Determination of Pyrethroid Metabolites in Human Urine by Capillary Gas Chromatography-Mass Spectrometry

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Key Words

Gas chromatography-mass spectrometry
Pyrethroid metabolites in urine
Urinary marker
4-fluoro-3-phenoxybenzoic acid
Cypermethrin and cyfluthrin

Summary

An analytical method for the simultaneous determination of the pyrethroid metabolites cis and trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid, cis 3-(2,2-dibromovinyl)-2,2-dimethylcyclopropane carboxylic acid, 3-phenoxybenzoic acid and 4-fluoro-3-phenoxybenzoic acid in human urine samples is described. The urine is subjected to acid-induced hydrolysis followed by exhaustive solvent extraction, covering both conjugated and free acids, followed by a common derivatisation step yielding the corresponding methyl esters. Quantitation was by diastereomeric, capillary gas chromatography-mass spectrometry. It appears that 4-fluoro-3-phenoxybenzoic acid is a characteristic urinary marker for cyfluthrin exposure. The limits of determination are 0.5–1.0 μg L^{-1} urine depending on the metabolites concerned. The applicability of the method was tested on urine samples from pest control operators exposed occupationally to cypermethrin and cyfluthrin.

Introduction

Synthetic pyrethroids like cyfluthrin, cypermethrin, permethrin and deltamethrin are increasingly used for indoor pest control. In formulations they appear either as a single isomer (deltamethrin, (S)-bioallethrin) or as isomeric mixtures containing two (α-cypermethrin), four (permethrin) or up to eight different stereoisomers (cypermethrin, cyfluthrin) depending on the number of chiral centres in the molecule. The presence of two chiral centres in the cyclopropane ring of, e.g., permethrin leads to two pairs of diastereomers, which are commonly designated as cis and trans. This nomenclature is based on the orientation of the C-1 and C-3 substituents in relation to the plane of the cyclopropane ring [1]. The stereochemistry of selected pyrethroids and their metabolic derivatives is shown in Figure 1.

Metabolism studies suggest that the primary alcohol esters of cyclopropane carboxylic acids with trans substituents such as isobutenyl or dichlorovinyl at C-3 in cyclopropane are mainly metabolized by esterases, whereas all esters of primary and secondary alcohols with cis-substituted cyclopropane carboxylic acids are mostly metabolized by mixed-functional oxidases [2]. Thus, in contrast to, e.g., permethrin, in mammals oxidative degeneration of deltamethrin is more important than hydrolytic ester cleavage [3]. After hydrolysis, the alcohol moiety of pyrethroids is similarly oxidized leading to a complex series of urinary products [4, 5]. Human oral-dose studies with cypermethrin (1:1 cis:trans isomers) showed that 78 % of the trans-DCCA and 49 % of the cis-DCCA were excreted in the urine during the first 24 h after exposure [6]. A similar study carried out with α-cypermethrin (one of the two diastereomeric pairs constitute cis-cypermethrin) confirmed the results [7]. However, while passing through the human body, both acid and alcohol moieties of the hydrolysed ester pyrethroids are converted into their corresponding acids, partly conjugated to, e.g., glucuronic acid and finally eliminated with the urine [8].

Knowledge of these biochemical pathways for detoxification of pyrethroids is used in the present study to develop an analytical method for the simultaneous determination of the important pyrethroid metabolites: cis and trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid (cis and trans-DCCA), cis-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropane carbo-

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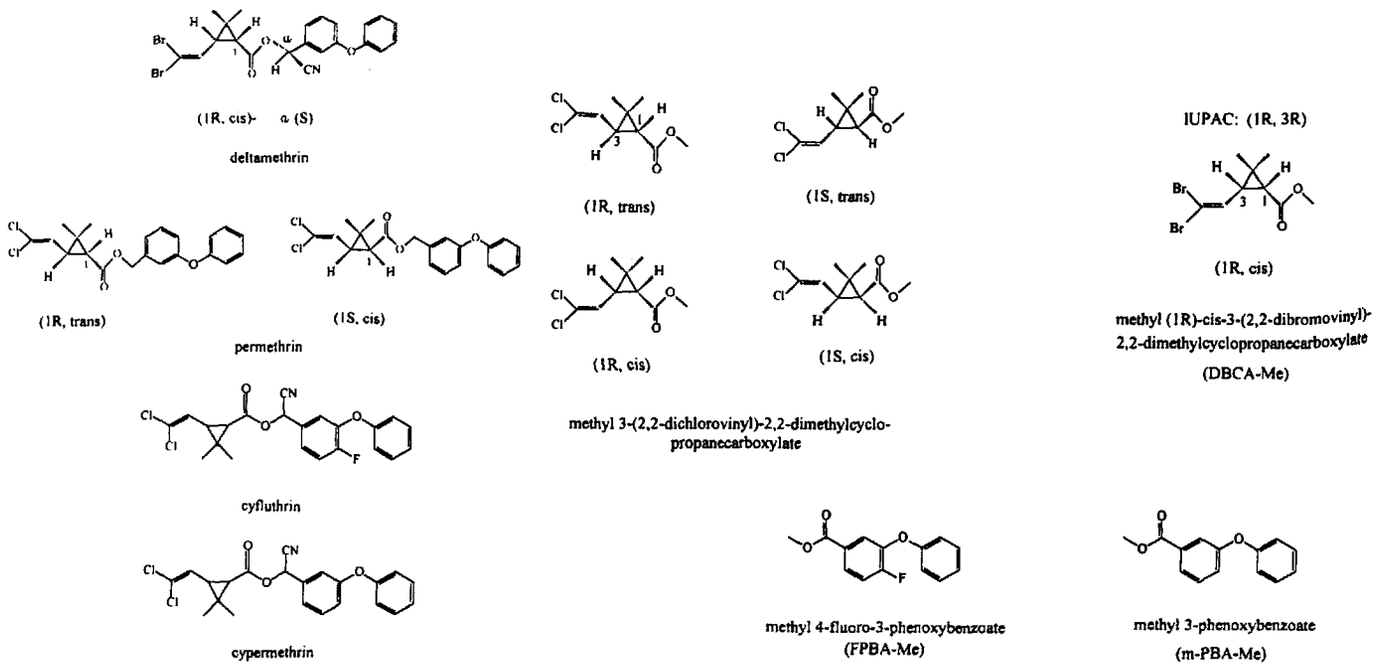

