A COMPARISON OF THE RESULTS OF FOUR *IN VITRO* ANTHELMINTIC TESTING TECHNIQUES

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Introduction

During the last decade the amount of interest in *in vitro* testing of compounds for anthelmintic activity has increased considerably, mainly because the need for better anthelmintics has become more and more evident, and to find them large numbers of compounds must be screened. Undoubtedly *in vitro* tests are fundamentally less satisfactory than are *in vivo* tests; however, a combination of several techniques obviates some of the objections, and allows more chemicals to be screened, on a preliminary basis, than would *in vivo* trials against a similar number of nematodes or platyhelminths. In all preliminary screening trials it is essential that the compounds should be tested for anthelmintic effect against several different helminths, because a compound may have no anthelmintic effect on some worms but be effective against others. It is also important, of course, when compounds are tested that none with anthelmintic activity should be missed in the preliminary screening. Furthermore, *in vitro* testing of compounds for anthelmintic activity has the advantage over *in vivo* testing in that compounds which would be lethal to the host can be compared, and, therefore, that data can be obtained on chemical structures inducing anthelmintic properties, which would be unobtainable from *in vivo* tests.

This paper compares the results obtained by four of the best known *in vitro* techniques with 52 compounds; it also gives the results from some known anthelmintics when they were tested *in vitro*.

Material and Methods

The *in vitro* techniques compared are those which employ the free-living stages of sclerostomes[1–6] (a technique which was originally designed to find compounds suitable for use against the pre-parasitic stages), sections of *Ascaris lumbricoides*[7], liver fluke (*Fasciola hepatica*)[8] and vinegar eelworms (*Turbatrix aceti*)[9]. In the technique which used the free-living stages of sclerostomes, the compounds were added to fresh horse fæces, and, after culturing, the numbers of larvæ from the treated fæces were compared with the numbers from the untreated fæces. Compounds were tested against *A. lumbricoides* and *F. hepatica* by attaching the anterior portion of the former and the whole of the latter to a lever, and recording the changes in their movements on a kymograph when the helminths were immersed in an aqueous solution, emulsion or suspension of the compound. The technique with *T. aceti* consisted of adding vinegar containing all ages of vinegar eelworms to an equal quantity of an aqueous solution, emulsion, or suspension of the compound, and observing the effect on the vitality of the eelworms.

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Most of the results quoted in this paper have already been published[7,8,10–14], but they are recorded in several journals and this is the first time that the anthelmintic effect of the compounds on the different helminths has been compared.

TABLE I

SHOWING THE LARGEST NUMBER OF PARTS OF FÆCES OR OF WATER WITH WHICH ONE PART OF THE COMPOUND WAS MIXED AND AN ANTHELMINTIC EFFECT WAS OBSERVED ON THE FREE-LIVING STAGES OF SCLEROSTOMES, ON VINEGAR EELWORMS (*T. aceti*), ON *A. lumbricoides* AND ON LIVER FLUKE (*F. hepatica*) *in vitro*

