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Metabolism of [^{14}C]Amitraz in Larvae of *Boophilus microplus*

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Abstract

Amitraz, 1,5-di(2,4-dimethylphenyl)-3-methyl-1,3,5-triazapenta-1,4-diene, labelled with ^{14}C in the 2-methyl groups was applied to *B. microplus* larvae by an immersion technique. The chemical penetrated readily but never appeared in large amounts internally due to rapid cleavage to *N*-2,4-dimethylphenyl-*N'*-methylformamidine. The expected complementary cleavage product 2,4-dimethylformanilide was not produced in equivalent quantity. However, large amounts of polar metabolite(s) were produced. Small quantities of 2,4-dimethylaniline and an unidentified non-polar metabolite were also produced. Of the identified chemicals only amitraz and *N*-2,4-dimethylphenyl-*N'*-methylformamidine were toxic to larvae. Piperonyl butoxide applied simultaneously with amitraz had only a slight effect on metabolism but had a three-fold synergistic effect. SKF 525-A similarly applied had a negligible effect on both metabolism and toxicity.

Introduction

The acaricide amitraz was released as an alkaline buffered formulation called Taktic in December 1975 for control of the cattle tick in Queensland, Australia. The chemical is highly effective against *B. microplus*, giving >99% control of parasitic ticks when applied at a concentration of 0·025% (w/v) to cattle (Wharton and Roulston 1976). The dual toxic effects produced by this acaricide of (1) causing detachment from the host and (2) outright killing, are the typical responses to formamidine acaricides (Roulston *et al.* 1971; Stone *et al.* 1974).

We undertook this study of [^{14}C]amitraz metabolism in larvae of *B. microplus* in order to gain basic information relevant to possible future resistance mechanisms, and also to increase the prospect of elucidating the still unknown lethal mechanism(s) of formamidines.

Materials and Methods

Radioactive Acaricide

Amitraz (compound I in Table 1) labelled with ^{14}C in the 2-methyl substituents had a specific activity of 1·22 mCi/mmol. It was purified by partitioning an n-hexane solution of the chemical against 0·001 M HCl followed quickly by aqueous sodium bicarbonate and then water. Contact of amitraz with acid media must in general be avoided due to acid instability of the chemical. Under our conditions the final hexane solution when dried with anhydrous sodium sulphate and chromatographed in a paper chromatographic system D, and a t.l.c. system A, contained amitraz with >99% radiochemical purity. Stock solutions were prepared by evaporating the n-hexane and dissolving the radiochemical in a 2% (v/v) solution of Triton X-100 in acetone.

Chromatographic Reference Compounds

A stock solution for ultraviolet visualization of compounds I–IV (Table 1) in t.l.c. sheets was prepared by dissolving the non-radioactive chemicals in acetone to give final concentrations of 4 mg of compound IV and 2 mg of compounds I, II and III per ml of solution. Then 5 μl of this solution were added routinely to samples before volume reduction and chromatography.

Tick Larvae

The unselected, acaricide-susceptible Yeerongpilly larvae were used 14 days after hatching. All stocks of larvae were the progeny of engorged adult female ticks dropped from artificially infested cattle. The adults, eggs and larvae were maintained in ventilated containers at 30°C and 90% R.H.

Dosage of Larvae

Aqueous colloids of ^{14}C or unlabelled chemicals were prepared by injecting 1 volume of stock acetone–Triton X-100 solution of the chemical into 99 volumes of water. Aqueous colloid (1 ml) was then added to 0·2 g larvae in a capped vial which was then shaken by hand for 5 min. The larvae were dried on wads of filter paper and either (1) transferred to ventilated storage vials until required for metabolism assays or (2) subsamples of *c.* 100 larvae were transferred with a small sable brush into filter paper packets and incubated for 24 or 72 h for toxicity tests. All samples were incubated at 30°C and 90% R.H.

Table 1. Formulae of amitraz and related reference compounds

Compound No.	Formula[A]	BTS No.[B]
I (amitraz)	$(R–N=CH)_2N–CH_3$	BTS 27419
II	$R–N=CH–NH–CH_3$	BTS 27271
III	R–NH–CHO	BTS 27919
IV	$R–NH_2$	BTS 24868

[A] R = 2,4-dimethylphenyl.
[B] BTS numbers assigned by The Boots Company Ltd.