Figure 1
Trivial names and relative stereochemistry of selected pyrethroids and metabolic derivatives according to Elliot [1].

xylic acid (cis-DBCA), 3-phenoxybenzoic acid (3-PBA) and 4-fluoro-3-phenoxybenzoic acid (FPBA) in urine of exposed subjects.

Urine samples were subjected to acid-induced hydrolytic cleavage to give free acid metabolites. After extraction, methylation and concentration, derivatives were separated by diastereoselective capillary gas chromatography on an apolar, HP Ultra 2 phase (Hewlett Packard) containing a poly(5 %-diphenyl-95 %-dimethylsiloxane) equivalent to DB 5 phase. For identification and quantitation mass spectra were obtained by electron impact (EI,70 eV) using selected ion monitoring (SIM) (Table I) or a narrow mass scan (120 – 260 amu) covering the most characteristic fragment ions (Figure 2 a–d).

Experimental

Reagents

Solvents of nanograde purity were from Promochem (Wesel, Germany). Sulphuric acid (Suprapur 96 %) and hydrochloric acid (36.5 – 38.0 %) were from Baker (Deventer, The Netherlands) and other chemicals (highest purity) were from Merck (Darmstadt, Germany).

The chemicals: 2- and 3-phenoxybenzoic acid were commercially available from Aldrich (Steinheim, Germany), cis and trans-permethrin were from Promochem (Wesel, Germany) and cis-DBCA was from Dr. Ehrensdorfer (Augsburg, Germany). All other free metabolites (cis and trans-DCCA, FPBA) were donated by Bayer (Leverkusen, Germany).

Sampling

Preferably, 24 h urine samples of exposed subjects were collected in polyethylene bottles. Until preparation, samples were stabilized with 1 % (v/v) chloroform [6]. Under storage conditions, 20 °C, samples were stable for a minimum of 5 weeks.

Analysis of Pyrethroid Metabolites in Human Urine Samples

5 mL urine sample was placed in a glass centrifuge tube equipped with screw cap. After adding 1 mL conc. HCl, the tightly sealed glass was placed in a water bath (90 °C) for 1 h. By this procedure the acid metabolites as well as their conjugates were converted into free acids. After cooling, the acidified urine sample was treated with 4 mL hexane. Then the tube was sealed, vigorously shaken for 1 min and rollermixed for 10 min. Following centrifugation (7 g, 10 °C) for 10 min, the organic layer was transferred to a second centrifugation glass containing 1 g anhydr. Na_2SO_4. This procedure was repeated twice. The combined organic layers were shaken, centrifuged (5 g, 10 °C) for 5 min and then transferred to a reaction vessel. Na_2SO_4 was washed with 4 mL hexane and the washing added to the extracts. Extracts were concentrated at reduced pressure in a water bath (60 °C) and reduced almost to dryness in a gentle stream of nitrogen. The residue was refluxed with 10 % (v/v) H_2SO_4 in methanol (10 mL) for 1 h. After cooling, the methylated species were transferred to a separation funnel, diluted with water (10 mL) and mixed with 1 M NaOH (15 mL). The methylated esters of phenoxybenzoic acid as well as of cyclopropane acids were extracted

Table I. Selected ion monitoring (SIM) program for quantitation of various pyrethroid metabolic derivatives covering fragment ions with least interference.