Compound	Free-living sclerostomes; concentration in fæces to kill 90 per cent.	Vinegar eelworms; concentration to kill 50 per cent.	*A. lumbricoides*		Liver fluke	
			Concentration	Effect	Concentration	Effect
Methyl iodide	50,000	16,000	1000	P	1000	L
Ethyl iodide	6700	1000	500	P	500	L
n-Propyl iodide	300	2000	500	P	1000	L
Methylene iodide	25,000	2000	2000	P	2000	P
Iodoform	16,700	16,000	1000	D	1000	SD
Carbon tetrabromide	5000	4000	2000	P	6000	L
					10,000	P
Allyl chloride	1000	2000	1000	D	1000*	N
Allyl bromide	14,000	4000	1000	P	5000	L
Allyl iodide	83,000	32,000 (24)	5000	P	5000	L
Allyl *iso*thiocyanate	100,000	32,000 (1)	4000	P	2000	L
					8000	P
Mercuric chloride	12,500	32,000 (24)	1000	SD	20,000	L
Ethyl mercuric chloride	25,000	32,000 (3)	2000	P	20,000	L
Ethoxyethyl mercuric chloride	16,700	32,000 (24)	1000	SD	16,000	L
Urea	200	U	1000*	N	1000*	N
Chlorobenzene	900	2000	1000	P	2000	L
Bromobenzene	1100	U	1000	P	2000	L
Iodobenzene	900	2000	1000	SD	3000	L
o-Dichlorobenzene	900	U	1000	P	1000	L
p-Dichlorobenzene	800	U	1000	D	1000	SD
Benzene hexachloride α-isomer	NE	U	1000*	N	1000*	N
,, ,, β-isomer	NE	U	1000*	N	1000*	N
,, ,, γ-isomer	NE	U	1000*	N	1000	D
,, ,, δ-isomer	NE	U	1000*	N	1000	L
					12,000	P
2:3:5:6-Tetrachloronitrobenzene	6	U	1000	D	1000*	N
Aniline	3300	U	1000*	N	1000*	N
Diphenylamine	8	32,000 (24)	1000	P	20,000	L
γ-Nitrophenol	400	4000	3000	P	1000	P
p-Nitrophenol	1000	U	1000	P	4000	L
					12,000	P
Thymol	100	2000	5000	P	10,000	L
4-*n*-Hexylresorcinol	16	8000	10,000 to 5000	P	10,000 to 5000	L
Gentian violet	80	16,000	2500*	N	5000	L
Essential oil ex *Artimisia maritima*, containing 65 per cent. β-thujone and 16 per cent. cineol-1:8	1000	U	1000	SD	2000	L
					3000	P
2-Mercaptobenzothiazole	3	1000	1000	SD	1000	L
					3000	P
Pyridine	600	U	5000	P	1000*	N
α-Picoline	1400	U	5000	P	1000	P
β-Picoline	900	U	1000	P	1000*	N
2:6-Lutidine	2000	U	2000	P	1000	SD
Quinoline	1100	U	1000	P	1000	SD
2:3-Dihydro-3-ketobenzo-1:4-thiazine	200	16,000	1000	D	2000	P
6:7-Dimethoxy-2:3-dihydro-3-ketobenzo-1:4-thiazine	2	U	1000	D	4000	P
6-Amino-2:3-dihydro-3-keto-benzo-1:4-thiazine	NE	U	1000	D	1000	P
Hydrochloride of 6-Amino-2:3-dihydro-3-ketobenzo-1:4-thiazine	13	U	1000*	N	3000	P
6-Chloro-2:3-dihydro-3-keto-benzo-1:4-thiazine	NE	16,000	1000	SD	8000	P
Phenothioxin	80	32,000 (24)	1000*	N	1000	P
Phenothiazine	3300	32,000 (48)	1000*	N	1000*	N
10-Acetyphenothiazine	NE	U	1000*	N	1000*	N
Phenothiazone	2000	32,000 (72)	10,000	P	8000	L
					16,000	P

TABLE I (*continued*)

Compound	Free-living sclerostomes; concentration in fæces to kill 90 per cent.	Vinegar eelworms; concentration to kill 50 per cent.	*A. lumbricoides*		Liver fluke	
			Concentration	Effect	Concentration	Effect
Thionol	NE	U	1000	N	1000	P
Phenothiazine sulphoxide ..	250	U	1000	SD	4000	P
Lauth's Violet	27	U	1000*	N	1000	D
Methylene blue	40	8000	1000*	N	2000	L
					3000	P
Arecoline hydrobromide ..	1700	2000	1000	P	10,000,000	P

NE = no lethal effect produced. U = over 50 per cent. were still alive after a week in 1 : 1000 concentration. N = little or no effect produced. P = paralysant. SD = strong depressant. D = depressant. L = lethal

In parentheses, the time taken to kill in hours when the concentration was lethal in less than a week.

* The most concentrated preparation which was tested.

A solution, emulsion or suspension of 1 : 1000 was the most concentrated preparation used against vinegar eelworms, *A. lumbricoides* and liver fluke, except that two compounds were tested at concentrations of 1 : 500 against *A. lumbricoides* and liver fluke.

RESULTS

Table I compares the anthelmintic effect of 52 compounds on the free-living stages of sclerostomes, on vinegar eelworms, on *A. lumbricoides* and on liver fluke.

In the column showing the effect of the compounds on sclerostomes the minimum concentration of compound to fæces which killed over 90 per cent. of the pre-parastic stages is shown. Some compounds were most effective when applied in the dry state or undiluted with water, and other compounds were most effective when applied in dilute, or moderately concentrated, or saturated aqueous solutions. When the compound produced no lethal effect it is shown by the letters "NE."

The effect of the same compounds on vinegar eelworms is shown by stating the minimum concentration which killed at least 50 per cent. in less than a week. The minimum concentration used was 1 : 32,000. When a compound at this concentration was lethal in less than a week, the time taken to kill the nematodes is shown in hours in parentheses. When over 50 per cent. were still alive after a week in a 1 : 1000 concentration it is indicated by a "U."

In the columns showing the effect on the anterior preparations of *A. lumbricoides*, the minimum concentration which had an effect in less than 30 minutes is given, and the extent of this effect is indicated as follows:—P, paralysant; SD, strongly depressant; D, depressant; N, little or no effect, and the most concentrated preparation which was tested is shown by an asterisk.

The effects of the compounds on liver fluke after 45 minutes are shown in the final column of Table I. The same symbols are used and in addition "L" signifies that the compound was lethal.

Table I includes some known anthelmintics; some others have been tested by one or more of the *in vitro* techniques; the results are given in Table II.

