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Capillary gas chromatographic determination of permethrin insecticide by transesterification[1]

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Abstract

A sensitive method was developed to determine permethrin extracted from phosphate buffer and cattle plasma by potassium cyanide catalyzed transesterification of this insecticide with refluxing ethanol and detection of the resulting ethyl esters by capillary gas chromatography with an electron capture detector. With a reflux time of 2 h and with 3-phenoxybenzyl 2-chlorobenzoate as an internal standard, linear calibration curves from buffer (5–250 ng) and plasma (5–100 ng) were obtained. Precision and accuracy of the method were ≤15%. The limit of detection was approximately 2.5 ng/ml (*cis*) and 1 ng/ml (*trans*) from buffer. In cattle sprayed along the back at 2 mg/kg, the concentration of *cis*- and *trans*-permethrin in plasma was below the detection limit (5 ng/ml).

Keywords: Permethrin

1. Introduction

Since the mid-1970s, analytical methods for the trace-level determination of permethrin [(3-phenoxyphenyl)methyl (±)-*cis,trans*-3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane carboxylate] have frequently relied on gas chromatography with an electron capture detector (GC–ECD). Applications in residue chemistry using packed [1,2] and capillary GC columns [3–6] can be found in the recent literature. Despite these applications, permethrin is not particularly amenable to GC analysis because of its relatively low volatility. Temperatures well in excess of 200°C are typically required for permethrin to elute from common GC columns.

The transesterification of esters in the presence of strong acids or bases as catalysts is a well-known reaction [7]. Using sodium L-menthate in benzene at room temperature, Chapman and Harris [8] found that permethrin gave stereoisomeric mixtures of the menthyl esters of permethrin acid which were analyzed by GC–ECD with a packed column. The ethyl esters of *cis*- and *trans*-permethrin acid are known to have excellent GC properties [9].

Permethrin is commonly used to control livestock pests, some of which have developed resistance to this insecticide [10,11]. Research on elucidating biochemical mechanisms that contribute to resistance will be aided by the availability of sensitive techniques for determination of permethrin in biological samples such as in the hemolymph of flies or in buffered enzyme suspensions. The potential absorption of this insecticide following topical application

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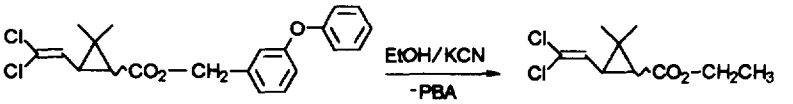


1a/1b 2a/2b

Fig. 1. Transesterification of *cis* (**1a**) and *trans* (**1b**) permethrin to *cis* (**2a**) and *trans* (**2b**) ethyl esters. PBA, 3-phenoxybenzyl alcohol.

to cattle underscores the need for specific methods of detection in blood samples to assess the extent of dermal absorption of permethrin in food-producing animals. Recently, high-performance liquid chromatography (HPLC) has been utilized for the determination of permethrin in rat plasma [12]. The limit of detection was 100 ng/ml.

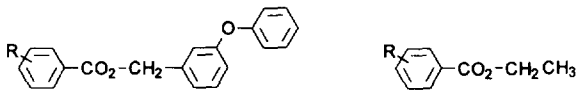
We report here the development of a method to transesterify the *cis* (**1a**) and *trans* (**1b**) isomers of permethrin with ethanol and potassium cyanide [13] and to determine the resulting *cis* (**2a**) and *trans* (**2b**) ethyl esters by capillary GC–ECD (Fig. 1). The 3-phenoxybenzyl ester of 2-chlorobenzoic acid (**3**) (Fig. 2) was shown to be a suitable internal standard for the determination of **1a** and **1b** in cattle plasma at concentrations of 5–100 ng/ml.

2. Experimental

2.1. Instrumentation and chromatographic conditions

A Hewlett-Packard Model 5890A GC instrument equipped with a ^{63}Ni electron-capture detector, a split-splitless injection port equipped with a silanized glass liner (HP Part No. 18740-80200), a Hewlett-Packard Model 7673A automatic sampler and a Hewlett-Packard Model 3392A reporting integrator were used. The injection port temperature was 225°C and the detector temperature 300°C. Helium was used as the carrier gas with the inlet pressure adjusted to give linear flow velocities for hexane of 28–29 cm/s at 35°C. Argon–methane (95:5) served as the make-up gas (60 ml/min) for the ECD. Fused-silica capillary columns were supplied by Hewlett-Packard (HP-5MS, 0.25 μm film thickness, 30 m× 0.25 mm I.D.). Samples (1 μl) were injected splitless (30 s) at an initial oven temperature of 50°C. After 1 min, the temperature rose at 25°C/min to 100°C, at 2°C/min to 120°C then at 8°C/min to 265°C. The column was held at 265°C for 10 min.

