work.

For the one-dose method a single group of rats was injected with a dose of the sample to be tested and another group, of the same sex and similar in respect to age and weight, was injected with a dose of the standard. In all assays 20 to 30 rats were used on each of the two groups. The slope of the dosage-response curve was assumed to be constant and curve numbers (4, 6) were used in calculating the results. For assays carried out up to 1938, curve numbers were taken from a dosage-response curve already published (2), and for those performed since

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that time they were computed from a dosage-response curve for sib-mated rats reported in this paper. The difference in the slopes of these curves was so small as to be statistically negligible, however, and curve numbers from either curve would have given satisfactory figures.

For the multiple-dose assays forty to sixty rats were divided into several similar groups. Each group received a different dose; usually two to four doses for each of the standard and sample were used. With this method the slope of the dosage-response curve was determined for every test. Results were computed according to the procedure of Bliss (7, 8).

The rats used were all bred and raised in this laboratory. Their care and the ingredients of the diet were not altered from 1930 to 1939.

RESULTS

The Canadian Standards.—Since 1930 a subsidiary standard for neoarsphenamine has been employed in Canada. The use of a supplementary standard in routine assays conserves the limited supply of the International Standard, and if the relative toxicity and activity of the two standards are known it is a simple matter to express the results in terms of the International Standard. The four successive Canadian Standards employed in the past ten years were batches of commercial material, and were not chosen because they represented the least toxic products on the market, but because they were believed to resemble most nearly the International Standard in their toxicity and physical properties.

The toxicity and trypanocidal value of the Canadian Standards have been determined in relation to the International Standard and the results are shown in Tables I and II.

The toxicities of lots 330, 133 and 336 shown in Table I, approximated 100 per cent, while that of 838 was definitely higher. These figures are weighted mean toxicities obtained from several assays performed on each standard over a period of from two to three years. The limits of error, within which the relationship to the International Standard is known, are given in column 3 of Table I and were computed according to Irwin (9). These limits of error are maximal since, for example, it can be shown to be statistically improbable that Canadian Standard 838 is less than 106 per cent or more than 116 per cent as toxic as the International Standard. The value reported in the table is, of course, the most probable value and is the proper one to use in calculating the toxicity of samples.

The fact that lot 838 is 111 per cent as toxic as the International Standard does not invalidate its usefulness as a comparative standard. A factor of 111 per cent is just as serviceable as one of 100 per cent, except that it necessitates another step in the calculation of results.

In Table II the average trypanocidal activities of only two of the Canadian Standards are shown, since the method used for determining therapeutic potency (4) had not been developed when standards 330 and 133 were employed. The potencies of the standards have been determined by capacity to clear the blood of trypanosomes in a five-hour period and also to cure trypanosomiasis when judged by an observation period of five weeks. The figures given are weighted means and the limits of possible error were calculated as for toxicity (9). It will be noted that the activity of the Canadian Standard determined by negative smears is four or five per cent less than by survivals. This difference was also obtained with most of the commercial samples tested, and could have been due to some characteristic of the International Standard.

Table I.—Toxicity of Canadian Standards for Neoarsphenamine in Terms of the International Standard

Standard Lot No.	Toxicity as Per Cent of International Standard	Maximum Limits of Error from True Value, as Per Cent when $P = 0.95$
330	103	±9
133	99	±8
336	97	±4
838	111	±5

Table II.—Trypanocidal Activity of Canadian Standards for Neoarsphenamine in Terms of the International Standard

Standard	Activity as Per Cent of International Standard: Negative Smears[c]	Activity as Per Cent of International Standard: Cures[d]	Maximum Limits of Error from True Value, as Per Cent when $P = 0.95$
336[a]	94		± 6
		99	± 8
Weighted mean	95		± 5
838[b]	78		±12
		82	±10
Weighted mean	80		± 8

[a] Figures obtained by direct comparisons with the International Standard.
[b] Figures obtained by comparison with Canadian Standard 336 and also by comparison with the International Standard.
[c] End point taken as negative blood smears five hours after injection of neoarsphenamine.
[d] End point taken as survival for five weeks after injection of neoarsphenamine.

The limits of error for the determinations of trypanocidal activity are greater than for toxicity because the inherent error of the method is greater, largely due to the more gradual slope of its dosage-response curve (Tables VI and VIII).

The weighted mean of the results in which both smears and survivals are included is also given. For routine assays it does not matter greatly which end point is employed, but it is better, particularly when comparing standards, to use the average from both criteria.

The Toxicity and Trypanocidal Activity of Market Samples.—Table III shows the toxicity of some market samples of neoarsphenamine. Two hundred assays are reported in all, and the products of manufacturers in England, France, the United States, Germany and Canada are included.