Metabolism Samples

At appropriate times after treatment, larvae were washed by agitating briefly once with 4 ml then once with 2 ml acetone. The washes were combined for scintillation counting and chromatography. The washed larvae were homogenized in 2 ml acetone using an ice-chilled glass homogenizer. The homogenate was centrifuged and the residue extracted twice more with 2 ml acetone and then with 2 ml 30% (v/v) methanol in acetone. The solid residue was air-dried and then digested in 0·5 ml of warm nitric acid, diluted with water to 2 ml and 0·2 ml was counted in Instagel to give a value for bound ^{14}C material. All scintillation counting was quench-corrected using the Automatic External Standard system. The acetone extracts were combined and the methanol–acetone extract kept separate. Aliquots of both extracts equivalent to 20 mg larvae were chromatographed in system A for routine assay. The radioactive counts of like peaks from chromatograms of the two types of extract were added to obtain the total for each metabolite or fraction. All results presented are averages of duplicates. Aliquots of acetone extracts equivalent to 10 mg of larvae were used for two-dimensional chromatograms in systems A and B or A and C for co-chromatographic identification of metabolites. The object of initially extracting larvae three times with acetone was to remove most of the amitraz without contact with methanol as amitraz is unstable in the latter solvent (communication from The Boots Company Ltd).

Chromatographic Systems

(i) *System A*

Merck t.l.c. aluminium sheets coated with 0·2 mm of silica gel 60 (DC-Alufolien Kieselgel 60) without fluorescent indicator were used in 20 by 20 cm size. Four samples were applied to each sheet as 2-cm streaks after reinforcement with reference compounds and, most importantly, with 5 μl of developing solvent (triethylamine–ether, 5:95 v/v). The basic solvent prevented breakdown of amitraz on the silica adsorbent. Failure to protect resulted in substantial cleavage of the acaricide.

The samples were applied 3 cm from the bottom of the sheet and developed for 15 cm. This system was used for routine metabolite assay.

(ii) *Systems A, B, and A, C*

Two systems of two-dimensional chromatography were employed both of which used the t.l.c. sheets and solvent of system A for the first development. The sample consisted of an aliquot equivalent to 10 mg larvae applied as a 6-mm spot. In system B the second developments were with 5% (v/v) methanol in toluene.

In system C the second developments were with the partition solvents of system D. Here the chromatographic sheet, after the first development, was dipped in 4% (v/v) polyethylene glycol 400 to just clear the radioactive strip, dried, and developed with mobile solvent. These systems were used to identify metabolites.

(iii) *System D*

Strips of Whatman No. 1 paper 3 cm wide were dipped in a 4% (v/v) solution of polyethylene glycol 400 in acetone and air-dried. The chromatograms were developed to 40 cm by descending solvent using a mixture of n-hexane, benzene, methanol and triethylamine (65:24:5:2 v/v). This system was used to purify ^{14}C reference compounds.

Recoveries of [^{14}C]Amitraz and Derivatives from Chromatograms

An aqueous colloid of [^{14}C]amitraz was hydrolysed partially with 0·1 M HCl and the products purified by liquid–liquid methods followed by chromatography in system D. The resulting compounds [^{14}C]I–IV were chromatographed in system A and the recoveries determined.

Assay of Chromatograms

Reference spots on one-dimensional chromatograms (system A) were located under short-wave ultraviolet light, marked with pencil, cut out, and eluted with 1·5 ml methanol in counting vials for 10 min. The coincident ^{14}C material was then counted after adding 10 ml Instagel. Remaining portions of the chromatograms were sectioned into *c.* 8 mm lengths and counted to give a complete profile. Two-dimensional chromatograms were similarly treated except that separate radioactive profiles were obtained for the origin, each reference spot and remaining areas along the second dimension so that the whole sheet was counted.

Toxicity Tests

The mortality of larvae was determined using as the criterion of death the inability of larvae to make forward movement. This was a practical necessity due to the slow killing action of formamidine acaricides wherein larvae pass through a protracted stage of uncoordinated leg movements before all movement ceases. LC_{50} values were determined from log-probit plots of chemical concentration and percentage mortality. Values for toxic compounds were recorded after incubation for 24 h. Chemicals which when used at 0·01% concentration produced negligible mortality in 72 h were rated non-toxic.

Synergism of amitraz by the mixed-function oxidase inhibitors SKF 525-A and piperonyl butoxide was tested by dosage of larvae with aqueous colloids containing various concentrations of amitraz and 0·01% (w/v) (a sublethal concentration) of either inhibitor. In this experiment we used the base form of SKF 525-A prepared from the Smith Kline and French product 2-diethylaminoethyl-2,2-diphenylvalerate, hydrochloride (Proadifen, hydrochloride).

