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BIOLOGICAL STANDARDIZATION OF "MSb POLYMER" (SODIUM *p*-MELAMINYL PHENYL STIBONATE)

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The polymer of sodium *p*-melaminyl phenyl stibonate, referred to by Friedheim (1948) as "MSb," was first described by Friedheim and Berman (1946). They observed its pronounced prophylactic effect against *Trypanosoma equiperdum* infections of the mouse, and this was confirmed for *T. rhodesiense* in mice by Rollo, Williamson, and Lourie (1949) and for *T. gambiense* by Le Rouzic (1949a). Williamson (1949) correlated the period of prophylaxis against *T. rhodesiense*, after a single injection of MSb, with the amount of antimony in the viscera. Rollo *et al.* (1949) found that the drug possessed a higher therapeutic ratio than tryparsamide in laboratory infections, and Le Rouzic (1949b) found it possessed therapeutic activity against all stages of *T. gambiense* infections. However, the main virtue of this drug is its prolonged prophylactic action which Rollo *et al.* (1949) found to extend for 41 weeks after a single injection to mice.

We first became interested in the biological standardization of MSb when it was required by the Colonial Office for clinical trial in Africa. Since the polymers are not a single chemical entity, and some variation can occur between batches, biological standardization was necessary. Because the chief use of the substance was for prophylactic treatment it was more appropriate to have a prophylactic than a curative laboratory test for trypanocidal potency. But tests involving a long delay are quite impracticable for routine purposes. Lourie (private communication) suggested an assay extending over a period of four weeks, and it was with this suggestion in mind that we elaborated a prophylactic test. In its final form, this is complete in only two weeks. We considered it advisable to standardize the product biologically as completely as possible, and consequently included toxicity and therapeutic determinations. In this paper the symbol MSb refers to the sodium salt of the polymer only and not to the monomer: the monomer is relatively inactive and possesses no prophylactic activity against *T. rhodesiense* in mice.

METHODS

Toxicity and Therapeutic Experiments.—Fawn mice, 13 to 15 g. body weight, from our inbred laboratory stock, which were deprived of food overnight and fed two hours before injection, were assigned at random to groups consisting of 10, or more usually 20. animals. In the toxicity experiments deaths were recorded for seven days after a single subcutaneous injection.

In the therapeutic experiments mice were infected intraperitoneally with *T. equiperdum*, and on the following day those showing an infection of 1 to 10 trypanosomes per high power microscope field were injected subcutaneously with the drug. Blood smears from the tail were examined on the fifth day, since the maximum response was found from preliminary experiments to occur then.

The graphical method of de Beer (1945) was used to evaluate the results in toxicity and therapeutic experiments.

Prophylactic Experiments.—Twenty fawn mice, of 15 to 20 g. body weight, were used on each dose of a three-dose experiment, each mouse receiving a single subcutaneous injection of the laboratory standard (sample 1) of MSb. One week later, each mouse was infected intraperitoneally with 0.2 ml. of a suspension of *T. equiperdum*, which contained approximately 40.000 trypanosomes. A blood smear from the tail of each mouse was examined one week after infection and the number of mice free of trypanosomes was recorded. Similar groups were treated with pentamidine isethionate in order that a comparison could be made with a trypanocidal drug of well-defined chemical structure. The whole experiment was repeated until sufficient data for statistical analysis had been obtained.

Solutions.—The sodium salt of the polymer dissolved in water gave a cloudy solution. Since weak solutions were unstable and could not be prepared directly, a 6% w/v solution of MSb was prepared initially by mixing solid MSb and urea in the proportion of 3:4 and adding sufficient water at about 37° C. to give a clear solution; this solution was then diluted with sufficient water to give the desired concentration of MSb. As the solutions were relatively unstable, either precipitation or turbidity occurring after several days at room temperature, all solutions were used

within 20 min. of preparation, the toxicity and therapeutic tests being carried out simultaneously.

The arbitrary laboratory standard (sample 1) gave cloudy solutions and precipitated overnight at a concentration of 6% w/v, but at 3% w/v in 4% w/v aqueous urea the solution was quite clear; the other samples used gave clear solutions at 6% w/v and no precipitation occurred overnight.

Results

Toxicity Experiments

Most of the deaths, after a single subcutaneous injection of MSb, occurred within four days, although the animals were observed for seven days, and the mortalities at this time were used to assess the results. In order to cover the range of toxic doses at least four doses of both the laboratory standard and test sample were injected, using a dose ratio of 1.25. Usually 20 animals received each dose. The results are shown in Table I.