The figures given in column 3 of the table are the average toxicities of the different batches of neo-

arsphenamine submitted by the manufacturers for test. The standard deviation represents the variations of the individual determinations from the mean, and includes the errors of the method as well as the variation in the toxicity of different batches. The error of a toxicity assay as measured by its standard error when 40 to 50 rats are used is about ±6 per cent,[1] and is about equal to the variation in the results shown for products of manufacturers 1, 2, 3 6 and 7. It appears, therefore, that the toxicity of batches produced by these companies is quite uni'orm. Variability is apparent, however, 'n the products of manufacturers 4, 5, 8 and 9 since the standard deviation of the individual results exceeds the standard error of the method.

Table III.—Toxicity of Neoarsphenamine Offered for Sale in Canada

Manufacturer	Number of Determinations	Average Toxicity as Per Cent of International Standard	Standard Deviation
1	16	80	7.9
2	34	85	6.9
3	32	85	7.3
4	5	90	13.1
5	49	91	13.0
6	6	100	8.5
7	11	102	5.8
8	20	103	10.3
9	27	115	10.1

In Table IV the average trypanocidal activity of neoarsphenamine from four manufacturers is shown. The limited number of samples assayed in each case makes it impossible to show that the standard deviations are significantly greater than could be anticipated by the error of the method, which is about ±11 per cent[2] when 40 to 50 rats are used, in contrast to the ±6 per cent for toxicity tests. For this reason it cannot be said categorically that there was a variation in the activities of the different batches of any brand.

Table IV.—Trypanocidal Activity of Neoarsphenamine Offered for Sale in Canada

Manufacturer	Number of Determinations	Average Activity as Per Cent of International Standard: Negative Smears[a]	Cures[b]	Standard Deviation
3	10	138		19.6
	7		140	10.3
7	7	96		17.2
	4		100	7.9
9	13	81		8.5
	8		97	11.9
5	15	72		8.9
	12		77	10.4

[a] End point taken as negative blood smears five hours after injection of neoarsphenamine.
[b] End point taken as survival for five weeks after injection of neoarsphenamine.

[1] Computed by equations 39 and 42 (9) for responses between 20 and 80 per cent.
[2] Computed by equations (39) and (42) (9) for responses between 20 and 80 per cent.

In Table V the therapeutic ratio and the therapeutic index for four brands of neoarsphenamine and two of the Canadian Standards are given. The therapeutic ratio is the average activity divided by the average toxicity, when both values are given as per cent of the International Standard. The therapeutic index (or curative ratio) is the ratio of the dose which killed 50 per cent of the rats to the dose curing 50 per cent of those infected with trypanosomes, and is a factor customarily used for assessing the usefulness of drugs. The therapeutic index may vary from colony to colony, depending on the resistance of the rats and trypanosomes to neoarsphenamine, but the therapeutic ratio, which depends only on the relationship of the sample to the International Standard, will be constant despite colony variations in resistance. The therapeutic ratios of the 5 brands of neoarsphenamine were found to vary from 0.76 to 1.64 and these differences emphasize the superiority of some brands of neoarsphenamine when judged by the biological tests used in this laboratory.

Table V.—Ratio of Trypanocidal Activity to Toxicity of Some Brands of Neoarsphenamine Sold in Canada

Manufacturer	Average Toxicity as Per Cent of International Standard	Average Activity[a] as Per Cent of International Standard	Therapeutic Ratio: Average Activity / Average Toxicity	Therapeutic Index
3	85	139	1.64	27
7	102	98	0.96	20
5	91	74	0.81	18
9	115	87	0.76	15
Canadian Standard 336	97	95	0.98	
Canadian Standard 838	111	80	0.72	

[a] The weighted mean of results given in Table IV for both end points.

The Slope of the Dosage-Response Curves and the Design of Tests.—Dosage-response curves for both the toxicity and trypanocidal tests have already been published (2, 4). After 1938 all of the animals used for routine tests were the offspring of brother-sister mating, and it was desirable to determine what effect had been produced by closer inbreeding on the slope of the dosage-response curve. Actually this type of breeding was deliberately adopted in an attempt to steepen the curve, and thus increase the accuracy of tests with a given number of animals (4). Seventy-three assays of the three-dose type were carried out during 1938–1939 and the slope and position of the regression lines computed by the usual methods (7, 8). Since there was no significant difference in their slopes,[3] the lines from individual

[3] Chi-square for b determined from equation (20a) (8).

tests were combined to produce the figures for the composite curves shown in Table VI. This table also compares the slope of the combined lines obtained in 1931–1932 from random-mated rats, with those from sib-mated animals in 1938–1939. The inbreeding has produced a slight increase in slope which, although not significant, does indicate that there is less individual variation in the response to neoarsphenamine.