Pyrethroids	Metabolites*	Retention time [min]	SIM [m/z]	Quantitation ion [m/z]	SIM programm [min]
Cyfluthrin α-Cypermethrin Cypermethrin Permethrin	cis-DCCA-Me	11.2	163, 165, 167, 187, 189	187	10.0 to 11.9
Cyfluthrin Cypermethrin Permethrin	trans-DCCA-Me	11.3			
Deltamethrin	cis-DBCA-Me	13.5	231, 233, 251, 253, 255	253	12.0 to 15.9
Cyfluthrin	FPBA-Me	17.0	133, 159, 187, 215, 246	246	16.0 to 17.2
Cyhalothrin α-Cypermethrin Cypermethrin Deltamethrin Permethrin	m-PBA-Me	17.4	115, 141, 169, 197, 228	228	17.3 to 18.0

*DCCA-Me: methyl 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate
DBCA-Me: methyl (1R)-cis-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropanecarboxylate
m-PBA-Me: methyl 3-phenoxybenzoate
FPBA-Me: methyl 4-fluoro-3-phenoxybenzoate

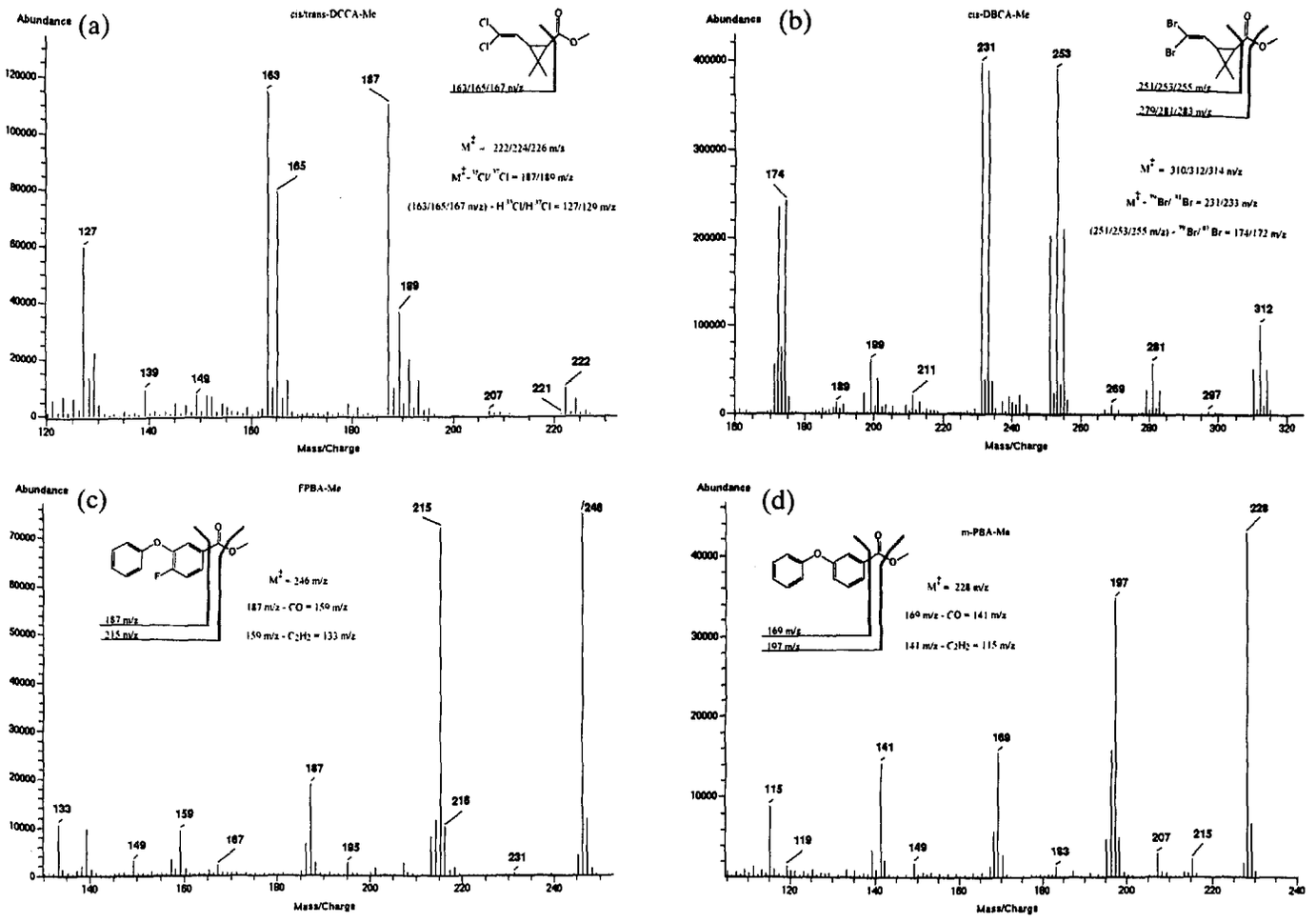

Figure 2a–d
Electron impact mass spectra (70 eV, 100 ng each, GC conditions as indicated) showing major key fragmentation patterns of cis and trans-DCCA-Me (a), cis-DBCA-Me (b), FPBA-Me (c) and m-PBA-Me (d).

three times with hexane (3 × 10 mL). Water residues within the collected organic layers were removed with anhydr. Na_2SO_4 (2 g). After filtration, concentration and reduction almost to dryness in a gentle stream of nitrogen, the residue was resolved in 0.5 mL iso-octane. An aliquot (1 µL) of this solution was subject to quantitative GC.