Electron impact mass spectra (70 eV) were obtained on a Hewlett-Packard Model 5989A GC–MS instrument. The transfer line was maintained at 280°C and the ion source at 250°C. Capillary columns of DB-5MS (15 m×0.25 mm) from J&W Scientific (Folsom, CA, USA) and HP-5MS (30 m×0.25 mm) from Hewlett-Packard were used. The samples were injected splitless with helium as the carrier gas.



3 R=2-Cl 5 R=2-Cl

4 R=4-Cl 6 R=4-Cl

Fig. 2. Structures of the chlorobenzoates investigated as internal standards.

2.2. Preparation of reference chemicals

A reference mixture of **2a** and **2b** was prepared by heating a mixture of permethrin (45% **1a**, 55% **1b**) and potassium cyanide in 95% ethanol for 16 h [13]. **Caution: KCN is a violent poison**. (3-Phenoxyphenyl)methyl 2-chlorobenzoate (**3**) was prepared from 2-chlorobenzoyl chloride and 3-phenoxybenzyl alcohol according to the procedure of Nakatsuka et al. [14]. (3-Phenoxyphenyl)methyl 4-chlorobenzoate (**4**) was prepared similarly, using 4-chlorobenzoyl chloride. Reference samples of ethyl 2-chlorobenzoate (**5**) and ethyl 4-chlorobenzoate (**6**) were prepared by esterification of the corresponding carboxylic acids with absolute ethanol and concentrated sulfuric acid [15].

2.3. Other chemicals and reagents

Reference samples of **1a** and **1b** were obtained from (±)-*cis*- and (±)-*trans*-permethrin acids [16] as previously reported [14,17]. Water was purified by a Barnstead NANOpure II system and double distillation. Phosphate buffer (pH 7, 0.1 *M*) was prepared with NaH_2PO_4 and Na_2HPO_4. Ethanol (95%) was distilled before use. Other organic solvents were OmniSolv glass distilled grade (supplied by BDH, Edmonton, Canada). Potassium cyanide obtained from Baker (Phillipsburg, NJ, USA) was used as a freshly prepared solution (0.2% w/v) in 95% ethanol. Excesses of ethanolic and aqueous solutions containing KCN were destroyed in a fumehood with sodium hypochlorite [18].

Stock solutions of **1a** and **1b** were prepared in 95% ethanol at concentrations of 0.2, 0.3, 0.4, 0.6, 0.8, 1.0 and 1.2 mg/ml. Stock solutions of **3** and **4** were prepared in 95% ethanol at a concentration of 0.8 mg/ml. Working solutions were appropriately prepared by dilution with 95% ethanol. All solutions were stored at 4°C.

2.4. Extraction and derivatization

Phosphate buffer (2.5 ml) in test tubes (100×13 mm) equipped with PTFE-lined screw caps was spiked with varying quantities of **1a** and **1b** and 50 ng of **3**. After vortexing, the samples were extracted with hexane (4×2 ml), using a recriprocal shaker (Ames aliquot mixer). The solvent was evaporated (Savant Model SVC-100H, Farmingdale, NY, USA) and the residue was dissolved in 95% ethanol (1 ml) containing 0.2% KCN. The mixture was heated for 2 h at 80°C (Pierce Reacti-Therm III module) and then water (2 ml) was added. After extraction with hexane (2×2 ml), the organic phase was washed with water (2 ml), concentrated under nitrogen to 0.5 ml with a Meyer N-EVAP apparatus (Organomation Associates, Berlin, MA, USA) and a portion was transferred to 250-μl glass inserts (HP Part No. 5181-3377) for GC–ECD analysis.

Equations describing the calibration curves were obtained by least squares linear regression analysis of the integrated peak-area ratio of **2a** (and **2b**) to **5** (*y*-axis) versus the amount of **1a** (and **1b**) added (*x*-axis).