Table VI.—Slope of the Regression Lines which Show Dosage-Mortality Relationship for the Determination of the Toxicity of Neoarsphenamine Using Albino Rats

Date	Type of Mating	b Slope of Line Weighted Mean	Standard Error	Material
1931–1932	Random	13.7	0.79	Standards and samples
1938–1939	Brother-Sister	15.3	0.93	Standards and samples
1938–1939	Brother-Sister	15.0	1.60	Standards only

Table VII.—Difference in the Slope of the Regression Lines for the Toxicity of Neoarsphenamine Due to the Sex of the Rats

Date	Sex	Type of Mating	b[a] Slope of Line Weighted Mean	Standard Error
1931–1932	Males	Random	13.0	0.95
1931–1932	Females	Random	15.4	1.40
1938–1939	Males	Brother-Sister	13.7	1.68
1938–1939	Females	Brother-Sister	16.0	1.11

[a] Results in each group were obtained from both standards and samples.

Table VIII.—Slope of Regression Lines Showing Dosage-Response Relationship in the Determination of the Trypanocidal Value of Neoarsphenamine Using Albino Rats

Sex of Rats	Type of Response	b Slope of Line Weighted Mean	Standard Error	Type of Breeding
Females	Smears[a]	11.3	1.11	Random 1935–1936
	Survival	6.2	0.60	
Males	Smears[a]	7.5	0.48	
	Survival	10.4	0.62	
Females	Smears[a]	8.2	0.79	Brother-Sister 1938–1939
	Survival	7.9	0.98	
Males	Smears[a]	8.2	0.61	
	Survival	10.5	0.80	
For brother-sister mating; all tests. Weighted mean		8.7	0.44	

[a] The negative smears end-point.

The figures given in Table VII show the influence of sex on the slope of these curves. Female rats are more uniform in their reaction to neoarsphenamine than males, but the difference is slight.

In Table VIII the regression coefficients for the trypanocidal activity obtained in 1935–1936 are compared with those from the sib-mated rats in 1938–1939. There is a slight but not significant increase for the sib-mated rats in all except the females with negative smears. Since no significant difference[4] can be shown in the four values for b determined in 1938–1939, a weighted mean for all tests has been computed and found to be 8.7 $\pm$0.44.

DISCUSSION

Assays of Commercial Samples.—The toxicities of the products of nine different manufacturers averaged from 80 to 115 per cent of the International Standard (Table III), and there can be no doubt that some neoarsphenamines are better than others as judged by these tests. There is no real difference in the average toxicities of the brands 1 to 5, but those of 6 to 9 are significantly greater. In fact, the average toxicity of the product of manufacturer 9 approaches the upper limit approved by the National Institute for Medical Research (3).

Another important point in estimating the reliability of the products is the uniformity of different batches. This is indicated by the size of the standard deviations shown in Table III. Here again the products of manufacturers 1, 2 and 3 were among the best. There could have been little real variation in the toxicity of batches submitted by these manufacturers since the error of the method would allow an apparent difference of $\pm$6 per cent in determination on the same material. On the other hand, manufacturers 4, 5, 8 and 9 were not so successful in producing neoarsphenamine with uniform toxicity. The evidence in Table III indicates that brands 1, 2 and 3 are definitely superior in that their toxicity is low and there is little variation from lot to lot.

Neoarsphenamine having a low toxicity need not necessarily have a low trypanocidal activity. Most of the brands assayed in this laboratory which showed a low toxicity had a high trypanocidal potency, and the most toxic were usually, although not invariably, the least active. This relationship is shown by the figures of Tables III and IV. Manufacturer 3 prepared a product

[4] See footnote No. 3, page 35.

with uniformly low toxicity and it was at the same time the most potent of those examined. In addition, one batch of neoarsphenamine from manufacturer 1 had a therapeutic activity of 120 per cent and a toxicity of 79 per cent of the International Standard. The most toxic product examined was that of manufacturer 9, and its activity was among the lowest. Number 7 stood in an intermediate position both as to toxicity and trypanocidal activity, being practically equivalent to the International Standard in both. The Canadian Standards also showed this relationship; Lot No. 838 was more toxic and less active than Lot No. 336 (Tables I and II).

It should be noted that neoarsphenamine 5 is an exception, and although relatively non-toxic has, on the average, a trypanocidal activity less than 80 per cent of the International Standard, which is lower than that recommended as a minimum (3).