Gas Chromatography-Mass Spectrometry

The methylated derivatives of 3-PBA, FPBA, cis-DCCA, trans-DCCA and cis-DBCA were simultaneously separated by diastereoselective capillary GC using a Hewlett-Packard MS-Engine with a GC 5890, an autoinjector 7673 and a 5989 A mass selective detector equipped with an apolar HP Ultra 2 fused-silica capillary (50 m × 0.2 mm i.d., 0.37 µm film; Hewlett Packard equivalent to DB-5) attached to a 1 m retention gap deactivated fused-silica (Hewlett Packard). Injection volume was 1 µL (on-column) with helium carrier gas at constant 2.2 mL min^{-1}.

The injector was set at 90 °C and programmed from 90 °C at 300 °C min^{-1} to 300 °C, holding for 22 min.

The column was maintained at 90 °C and programmed from 90 °C at 40 °C min^{-1} to 130 °C, holding for 2 min and then at 10 °C min^{-1} to 270 °C, holding for 5 min.

The ion source was set at 200 °C and the MS interface held at 280 °C.

Electron impact ionisation (EI) was at 70 eV.

To enhance the sensitivity of the instruments and thus lower determination limits, the quadrupole-MS worked in the selected ion monitoring mode (SIM) with an EMV 400 (relative) and a dwell time of 100 ms. The major key fragmentation patterns used for identification and quantitation are in Table I and Figure 2 a–d.

External standard solutions of the free acid metabolites in methanol were prepared and aliquots were added to 5 mL water samples. Concentration was adapted to the determination limits and sample blanks of the compounds concerned. For calibration purposes each of these samples were treated like the urine samples. This procedure covers all possible losses during the whole workup process. Since only one internal standard is commercially available (o-PBA), quantitation was achieved by external calibration using peak areas of the fragmentation ion of interest.

Calibration curves of the methylated acid metabolites were linear from 0.5–500 µg L^{-1}.

Limits of determination were 0.5 µg L^{-1} for cis and trans-DCCA (187 m/z), and cis-DBCA (253 m/z) and 1 µg L^{-1} for 3-PBA (228 m/z) and FPBA (246 m/z). The detection limits (signal-to-noise rate: 3:1) were 0.2 µg L^{-1} for cis and trans-DCCA (187 m/z) and cis-DBCA (253 m/z) and 0.3 µg L^{-1} for 3-PBA (228 m/z) and FPBA (246 m/z). Both, limits of detection and determination were calculated according to VDI 2449 with an accuracy of p = 95 % [9].

For the metabolites cis and trans-DCCA, 3-PBA and FPBA (concentration 3-12 µg L^{-1}), a typical in-run coefficient of variation of real urine samples was 15 % (n = 6) and the average between-run coefficient of variation was 18 % (n = 8). The analysed samples were taken from people occupationally exposed to cyfluthrin and cypermethrin. Due to the lack of commercially available methylated acid metabolites, no additional calculation of the recovery could be performed.

Results

Isotopic Ratios of Cyclopropane Carboxylic Acid Moieties for Quality Assurance

Useful tools for unambiguous identification of halogen bearing acids, cis and trans-DCCA and cis-DBCA, are their characteristic isotopic patterns formated during EI. Identification of the characteristic isotopic patterns in real urine samples can be used for quality assurance.

Figures 3a and 3b show the mass chromatograms of standards bearing the isotopes $^{79}Br/^{81}Br$ and $^{35}Cl/^{37}Cl$. The abundance of the element chlorine is for a(^{35}Cl) ≈ 3.1 (75.5 %) and for b(^{37}Cl) ≈ 1.0 (24.5 %). Thus, the peak intensity distribution for 2 chlorine atoms is given by $(a + b)^2 = a^2 + 2ab + b^2$. Details of the calculation are given elsewhere [10]. Table II shows calculated and observed SIM peak intensities for the fragment ion indicated in Figure 3a. Within the precision of the instrument employed, calculated and observed peak intensities match well. Real urine samples from people exposed to cypermethrin and cyfluthrin were tested for their isotopic fragmentation patterns. The results demonstrate that the isotopic fragmentation patterns also match well, (Table II). It is also possible to derive other isotopic fragmentation patterns (e.g. 187–189 m/z with an isomeric ratio 3:1) from the mass spectra of Figure 2. SIM programs for metabolites of cyfluthrin and other important pyrethroids are indicated in Table I.

Efficiency of Hydrolytic Cleavage

To test the necessity for acid induced hydrolysis, a real urine sample from a subject exposed to cyfluthrin was split into four 5 mL aliquots (Figure 4a–d). The time of hydrolysis was varied between 5 and 120 min and the heating procedure was varied between no heating up to 90 °C. All other analytical steps remained the same. In Figure 4 it can clearly be seen that the optimal condition is hydrolysis time about 45–60 min and a heating temperature of 90 °C (Figure 4c). Further heating leads to a significant loss of trans-DCCA and FPBA. Without thermally induced hydrolysis the recovery of the cyclopropane moiety is only 2–4 %. Therefore, heating during hydrolysis is necessary for adequate recovery and for obtaining correct diastereomeric ratios.