TABLE II

EFFECT OF SOME KNOWN ANTHELMINTICS, WHICH ARE NOT INCLUDED IN TABLE I, ON THE FREE-LIVING STAGES OF SCLEROSTOMES, *A. lumbricoides* AND LIVER FLUKE *in vitro*; THE SAME METHOD AND LETTERS ARE USED TO SHOW THE EFFECTS AS WERE USED IN TABLE I

Compound	Sclerostrome larvæ; concn. in fæces to kill 90 per cent.	*A. lumbricoides*		Liver fluke	
		Concentration	Effect	Concentration	Effect
Nicotine sulphate 40 per cent.	350	—	—	—	—
Nicotine	—	2000 to 1000	P	200,000	P
Carbon tetrachloride	80	2000 to 1000	P	1000	L
Oil of Chenopodium	1000	5000	P	5000	L
" " "	—	—	—	20,000 to 10,000	P
Copper sulphate	23	—	—	—	—
"Iodine vermicide"	1250	—	—	—	—
Turpentine	180	—	—	—	—
Powdered areca nut	NE	—	—	—	—
Chloroform	84	—	—	—	—
Hexachloroethane	830	—	—	1000	L
Tetrachloroethylene	320	2000 to 1000	P	1000	L
Toluene	625	—	—	—	—
β-Naphthol	8	5000 to 2000	P	4000 to 2000	L
Carbon disulphide	310	—	—	—	—
Sodium fluoride	1700	—	—	—	—

Some other compounds which have been tested or used occasionally as anthelmintics are included in the tables of Parnell and Mackie.[3]

DISCUSSION

The results emphasise the need for screening compounds against a variety of helminths rather than against only one, as the tables show that the potency of many compounds varies considerably against the different helminths. In tests against sclerostome larvæ a few compounds are more effective against some genera than against others. Similarly, it is, of course, well known that *in vivo* some anthelmintics are very effective against some nematodes, but have little effect on others, and, perhaps, no effect on other helminths.

It appears, however, from Table I that if a compound has no effect on sclerostomes, at 1:100 concentration, or on liver fluke, at 1:1000 concentration, it has, in general, little or no effect against the other helminths *in vitro*, at 1:1000 concentration. If this could be established on a larger scale, much work might be saved in future preliminary screening trials. The tables also suggest that, if the known anthelmintics had been subjected to *in vitro* testing before they were used as anthelmintics, they would not have been rejected during the preliminary screening, with the possible exception of areca nut, which is used only against tapeworms, and was tested only against sclerostomes.

Sclerostome larvæ, although pre-parasitic stages are used, may be particularly valuable for screening, because the greatest need in agricultural helminthology is for an anthelmintic, which will be effective against the immature parasitic stages of bursate nematodes, as well as against the adults, and which will have little, if any, effect on the host. It would be an additional advantage if the anthelmintic were suitable for incorporating in mineral licks or mixtures or in feeding stuffs, so that daily doses could reduce the damage to the host by the immature stages, which at present is usually unpreventable. However, tests to find suitable anthelmintics should, perhaps, use infective or exsheathed infective larvæ, rather than all free-living stages.

Table I suggests that the aliphatic compounds containing iodine or bromine may be particularly effective against sclerostome larvæ. On the other hand, the aromatic halogen compounds are much less potent, except against liver fluke. The volatility of methyl iodide may account for the low values with *A. lumbricoides* and liver fluke, since they were tested in open containers, while the compound acted upon the sclerostome larvæ and vinegar eelworms in covered vessels. Allyl iodide and *iso*-thiocyanate are very effective against all four helminths, and are more potent than allyl bromide or chloride. It is noteworthy that diphenylamine, 4-*n*-hexylresorcinol and gentian violet have so little effect against sclerostomes compared with the other three helminths. The lack of activity of the pyridines and quinoline towards vinegar eelworms, compared with their effect on the other nematodes, is interesting.

A comparison of the benzo-1 : 4-thiazine derivatives indicates that they are all paralysant, but not lethal, against liver fluke; however, 2 : 3-dihydro-3-ketobenzo-1 : 4-thiazine and its 6-chloro-derivative were lethal against vinegar eelworms, whereas only the former was effective against sclerostomes.

Only the sclerostome and vinegar eelworm techniques show phenothiazine to be lethal, whilst phenothiazone is shown to be effective by all four techniques. Few of the other related compounds were outstanding in their *in vitro* anthelmintic potency.

Summary

1. The results of four *in vitro* anthelmintic screening techniques are compared.

2. Only 13 out of the 52 compounds showed distinct anthelmintic activity in all four *in vitro* techniques. Thirty-four of the compounds were effective against free-living sclerostomes at concentrations of 1 : 100 or less; 28 were lethal to vinegar eelworms; 24 caused paralysis in *A. lumbricoides*, and 35 were lethal or paralysant to liver fluke.

3. Some results obtained by known anthelmintics with these *in vitro* techniques are included.

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