TABLE I

MSb POLYMER—ASSAY IN MICE OF SUBCUTANEOUS TOXICITY OF LABORATORY STANDARD (SAMPLE 1) AND OF UNKNOWN SAMPLES IN TERMS OF THIS STANDARD

Sample No.	Expt. No.	No. of Mice	LD50 (mg./kg.)	% Limits (P=0·05)	b	Potency	% Limits (P=0·05)
1	1	60	1,700	88·5–113·0	8·2		
	2	70	2,600	89·5–112·0	7·7		
	3	80	2,800	87·5–114·0	5·4		
	4	70	2,170	90·5–110·5	7·7		
	5	70	2,200	89·0–112·5	6·3		
	6	80	1,520	87·0–115·0	5·4		
	7	80	1,900	86·5–116·0	4·6		
	7*	80	2,040	92·0–109·0	8·7	1·10	86·0–116·0
2	3	100	2,510	91·0–110·0	6·7	1·11	85·5–117·0
3	4	90	2,790	88·5–113·0	5·6	0·81	86·0–116·5
4	5	90	2,580	90·0–111·5	6·3	0·86	85·0–117·5
5	6	100	2,120	83·5–119·5	3·4	0·66	80·5–124·0

* = Bulk standard as opposed to ampouled standard.

χ^2 tests for linearity of the regression lines (first approximation) and for homogeneity of the various estimates of slope were applied. These tests showed that the responses in any one regression line did not deviate significantly from linearity, and that the slopes of the regression lines obtained on various occasions with the laboratory standard and with the different test samples were homogeneous.

Therapeutic Experiments

Preliminary experiments, using a total of 280 mice, were carried out to investigate the effect of urea in the solutions against both *T. rhodesiense* and *T. equiperdum*. It was found that the addition of urea had no consistent effect upon the response of the trypanosomes. Urea was therefore used in all subsequent therapeutic tests as its presence was essential for the satisfactory preparation of the stronger solutions used in the toxicity experiments. The difference between the responses of the two species of trypanosome was just significant ($P \ll 0.05$). *T. equiperdum* was used in all later work because this organism has been extensively used for the biological standardization of organic arsenicals and trypanocides.

In the therapeutic experiments (3 + 3) dose assays were used with 20 infected animals on each dose. The results are shown in Table II. Again none of the regression lines deviated significantly ($P > 0.05$) from linearity; the various lines obtained with the standard formed a homogeneous series, as did those of the various test batches.

TABLE II

MSb POLYMER—ASSAY IN MICE OF THE EFFECTIVE POTENCY OF LABORATORY STANDARD (SAMPLE 1) AND OF UNKNOWN SAMPLES IN TERMS OF THIS STANDARD USING *T. EQUIPERDUM*. SAMPLES INJECTED SUBCUTANEOUSLY

Sample No.	Expt. No.	No. of Mice	ED50 (mg./kg.)	% Limits (P=0·05)	b	Potency	% Limits (P=0·05)
1	1	60	10·50	93·0–107·5	12·0		
	2	60	9·60	89·5–112·0	7·6		
	3	60	14·50	92·0–108·5	11·1		
	4	60	9·05	89·0–112·0	7·8		
	5	60	7·65	85·5–117·0	6·0		
	6	70	8·00	88·0–114·0	6·5		
	6*	70	8·00	88·0–114·0	6·5	1·00	83·5–120·0
2	2	60	8·60	88·5–113·0	7·6	1·12	84·5–118·5
3	3	60	14·30	92·5–108·0	11·7	1·01	89·0–112·0
4	4	60	10·25	92·0–108·5	11·0	0·93	87·5–114·0
5	5	60	10·35	93·0–107·5	12·5	0·92	87·0–115·0

ED50 = Dose which cleared trypanosomes from peripheral bloodstream in 50% of mice after five days.
* = Bulk standard as opposed to ampouled standard.

Prophylactic Experiments

The results of these experiments are shown in Table III. Untreated, infected control mice died within five days. Groups of animals were similarly tested with pentamidine isethionate, in order to test the method with a prophylactic drug of definite chemical structure.

The data from the five separate experiments (Table III) were adequate to warrant a thorough statistical analysis by the method of maximum

TABLE III

MSb POLYMER AND PENTAMIDINE ISETHIONATE. SUBCUTANEOUS PROPHYLACTIC EXPERIMENTS.

Mice infected with *T. equiperdum*

	MSb Polymer						Pentamidine Isethionate					
Dose (mg./kg.)	10·6	7·5	5·3				4·24	3·00	2·12			
Expt. No.	Mice Cleared of Trypanosomes on 7th Day/Mice Used			Slope (b)	PD50 (mg./kg.)	% Fiducial Limits (P=0·05	Mice Cleared of Trypanosomes on 7th Day/Mice Used			Slope (b)	PD50 (mg./kg.)	% Fiducial Limits (P=0·05)
1	17/20	12/20	6/16	4·49	6·36	59·3–119·8	14/20	13/20	9/20	2·15	2·27	
2	16/20	8/20	2/20	7·06	8·09	88·3–115·5	—	—	—	—	—	—
3	—	—	—	—	—	—	17/20	13/20	1/20	8·48	2·97	89·2–111·8
4	20/20	16/19	8/20	9·06	5·69	86·8–115·1	18/20	15/20	4/20	7·33	2·63	85·9–112·9
5	17/20	6/20	0/20	11·39	8·49	91·2–110·4	18/20	14/20	6/20	6·08	2·54	81·1–115·0
	$b_c = 7·06$ Slopes $\chi^2_{[3]} = 6·739$ (P=0·05–0·10)						$b_c = 5·49$ Slopes $\chi^2_{[3]} = 10·148$ (P=0·01–0·02) Omitting Expt. No. 1: $b_c = 7·22$ Slopes $\chi^2_{[2]} = 1·007$ (P=0·50–0·70)					

PD50 = median protective dose (see text).