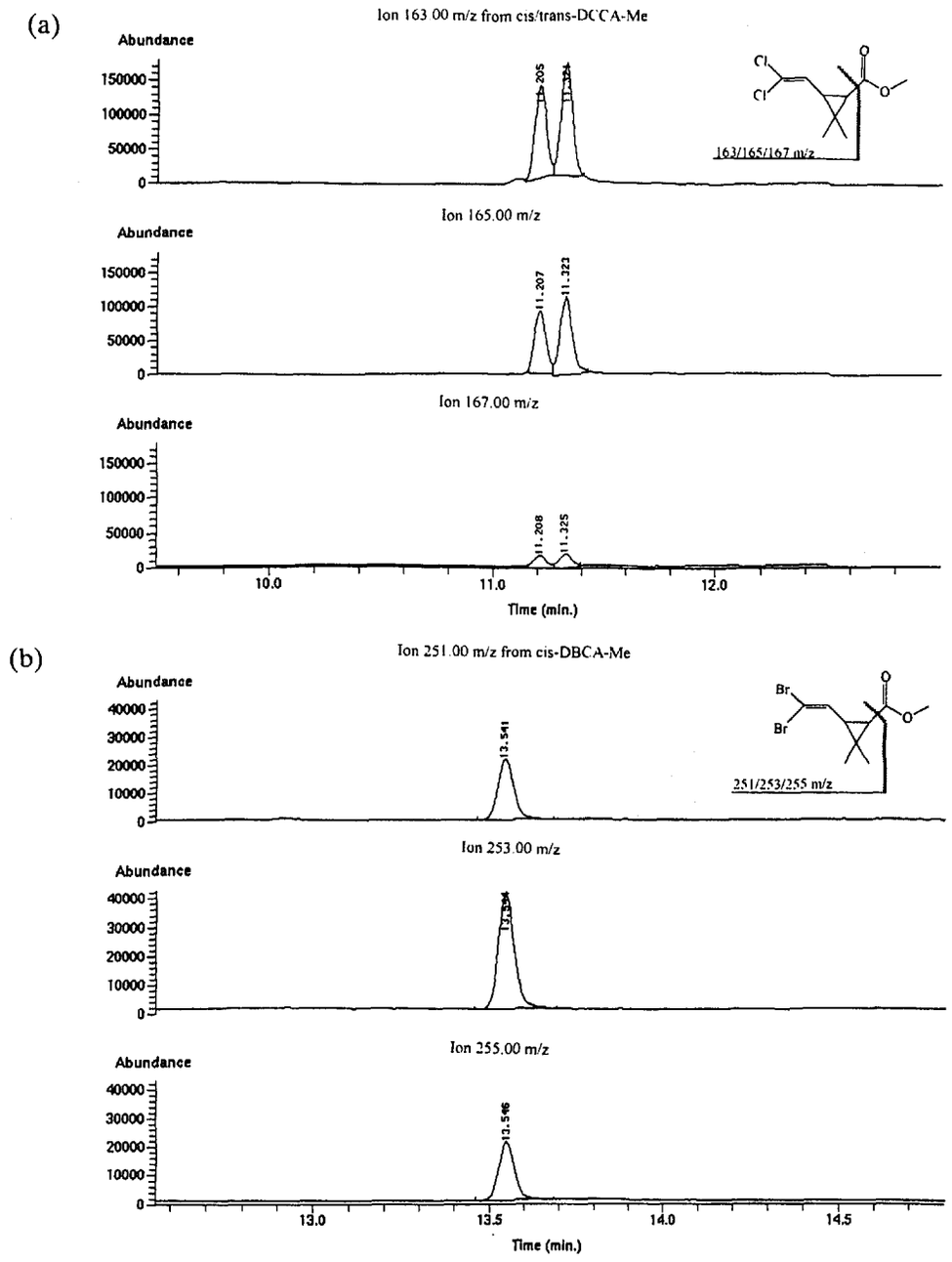

Figure 3a, b

Mass chromatograms of cis and trans-DCCA-Me standards (EI, 10 ng each) showing for ^{35}Cl:^{37}Cl isotope ratios 9:6:1 (a) and of cis-DBCA-Me standard (1 ng) showing for ^{79}Br:^{81}Br isotope ratios 1:2:1 (b). Details see text.

Table II. Calculated and observed SIM peak intensities for fragment ion 163, 165, 167 m/z in Figure 2a.