2.5. Validations

An intra-assay experiment was performed by spiking phosphate buffer (2.5 ml) with 25 ng and 100 ng of **1a**, 25 ng and 100 ng of **1b** and 50 ng of **3**. The samples were extracted with hexane and derivatized as described in Section 2.4. The final hexane extracts were concentrated to 0.5 ml and analyzed along with samples of the calibration curve, prepared on the same day by spiking phosphate buffer with 5–250 ng of **1a**, 5–250 ng of **1b** and 50 ng of **3**. Inter-assay experiments were performed in a similar manner by spiking phosphate buffer (in duplicate) at the same levels (25 and 100 ng) and analyzing the samples. Four experiments were done in different weeks. New calibration curves consisting of eight points for each isomer were prepared in singlicate on the day of the analysis. The amount of **1a** (and **1b**) found in the spiked samples was calculated by reference to the equations describing the calibration curves of **2a** (and **2b**).

Control plasma (1 ml), obtained before treatment of the cattle and stored at −80°C in polypropylene tubes, was spiked with 10, 25 and 75 ng of **1a** and **1b** and 50 ng of **3**. In the intra-assay experiment, the samples were processed immediately after spiking the plasma. In the inter-assay experiments, control plasma was spiked with **1a** and **1b** then the samples were stored at −80°C. On each day of the analysis, two samples at each concentration were thawed, **3** (50 ng) was added and the samples were extracted with hexane, derivatized, re-extracted with hexane and a portion (250 μl) of the concentrated hexane extract was transferred to an autosampler vial. Calibration curves were likewise obtained on each day of the analysis by spiking control plasma with **1a** (5–100 ng), **1b** (5–100 ng) and **3** (50 ng).

2.6. Limit of detection

Phosphate buffer (2.5 ml) and cattle plasma (1 ml) were spiked with **1a** and **1b** at levels of 0 (blank), 1, 2.5 and 5 ng. The samples (in duplicate) were extracted and derivatized as described in Section 2.4. Peak heights of the expected signals from **2a** (15.9 min) and **2b** (16.3 min) were measured and compared to the largest matrix signal (noise) eluting in the window of 15.7–16.5 min of a corresponding

blank. Samples that gave a signal to noise ratio of ≥3 were considered to be above the limit of detection. The limit of detection was determined with control plasma from four Hereford heifers.

2.7. Animal treatments

Two Hereford heifers maintained in a controlled environment room as previously described [19] were used. The animals weighed 428 kg and 476 kg at the time of topical treatment with permethrin (Ectiban 25 Fly Killer surface spray, supplied by Coopers Agropharm, Ajax, Canada). This emulsifiable concentrate contained permethrin (40% *cis*: 60% *trans*) at 240 g/l and was diluted with water so that 50 ml delivered a topical dose of 2 mg of permethrin per kg of body weight. Prior to treatment, an area along the backline, 80 cm long with a width (in mm) equal to the body weight in kg, was marked. With one animal, the haircoat of the treatment area was removed with Oster clippers (Model A5-08) whereas the haircoat was of normal length with the second animal. On both animals, the hair on a 10 cm border surrounding the spray area was clipped. The border acted as a barrier to spreading of the treatment solution.

The animals were treated by evenly spraying the prescribed area with a hand-held household spray atomizer. The container was rinsed with water (5 ml) and the rinse was also applied. The midpoint of the spray time (2–3 min) was considered as time 0. Post-treatment bleeding times were at 0.5, 1, 2, 4, 6, 8, 12, 24, 48, 72 and 144 h. Blood was collected from a jugular vein into gray-stoppered 10-ml tubes containing potassium oxalate and sodium fluoride (Becton Dickinson Canada, Mississauga, Canada). The plasma was obtained by centrifuging at 700 *g* for 10 min and stored at −80°C for 2 weeks before analysis.

Plasma (1 ml, in duplicate) of each blood sample collected from the treated heifers was extracted and derivatized as described.

3. Results and discussion

Authentic mixtures of **2a** and **2b** in hexane were readily separated on a capillary column of HP-5MS. Under splitless conditions, the elution temperatures during capillary GC–ECD were 147°C (**2a**) and 149°C (**2b**), corresponding to retention times of 15.9 and 16.3 min. The *trans* ester showed nearly twice the response to the ECD compared to the *cis* ester. Permethrin eluted after the column was held at the upper temperature (265°C), giving retention times of 35.3 (**1a**) and 35.7 min (**1b**).