Metabolism to $^{14}CO_2$

Larvae dosed with [^{14}C]amitraz were transferred to an apparatus which allowed the collection of respired CO_2 (Schuntner *et al.* 1972).

Results and Discussion

Chromatography of Larval Extracts

System A chromatograms of acetone and methanol–acetone extracts of larvae 3 h after treatment with 0·008% [^{14}C]amitraz are shown in Fig. 1. Most of the

extractable material was in the acetone extracts but significant amounts of polar material ($R_F = 0$) were extracted with the methanol–acetone solvent. Recoveries of purified [^{14}C]I, II and III in this system were quantitative (97–100%), whilst the rather volatile compound IV was more variable (79–93%).

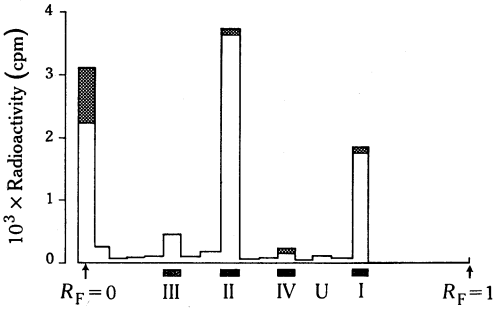

Fig. 1. Radioactive zones obtained by t.l.c. in system A of extracts of larvae 3 h after dosage with 0·008% (w/v) [^{14}C]amitraz as aqueous colloid. Unshaded histograms, acetone extract; shaded histograms, 30% (v/v) methanol in acetone extract; solid bars, visualized reference compounds; U, unidentified metabolite.

Identity of Metabolites

The results of quantitative assay of two types (A, B, and A, C) of two-dimensional chromatography of an acetone extract of [^{14}C]amitraz-treated larvae are shown in Table 2. The final percentage of activity in visualized spots or non-visualized radioactive zones and the R_F values are indicated. Co-chromatographic identity is established with compounds I–IV. The homogeneity of the immobile polar fractions was investigated by further t.l.c. using 30% (v/v) methanol in chloroform as solvent; most of the activity migrated to *c.* R_F 0·5 as a single spot and the remainder appeared as a minor fraction of lower mobility.

Table 2. Concentration of ^{14}C label in visualized reference spots or in unidentified radioactive spots (U) as a percentage of total activity along the second dimension of two-dimensional chromatograms (A, B, and A, C) of an acetone extract of larvae 3 h after dosage with 0·008% [^{14}C]amitraz

The concentration in the spots (and in an 8 mm zone either side of the spots—in parentheses) is given as the percentage of total activity. R_F values for solvent systems A, B and C are also shown

Zone	Concn of ^{14}C (%)		R_F		
	A, B	A, C	A	B	C
Polar ($R_F = 0$)	80 (88)	89 (99)	0·0	0·0	0·0
III	84 (90)	94 (99)	0·22	0·16	0·06
II	94 (100)	97 (99)	0·37	0·25	0·11
IV	85 (90)	74 (85)	0·51	0·43	0·30
U	80 (88)	89 (99)	0·60	0·61	0·54
I	97 (98)	98 (99)	0·71	0·70	0·76

Metabolism of [^{14}C]Amitraz at Two Doses

The metabolism of [^{14}C]amitraz in larvae after dosage with 0·008 and 0·0004% colloids is shown in Table 3 as the percentages of total internal ^{14}C material including residual activity, whilst the total internal and external ^{14}C material is shown as microgram equivalents of [^{14}C]amitraz. The decidedly lethal treatment with 0·008% amitraz was initially used to obtain high activity samples for development of analytical

methods and also for two-dimensional co-chromatographic identification of metabolites. The lower concentration was regarded as the practical lower limit for counting purposes and was reasonably close to the 24 h LC_{50}. The penetration of amitraz was quite rapid with about half the external material penetrating in 3 h for both doses as judged from total internal and external ^{14}C material. Qualitatively there was no difference in metabolism at the two doses.