		Calculated values*		Cyfluthrin**		Cypermethrin**	
Isotopic combinations	Terms*	Relative peak intensities	Normalized [%]	cis-DCCA-Me [%]	trans-DCCA-Me [%]	cis-DCCA-Me [%]	trans-DCCA-Me [%]
$^{35}Cl/^{35}Cl$	a^2	9.6	57.1	61.8	57.4	58.9	61.6
$^{35}Cl/^{37}Cl$	2ab	6.2	36.9	31.8	36.9	35.8	32.4
$^{37}Cl/^{37}Cl$	b^2	1.0	5.9	6.4	5.7	5.3	6.0
sum	$(a + b)^2$	16.8	100	100	100	100	100

Mass spectra of various compounds generated with amounts 50–100 pg.

* calculated from: $(a + b)^2 = a^2 + 2ab + b^2$

** real 24 h urine samples from persons exposed to cyfluthrin and cypermethrin

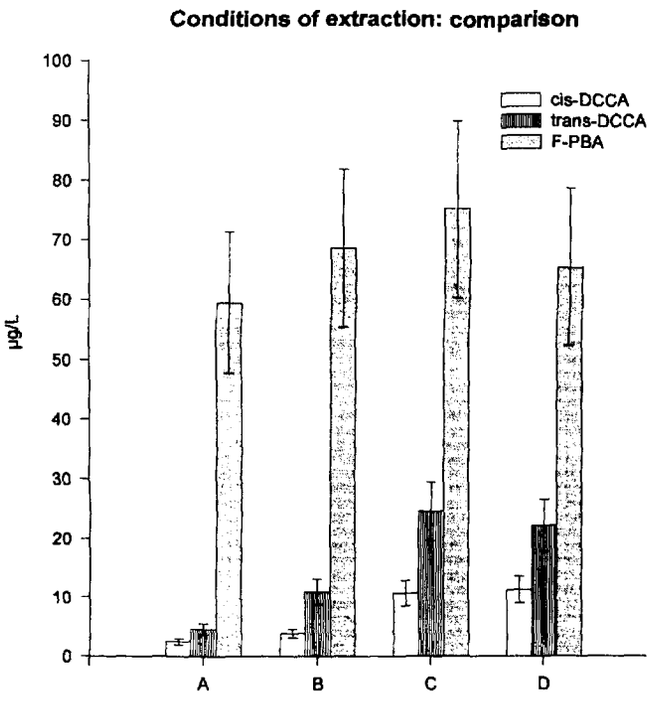

Figure 4
Acid induced hydrolysis of urine sample of subject exposed to cyfluthrin split into four 5 mL aliquots, (a) 25 °C, 1 mL conc. HCl, extraction after 5 min stirring, (b) 25 °C, 1 mL conc. HCl, extraction after 45 min stirring, (c) sample work-up according to present method, (d) 90 °C, 1 mL HCl, extraction after 120 min stirring. After extraction all samples treated as (c). Details see text.

Examples for Application of Analytical Method

In Figure 5a–c a typical SIM chromatogram of a 24 h urine sample from a pest control operator exposed to the pyrethroid cyfluthrin during indoor application is shown. The sample was monitored for the major cyfluthrin metabolites. The following concentrations were found: cis-DCCA 15.3 µg L^{-1} trans-DCCA 35.7 µg L^{-1} and FPBA 110.0 µg L^{-1}. The second mass chromatogram (Figure 6a–c) shows a 24 h urine sample from a pest control operator exposed to the pyrethroid cypermethrin during wood treatment. In this case, the following concentrations of metabolites were measured: for cis-DCCA 85.0 µg L^{-1}, for trans-DCCA 180.0 µg L^{-1} and for 3-PBA 475 µg L^{-1}.

All urine samples were taken after completion of the day's work in the exposure free time over the weekend according to the suggestions of Woolen [11] for accurate biological monitoring.

Discussion

It has been emphasized by Kutter and Class that the choice of moderate gas chromatographic conditions for diastereoselective separation of unchanged pyrethroids is essential, because natural pyrethrines and their synthetic analogues containing an α-cyano group like cypermethrin, cyfluthin and deltamethrin are sensitive to thermal isomerisation and peak broadening [12]. This complicates a useful interpretation of the results. However, in the present study it was shown that stereoisomers of the methyl esters of the metabolites 3-phenoxybenzoic acid (3-PBA-Me), 4-fluoro-3-phenoxybenzoic acid (FPBA-Me), cis and trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid (cis-trans-DCCA-Me) and cis 3-(2,2-dibromovinyl)-2,2-dimethylcyclopropane carboxylic acid (cis-DBCA-Me) could be chromatographed without problems. Diastereometric selectivity was generally achieved for the enantiomeric pairs of cis and trans-DCCA-Me and cis-DBCA-Me on nonpolar HP Ultra 2 or on various equivalent capillaries (DB 5 ms, SE-54, length 20–60 m, film 0.1–0.37 µm). The elution order was determined by injection of methylated single isomeric pairs. According to observations with pyrethroids like cis-permethrin [(1 RS, cis)-isomers] and trans-permethrin [(1 RS, trans)-isomers] [12] the enantiomeric pairs of DCCA-Me with the absolute configuration (1 RS, cis) at the C-1 cyclopropane atom elutes earlier than corresponding (1 RS, trans)-isomers. The (1 R, cis)-DBCA-Me is derived from deltamethrin (1R, cis, αS)-isomer, which is used as the single, most active isomer in pesticide formulations. The other, less polar esters 3-PBA-Me and FPBA-Me could also be chromatographed without problems.