Phosphate buffer of pH 7 was initially spiked with **1a** (20 μg) and **1b** (20 μg). After extraction and transesterification, yields were estimated by GC–ECD by comparison to the area counts of a reference mixture of **2a** and **2b** in hexane. Optimum yields of the ethyl esters were obtained with a reflux time of 2–3 h. Both **2a** and **2b** tended to degrade with prolonged refluxing.

To identify an internal standard, we studied the conversion of **3** to **5** and the conversion of **4** to **6** (Fig. 2). Optimum conditions for the transesterification of **3** were similar to those for **1a** and **1b**. In contrast, **4** was readily transesterified at room temperature. Ethyl ester **6** decomposed rapidly under refluxing conditions. Ethyl ester **5** also decomposed on prolonged refluxing but the yield was approximately 50% after 2 h. Since this compared favourably to yields of **2a** (75%) and **2b** (65%) after a 2-h reflux, **3** was selected as the internal standard.

This method was examined for determination of permethrin at low concentrations. Phosphate buffer spiked with 1 ng of **1a** and **1b** gave small signals at 15.9 and 16.3 min if the final hexane extracts were evaporated to a small volume (<100 μl). With a final volume of 0.5 ml, spikes of 2.5 ng of **1a** and 1 ng of **1b** were detected at a signal-to-noise ratio of ≥3, which corresponded to on-column sensitivities 5 pg (*cis*) and 2 pg (*trans*).

Samples representing calibration curves were obtained by spiking phosphate buffer with 5–100 ng and 5–250 ng of **1a** and **1b** (50 ng of **3**). After derivatization and examination of the concentrated hexane extracts by GC–ECD, the correlation coefficients for the calibration curves of **2a** and **2b** were ≥0.997.

Chromatograms from GC–ECD analysis of hexane extracts from the transesterification experiments were free of interfering peaks at the retention times of **2a**, **2b** and **5**. 3-Phenoxybenzyl alcohol eluted several minutes after these ethyl esters during GC–MS but did not respond to the ECD.

Table 1
Description of low concentration calibration curves derived from transesterification of permethrin and internal standard residues extracted from phosphate buffer and cattle plasma

	Buffer	Plasma		
Spikes[a]				
1a	5–250 ng		5–100 ng	
1b		5–250 ng		5–100 ng
3	50 ng	50 ng	50 ng	50 ng
Equations[b]				
Slope	0.0068±0.0009	0.0109±0.0015	0.0064±0.0006	0.0115±0.0013
y-Intercept	−0.0204±0.0208	−0.0145±0.0339	0.0034±0.0060	0.0093±0.0128
r^2	0.9977±0.0027	0.9972±0.0027	0.9957±0.0043	0.9971±0.0019

[a] **1a** and **1b** correspond to *cis*- and *trans*-permethrin; **3** represents 3-phenoxybenzyl 2-chlorobenzoate, the internal standard.
[b] Obtained by unweighted least-squares linear regression of the peak area ratios of **2a** (and **2b**) to **5** versus the amount of **1a** (and **1b**) added. Mean±S.D., $n=5$.

Spiking experiments with cattle plasma showed that the limit of detection ranged from 2.5–5 ng/ml for **1a** and from 1–2.5 ng/ml for **1b**. Data from linear regression analysis of five calibration experiments covering the ranges of 5–250 ng (buffer) and 5–100 ng (plasma) was tabulated (Table 1).

Intra-day precision (R.S.D.) and accuracy were assessed by analysis of five samples at two spiking levels for buffer and at three spiking levels for plasma. Precision and accuracy for all samples analyzed were within 10% of the nominal values (Table 2).

Inter-day precision and accuracy were determined by analysis of two samples at each spiking level on four separate days. Precision (3.4–15.2%) and accuracy (0.6–11.0%) were within acceptable limits for validation of a chromatographic assay [20].

The method was applied to the analysis of plasma samples from cattle treated topically with a commercial formulation of permethrin registered for use on livestock. After a single application of 2 mg/kg to the back, approximately twice the dose recommended for protection of cattle from black flies in Canada [17], **1a** and **1b** could not be detected in blood samples collected for up to 6 days post-treatment. Fig. 3 shows a chromatogram from a treated heifer compared with that of spiked plasma and control plasma.