Methods of Assay.—Single-dose and multiple-dose variations of the methods for toxicity and trypanocidal activity have been employed in this work. The advantages and disadvantages of each of these methods of arranging the doses have been discussed elsewhere (4, 5, 10). The theoretical basis for the argument for the use of the one dose method centers largely around whether or not the slope of the dosage response curves remains constant. In routine assays the figures obtained for the slope will differ from test to test, because the individual variation in the whole colony of rats to the treatment cannot be determined exactly from the necessarily small samples of animals used each time. However, it is possible to decide whether or not the differences observed in the values of b computed from the individual assays are due only to this sampling variation (8). The necessary computations for this purpose have been done with the regression lines used in this work and, as no significant differences could be shown among them, they were combined to give composite regression lines with the slopes shown in Tables VI, VII and VIII.

These values for b, acquired from a large number of animals and separate tests, are much closer approximations to the true values for our colony than those obtained with the few rats used in each routine test. The error contributed by uncertainty in the determined value of the slope is consequently reduced by using the composite b (Tables VI, VII and VIII) in calculating the results of assays.

The use of one dose and a composite b is satisfactory in these methods since the slopes cannot be shown to change significantly from time to time. The simplicity of the one-dose technique, particularly in the calculation of results, is a strong recommendation in its favor, especially in routine assays where a large number of tests must be done each week (10). It is not advisable, however, to adopt this method until experience gained with the animals and drugs used has indicated that it is feasible, and it should not be employed when investigating new substances or in research problems.

Steeper dosage-response curves yield more accurate assays. They imply less individual variation in the test animals, and every effort consistent with economy of labor and material, should be made to increase their slopes. Individual variation may be reduced by maintaining a satisfactory and constant environment, and by close inbreeding.

Since the environment appeared to be satisfactory and could be kept constant, an attempt was made to increase b by closer inbreeding of the rats. It was hoped that the results would indicate the influence of the genetic factor in individual variation to a drug. The same colony of rats was employed as in 1931–1932 together with the same diet and care. Matings, however, were all brothers to sisters and the figures for 1938–1939 reported in this paper were obtained with rats which had been inbred for at least six successive generations, and whose origin could be traced to one pair. Tables VI and VII show that there was a slight increase in b which, however, could not be proved to be statistically significant. It was concluded that the accuracy gained did not justify the somewhat greater work necessary for this type of mating.

It is probable, of course, that the rats used

in 1931–1932 had been inbred for some time before they were received in this laboratory in 1928. From then until 1935 they were mated in a random fashion by selecting healthy stock without regard to relationship.

In spite of the failure to significantly increase uniformity in our colony by this means, it has been definitely shown by others that considerable improvement in accuracy results from the use of a carefully bred and tended stock of animals (5, 11).

The slopes of the regression lines for sulpharsphenamine, arsphenamine and mapharsen have been computed for a few assays using male rats in the majority of tests, and are given here as a matter of interest. The average values of b were as follows; for sulpharsphenamine 9 $\pm$2.1, for arsphenamine 10 $\pm$1.3 and for mapharsen 9 $\pm$0.97.

SUMMARY

1. Two hundred tests for the toxicity, and seventy-six tests for the trypanocidal activity of commercial samples of neoarsphenamine are reported. Toxicity and trypanocidal activity are expressed in terms of the International Standard Neoarsphenamine.

2. Differences are shown to exist in the toxicity and activity of the products of different manufacturers.

3. The least toxic neoarsphenamines frequently have the highest trypanocidal activity, as judged by these assays.

4. Some brands are more variable from batch to batch in their toxicity than others.

5. The toxicity of four lots of Canadian Standard Neoarsphenamine, and the trypanocidal activity of two lots are reported.

6. A one-dose method of assay, using curve numbers in calculating results, may be employed with the rats used in this laboratory.

7. The effect of inbreeding on the slope of the dosage-response curve is demonstrated.

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Intravenous Toxicity of Heparin-Sodium Sulfapyridine Combinations and Protective Action of Barbiturates*

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INTRODUCTION

Murray and Best (1) first demonstrated the usefulness of heparin in the prevention of post-operative thrombosis. Since then studies on the use of heparin in subacute bacterial endocarditis have also been reported (2, 3). In this condition it would seem desirable to give one of the newer chemotherapeutic agents simultaneously with the intravenous administration of heparin in order that the blood stream may be cleared of the invading micro-organisms and preventing at the same time further deposition of thrombotic masses on the heart valves. However, no experimental information has thus far been reported indicating the safety of such a procedure.

EXPERIMENTAL

Prior to the clinical use of heparin and sodium sulfapyridine mixtures intravenously in cases of

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