Table 3. Distribution of ^{14}C label in tick homogenates at various times after dosage with 0·008 or 0·0004% [^{14}C]amitraz, as the percentage of total internal activity, including the residue

Total internal and total external ^{14}C materials are shown as microgram equivalents of [^{14}C]amitraz. Extracts were chromatographed in system A

Fraction	Distribution of ^{14}C label (%)			
	1 h	3 h	6 h	16 h
	(*a*) 0·008% [^{14}C]amitraz			
Polar (R_F = 0)	15·8	24·4	28·1	27·4
III	3·6	5·4	6·4	7·4
II	21·7	30·9	35·2	36·6
IV	1·9	3·2	3·1	4·8
U	3·3	1·8	1·0	0·6
I	48·0	21·7	12·0	2·7
Residue	5·6	12·6	14·2	20·6
Internal (μg equiv.)	51·9	101·2	131·5	156·1
External (μg equiv.)	170·9	112·0	76·9	20·2
	(*b*) 0·0004% [^{14}C]amitraz			
Polar (R_F = 0)	22·4	26·7	22·5	28·3
III	5·3	6·5	5·3	7·2
II	26·0	26·7	27·7	21·5
IV	2·3	2·3	3·3	3·0
U	2·3	0·8	1·0	2·0
I	28·0	10·7	5·3	1·1
Residue	13·7	26·4	34·8	37·0
Internal (μg equiv.)	3·73	5·36	8·03	8·13
External (μg equiv.)	8·41	5·49	3·34	0·78

The early metabolism of the higher dosage was slower especially at 1 h, where 48% of the total internal label was amitraz compared with 28% at the lower dosage. Thereafter internal amitraz levels at both doses declined rapidly. The major metabolic products were compound II and the polar fraction. Both increased steadily at the higher dosage, but at the lower dosage compound II appeared to undergo subsequent breakdown after 6 h. The apparent greater stability of compound II at the higher dosage probably resulted from the larger reserve of amitraz. Compounds III and IV never reached high levels, and an unidentified metabolite (U) appeared insignificant throughout. The relatively small quantities of compound III were contrary to the expectation that the cleavage of the –N–CH= bond would have resulted in equivalent production of compounds [^{14}C]II and [^{14}C]III. The deficit in compound III was largely accounted for by the polar fraction.

Metabolism of Amitraz to CO_2

Samples of larvae were dosed with 0·0004 and 0·008% [^{14}C]amitraz, and the respired $^{14}CO_2$ was collected for consecutive 30-min periods up to 6 h. The larvae were then washed with acetone and homogenized in methanol to determine the total internal activity in the homogenate. $^{14}CO_2$ was produced at a fairly uniform rate but amounted to only 1·3% of the total internal label 6 h after dosage with 0·0004% [^{14}C]amitraz. A little more was produced from larvae dosed at 0·008%, but this was only 0·17% of the total internal label at 6 h. Overall this oxidation of the 2-methyl groups appears unimportant as a degradative route.

Toxicity of Amitraz and Some Metabolites

The results of toxicity tests of compounds I–IV are shown in the following tabulation:

	I	II	III	IV
24 h LC_{50}	0·00018%	0·0070%	Non-toxic	Non-toxic

Only compounds I and II exhibited significant toxicity, compound I being by far the more toxic. However, a study of deposition and penetration of compounds [^{14}C]I and [^{14}C]II indicated that the 'real' difference was somewhat less than indicated. We calculated that the topical toxicity of compound I was approximately five times that of compound II.

Synergism of Amitraz

Larvae were dosed by immersion in aqueous colloids containing various concentrations of amitraz alone or with 0·01% (w/v) SKF 525-A or piperonyl butoxide and the synergistic ratios of the inhibitors were calculated. These are shown in the following tabulation:

	I	I+SKF 525-A[A]	I+piperonyl butoxide[A]
24 h LC_{50}	0·00018%	0·00018%	0·000060%
Synergistic ratio		1·0	3·0

[A] SKF 525-A alone and piperonyl butoxide alone caused < 1% mortality in 24 h at 0·01% (w/v).

There was no change in toxicity due to SKF 525-A, but there was a three-fold synergistic effect of piperonyl butoxide. The metabolism of 0·0004% [^{14}C]amitraz alone and in the presence of simultaneously applied inhibitors at 0·01% (w/v) concentration was studied. The results (calculated as the percentage of total extract), 6 and 16 h after dosage, show that neither compound had much effect on the total metabolism of amitraz, but both appeared to inhibit the subsequent metabolism of compound II to a small extent (Table 4). It is difficult to explain the synergistic effect of piperonyl butoxide together with the ineffectiveness of SKF 525-A from the metabolic data. Although 0·01% (w/v) piperonyl butoxide is a sublethal dose, at higher concentrations this compound is toxic to larvae (and to parasitic ticks) (Schuntner *et al.* 1974). Also it has been found that formamidine acaricides and especially amitraz are mixed-function oxidase inhibitors themselves and it was suggested that this activity produced the initial lesion in formamidine lethality (Schuntner and Thompson 1976). If this is true then additive toxicity may explain the apparent synergism by piperonyl butoxide.