Table 2
Assay validation data for the determination of permethrin by transesterification[a]

	Buffer				Plasma					
	1a	**1b**	**1a**	**1b**	**1a**	**1b**	**1a**	**1b**	**1a**	**1b**
Intra-day ($n=5$)										
Added (ng)	25	25	100	100	10	10	25	25	75	75
Found (ng)	26.5	25.8	95.5	94.9	9.8	10.5	26.1	24.9	82.4	70.1
R.S.D. (%)[b]	9.5	7.1	7.3	5.7	3.2	2.0	8.6	8.7	2.3	3.2
Accuracy (%)[c]	5.9	3.1	−4.5	−5.1	−1.5	5.5	4.6	−0.3	9.9	−6.5
Inter-day ($n=4$)										
Added (ng)	25	25	100	100	10	10	25	25	75	75
Found (ng)	27.1	26.8	97.2	96.4	8.9	9.9	24.4	26.5	73.3	75.5
R.S.D. (%)[b]	4.1	3.4	13.4	8.5	10.7	6.9	8.2	4.5	15.2	7.7
Accuracy (%)[c]	8.5	7.4	−2.8	−3.6	−11.0	−1.3	−2.2	5.9	−2.2	0.6

[a] **1a** and **1b** correspond to *cis*- and *trans*-permethrin.
[b] R.S.D.=(S.D./mean)×100.
[c] Accuracy=[(found−added)/added]×100.

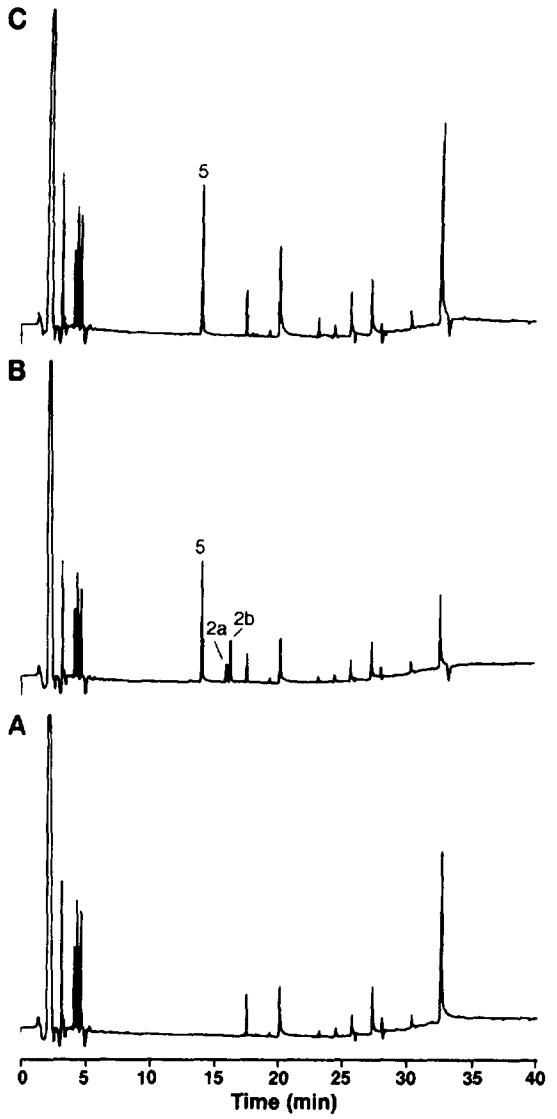


Fig. 3. Capillary GC–ECD chromatograms of cattle plasma samples after extraction and transesterification. (A) Plasma blank; (B) plasma spiked with 50 ng of **1a**, **1b** and **3**; (C) plasma 4 h after spray application to which **3** (50 ng) was added before extraction.

The distribution and metabolism of radiolabeled permethrin has been studied after oral treatment of cows [21] and goats [22] but similar studies in ruminants following topical application could not be found in the literature. Residues of topically applied fenvalerate [23] and deltamethrin [24] in dairy cattle have been reported to be very low. The transdermal absorption of permethrin in scabies patients is also very low, approximately 0.5% of the applied dose [25]. Wintersteiger et al. [26] were unable to detect by HPLC topically applied pyrethrins in human plasma.

Because the present experiments were aimed at detecting the parent compound only, it is possible that permethrin penetrates cattle skin, as it does the skin of rats and monkeys [27], but undergoes complete metabolism during or after absorption. On the other hand, the skin may act as an effective barrier to absorption when permethrin is applied as an emulsifiable concentrate diluted with water. Topical drug delivery to cattle is influenced by many factors [28], including the formulation solvent. Further research is needed on permethrin applied in different formulations to various breeds of cattle.

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