The present analytical method uses a general liquid-liquid-extraction technique for simultaneous determination of major urinary pyrethroid metabolites. Furthermore, this simple method guarantees a noticeable decrease in work load and it is possible to monitor additional metabolites for which the older methods [6–8] were not intended.

The results of this study can be compared with those of other human studies with cypermethrin [6–8]. Moreover, this general method was used for the recently introduced ester pyrethroid cyfluthrin. Results obtained with this are comparable with those of the structurally related cypermethrin. Additionally, it has been demonstrated that FPBA is a characteristic urinary marker for cyfluthrin exposure

In agreement with Eadsford et al. [6, 7], preparation of the methyl esters with acidified methanol is cheap and efficient. Previous studies reported a detection limit of 0.5 µg L^{-1} [7, 8], whereas the present study yields a value of 0.2 µg L^{-1}.

Application of proper internal standards for lowering the coefficients of variations has only been solved for 3-PBA and remains a goal for all other metabolites.

Considering previous oral exposure studies to cypermethrin with volunteers conducted by Eadsford et al. and Woolen et al., approximately twice as much trans-DCCA was excreted in urine compared to cis-DCCA [6–8]. This is in agreement with excretion profiles in 12 to 24 h urine from pest control operators, obtained in this study. In the case of cypermethrin (pesticide formulation containing 50.5 % cis and 49.5 % trans-cypermethrin) the average urinary cis:trans-DCCA ratio was

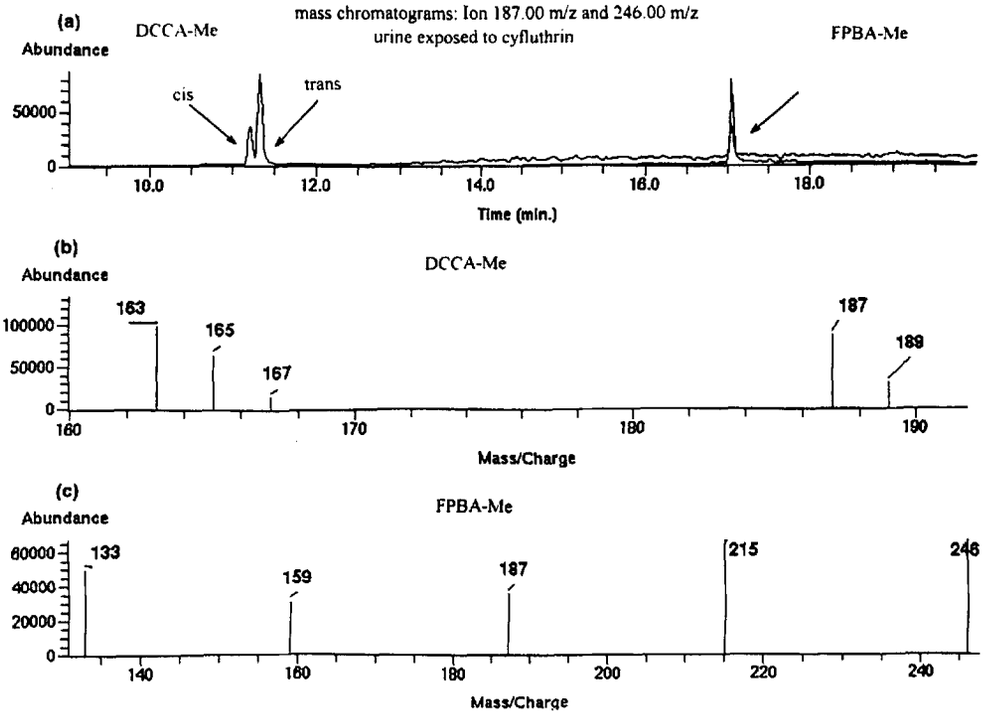

Figure 5a–c

Selected ion monitoring chromatograms of real 24 h urine sample from pest control operator exposed to cyfluthrin (SIM according to Table I): overlayed mass chromatograms for quantitation ions 187 m/z and 246 m/z (a); SIM program for quantitation of cis and trans-DCCA-Me (cis-DCCA: 15.3 μg L^{-1} trans-DCCA-Me: 35.7 μg L^{-1}, cis:trans: 1:2.3) (b); SIM program for quantitation of FPBA-Me (FPBA: 110 μg L^{-1}) (c); GC conditions as indicated.