Degradative Mechanisms and Future Resistance

Piperonyl butoxide is maximally effective as an inhibitor of ring hydroxylation of carbaryl, oxidative desulphuration of thiophosphate acaricides, *N*-demethylation of chlordimeform and of chloromethiuron in tick larvae when applied at a concentration of 0·01% (w/v) (unpublished data). The lack of effect of this inhibitor and of SKF 525-A on metabolism of amitraz and the small effect on metabolism of compound II indicates that mixed-function oxidases, phenolase (also inhibited by

Table 4. Distribution of ^{14}C label in acetone extracts of larvae 6 and 16 h after dosage with 0·0004% [^{14}C]amitraz alone or with 0·01% (w/v) SKF 525-A or piperonyl butoxide, as the percentage of total extracted label

Total radioactive material extracted is shown as microgram equivalents of [^{14}C]amitraz. Extracts were chromatographed in system A

Fraction	Distribution of ^{14}C label (%)		
	I	I+SKF 525-A	I+pip. but.
	(*a*) 6 h		
Polar (R_F = 0)	38·8	34·8	29·9
III	10·1	8·1	10·8
II	33·6	36·0	34·8
IV	5·5	7·7	7·1
U	2·6	2·2	2·8
I	9·4	11·1	14·5
Total (μg equiv.)	6·13	6·13	5·36
	(*b*) 16 h		
Polar (R_F = 0)	36·5	35·3	26·6
III	13·1	8·9	16·2
II	30·7	35·0	39·3
IV	9·0	8·1	8·3
U	3·2	4·4	3·1
I	7·5	8·3	6·5
Total (μg equiv.)	7·08	6·97	8·22

piperonyl butoxide), and monoamine oxidase (also inhibited by SKF 525-A) are not specifically able to degrade amitraz or compound II. Furthermore the stability of compound II in larvae suggests that they possess no effective system for the metabolism of this compound. The rapid initial cleavage of amitraz was little affected by increasing the concentration of it from 0·0004 to 0·008%. Usually such a change in any acaricide concentration results in a marked decrease in percentage metabolism in a given time in larvae, e.g. coumaphos (Roulston *et al.* 1969). This suggests the possibility that the cleavage of amitraz may be non-enzymic.

From our present data likely resistance mechanisms would involve (1) an enzymic attack on the intact amitraz, (2) a more rapid (enzymic) degradation of compound II, or (3) the development of an insensitive target enzyme (possibly a mixed-function oxidase). If and when pure resistant strains are available it will be a matter of considerable practical and academic interest to determine whether sensitivity can be restored by alteration of the toxicant's structure or by 'synergism'.

Acknowledgments

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References

Roulston, W. J., Schuntner, C. A., Schnitzerling, H. J., and Wilson, J. T. (1969). Detoxification as a mechanism of resistance in a strain of the cattle tick *Boophilus microplus* (Canestrini) resistant to organophosphorus and carbamate compounds. *Aust. J. Biol. Sci.* **22**, 1585–9.

Roulston, W. J., Wharton, R. H., Schnitzerling, H. J., Sutherst, R. W., and Sullivan, H. D. (1971). Mixtures of chlorphenamidine with other acaricides for the control of organophosphorus-resistant strains of cattle tick *Boophilus microplus*. *Aust. Vet. J.* **47**, 521–8.

Schuntner, C. A., Roulston, W. J., and Wharton, R. H. (1974). Toxicity of piperonyl butoxide to *Boophilus microplus*. *Nature* (*London*) **249**, 386.

Schuntner, C. A., Schnitzerling, H. J., and Roulston, W. J. (1972). Carbaryl metabolism in larvae of organophosphorus and carbamate-susceptible and -resistant strains of cattle tick *Boophilus microplus*. *Pest. Biochem. Physiol.* **1**, 424–33.

Schuntner, C. A., and Thompson, P. G. (1976). Inhibition of a carbaryl oxidising enzyme as the primary lesion in the lethal action of formamidines in *Boophilus microplus*. *J. Aust. Entomol. Soc.* **15**, 388.

Stone, B. F., Atkinson, P. W., and Knowles, C. O. (1974). Formamidine structure and detachment of the cattle tick *Boophilus microplus*. *Pest. Biochem. Physiol.* **4**, 407–16.

Wharton, R. H., and Roulston, W. J. (1976). Acaricide resistance in *Boophilus microplus* in Australia. Paper presented at Seminario sobre Hemoparasitos, Centro Internacional de Agricultura Tropical (CIAT), Cali, March 1975. (Mimeograph.)

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