Potency of MSb polymer in terms of pentamidine isethionate based on experiments 4 and 5 (maximum likelihood method):
Potency = 37.55%.
5% fiducial limits = 33.31-42.34%.

likelihood. In no experiment was there a significant curvilinear component in the regression line (P>0.05). But a χ^2 test on the variation in slope encountered with pentamidine isethionate showed a significant heterogeneity. This was attributable to the results in Experiment 1. Omitting this experiment the common slope was 7.22. None of the regression lines obtained with MSb showed a significant departure from rectilinearity (P>0.05), and the various estimates of slope were homogeneous. The common slope was 7.06. The potency of MSb in terms of pentamidine isethionate, based on experiments 4 and 5, was 37.55%, the fiducial limits (P=0.05) of this estimate being 33.31% to 42.34%.

DISCUSSION

In drafting biological standards for the quality control of samples of MSb, the following recommendations may be made:

Toxicity Test.—A toxicity test based on Gaddum's method (1943) could be used. The weighted arithmetic mean of the slope of the regression line is low (b=5.83), and a test using a dose ratio (S/U) of 1.25 with 20 animals on "standard" and "unknown" is fairly lenient (Table IV) although it would serve to eliminate any very toxic batches. On the other hand there are objections to this (1+1) dose assay (Perry, 1950), and it is better to use a (2+2) dose assay. A suitable dose ratio would be 1.8, and the results could be calculated by one of the recognized procedures.

Therapeutic Test.—For the therapeutic test, the (1+1) dose assay (Hawking, 1943) is slightly more stringent, because the common regression coefficient is higher (b=8.58). However, since the therapeutic test is just as, if not more, important than the toxicity test, a (2+2) dose assay should be adopted, with 20 animals receiving each dose. A suitable dose ratio would be 1.5 and the potency could be calculated by any suitable method.

TABLE IV

PERCENTAGE BATCHES WHICH WOULD FAIL A TOXICITY TEST IN A (1+1) DOSE ASSAY

n=20, b=5·83

S/U	Percentage Toxicity					
	100	110	120	130	140	150
	Percentage Failures					
1·15	16·5	34	53	70	83	91·5
1·20	12	26	45	62	77	87·5
1·25	8	19	35	53	69	81·5
1·30	5	13	27	44	60	74

Prophylactic Test.—A (2+2) dose assay for the prophylactic testing of MSb, using 20 animals on each dose, is adequate since the log. dose-probit regression line is rectilinear. A suitable dose ratio would be 1.8. The "PD50" would be defined as that dose which, when given subcutaneously one week before the intraperitoneal injection of a standard number of trypanosomes, prevented the appearance of trypanosomes in the peripheral blood in 50% of the mice for a further week; and the potency could be calculated by any suitable method.

Though Lourie suggested to us a prophylactic test extending over a period of four weeks, we have used one lasting only two weeks. Since neither procedure gives information on the duration of prophylaxis that might be expected, the shorter test possesses advantages as a routine assay.

Summary

1. The conditions for carrying out assays in mice for toxicity, therapeutic, and prophylactic potency against *T. equiperdum* using the polymer of sodium *p*-melaminyl phenyl stibonate (MSb) are described.

2. Statistical analysis showed that the regression lines in each of the three types of assay were without significant departure from rectilinearity, and the lines obtained in each type of test formed a homogeneous series.

3. For testing toxicity, a (1 + 1) dose assay could be used with a dose ratio (S/U) of 1.25; but a (2 + 2) dose assay is preferable, with a dose ratio of 1.8. For the therapeutic and prophylactic tests, a (2 + 2) dose assay ought to be employed, with dose ratios of 1.5 and 1.8 respectively. In each test 20 mice per dose should be used.

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References

de Beer, E. J. (1945). *J. Pharmacol.*, **85**, 1.
Friedheim, E. A. H. (1948). Communication to International Trypanosomiasis Conference, Brazzaville: February, 1948.
—— and Berman, R. L. (1946). *Proc. Soc. exp. Biol., N.Y.*, **62**, 131.
Gaddum, J. H. (1943). *Quart J. Pharm.*, **16**, 78.
Hawking, F. (1943). Ibid., **16**, 13.
Perry, W. L. M. (1950). *The Design of Toxicity Tests*, London: H.M.S.O.
Le Rouzic, J. (1949a). *Bull. méd. A.O.F.*, No. Spec. 53.
—— (1949b). Ibid., No. Spec. 63.
Rollo, I. M., Williamson, J., and Lourie, E. M. (1949). *Ann. trop. Med. Parasit.*, **43**, 194.
Williamson, J. (1949). *Ann. trop. Med. Parasit.*, **43**, 209.