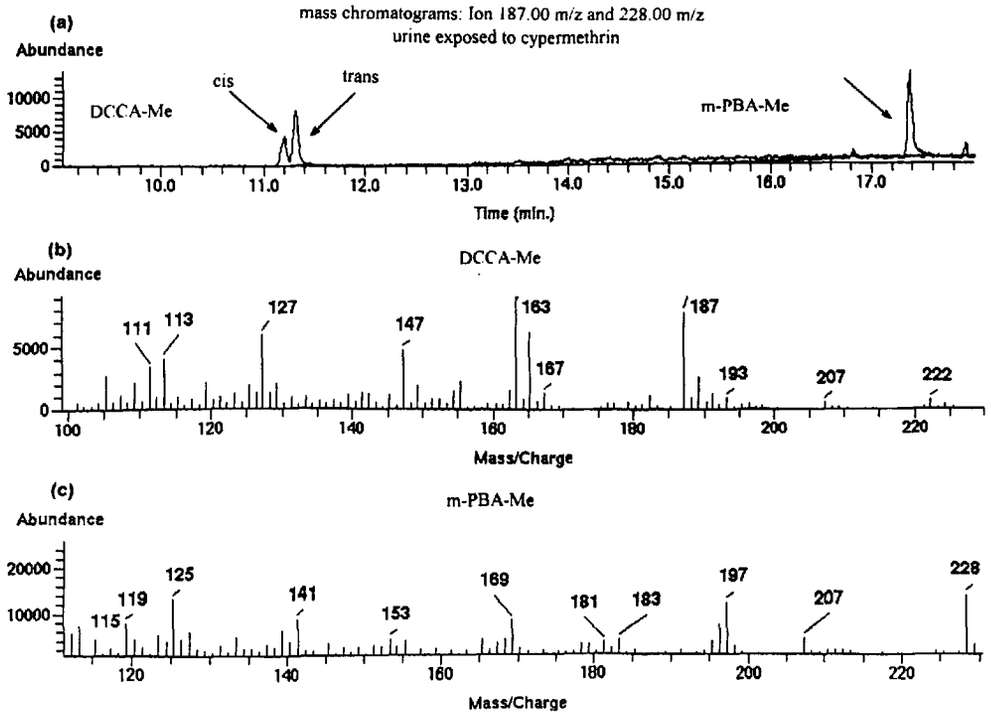

Figure 6a–c

Narrow mass range (EI 70 eV, 100–230 amu) chromatograms of real 24 h urine sample from pest control operator exposed to cypermethrin: overlayed mass chromatograms for quantitation ions 187 m/z and 228 m/z (a); mass spectrogram of cis and trans-DCCA-Me (free cis-DCCA: 85 μg L^{-1} trans-DCCA-Me:180 μg L^{-1}, cis/trans: 1:2.1) (b); mass spectrogram of m-PBA-Me (free m-PBA: 475 μg L^{-1}) (c); GC conditions as indicated.

1:2.1 (Figure 5) and for cyfluthrin (pesticide formulation containing 42 % cis and 58 % trans-cyfluthrin) 1:2.3 (Figure 6). As no cis-to-trans-conversion can be observed during acid hydrolysis and chromatography, a large excretion of trans-DCCA could be a clear sign of a significant oral or inhalation intake during pesticide application [6, 8].

Generally, an analytical method for biological monitoring of pyrethroid exposure under environmental as well as occupational conditions should yield correct values and, ratios of the different diastereomers concerned. Cis and trans-DCCA especially are the most useful urinary metabolites since their ratio depends highly on the route of administration (oral and inhalation versus dermal). Moreover, these metabolites were not detectable (limit of detection: 0.2 $\mu g\ L^{-1}$ signal-to-noise ratio 3:1) in urine samples of non-exposed subjects. Due to the fact that one trans-DCCA molecule is obtained from one trans-cypermethrin molecule, it is possible to calculate the pyrethroid equivalents eliminated. Whereas the phenoxybenzoic moiety is, for example, derived from all the cypermethrin stereoisomers. In the case of cyfluthrin, FPBA is specifically related only to this compound, whereas m-PBA can be obtained from different pyrethroids (permethrin, cypermethrin, deltamethrin). This information can be used additionally for an estimation of the extent of cyfluthrin exposure under the likely event of an application of formulations containing pyrethroid mixtures.

Conclusion

- Sensitive diastereoselective chromatography (GC-MS) of metabolic derivatives is adequate for the quantitation of pyrethroid metabolites in urine.
- Since in the case of non-exposure the urinary concentrations of the pyrethroid metabolites discussed are below limits of detection, they are suitable for biological monitoring of exposed persons.
- The major metabolites of the pyrethroids cypermethrin and cyfluthrin are detectable in urine samples collected 0–24 h after exposure.
- 4-fluoro-3-phenoxybenzoic acid (FPBA) is a urinary marker characteristic of exposure to cyfluthrin.
- The urinary metabolite profile of cyfluthrin obtained under occupational exposure conditions is comparable to cypermethrin.

Biological monitoring of pyrethroids based on urine measurements should be the preferred method for field studies designed to assess the dose of pyrethroids absorbed from various routes of exposure.

Acknowledgements

This study was supported by Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie (BMBF), Grant No. 07 INR 30. We thank Dr. Lewalter (Bayer AG, Leverkusen, Germany) for the donation of the metabolites, Mrs. Geicke for her excellent technical assistance and Dr. A. Leng for fruitful discussions.

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Received: May 13, 1996
Accepted: Jul 17, 1996