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Journal of Chromatography B, 778 (2002) 121–130

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

New gas chromatographic–mass spectrometric method for the determination of urinary pyrethroid metabolites in environmental medicine

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Abstract

We have developed and validated a new, reliable and very sensitive method for the determination of the urinary metabolites of the most common pyrethroids in one analytical run. After acidic hydrolysis for the cleavage of conjugates, the analytes *cis*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid (*cis*-Cl_2CA), *trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid (*trans*-Cl_2CA), *cis*-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid (Br_2CA), 4-fluoro-3-phenoxybenzoic acid (F-PBA) and 3-phenoxybenzoic acid (3-PBA) were extracted from the matrix with a liquid–liquid extraction procedure using *n*-hexane under acidic conditions. For further clean-up, NaOH was added to the organic phase and the carboxylic acids were re-extracted into the aqueous phase. After acidification and extraction into *n*-hexane again, the metabolites were then derivatised to volatile esters using *N-tert.*-butyldimethylsilyl-*N*-methyltrifluoroacetamid (MTBSTFA). Separation and detection were carried out using capillary gas chromatography with mass-selective detection (GC–MS). 2-Phenoxybenzoic acid (2-PBA) served as internal standard for the quantification of the pyrethroid metabolites. The limit of detection for all analytes was 0.05 μg/l urine. The RSD of the within-series imprecision was between 2.0 and 5.4% at a spiked concentration of 0.4 μg/l and the relative recovery was between 79.3 and 93.4%, depending on the analyte. This method was used for the analysis of urine samples of 46 persons from the general population without known exposure to pyrethroids. The metabolites *cis*-Cl_2CA, *trans*-Cl_2CA and 3-PBA could be found in 52, 72 and 70% of all samples with median values of 0.06, 0.11 and 0.16 μg/l, respectively. Br_2CA and F-PBA could also be detected in 13 and 4% of the urine samples.

Keywords: Biological monitoring; General population; Pyrethroid; Pesticides

1. Introduction

Synthetic pyrethroids are among the most effective insecticides used worldwide with a broad spectrum of applications. They are synthetic analogues of the pyrethrins, the naturally occurring insecticidal constituents of the flowers of *Tanacetum cinerarieafolium.* In formulations, they are usually present as a mixture of several stereo- and optical isomers [1]. In addition to their extensive use in agriculture, they are applied for the conservation of wool carpets, as wood preservative and in indoor pest control. They are also important in public health and veterinary medicine, where they are used as ectoparasiticides [1,2].

Pyrethroids are very lipophilic components with

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1570-0232/02/$ – see front matter
PII: S0378-4347(01)00452-2

neurotoxic properties, interacting with the sodium channel both in insects and mammals. In mammals, they are rapidly metabolised by cleavage of the central ester linkage, leading to non-toxic metabolites [3]. Consequently, the toxicity of pyrethroids for mammals is classed as low and the WHO has set acceptable daily intakes for pyrethroids like permethrin and cypermethrin of 0.05 mg/kg body weight [4].

However, recent investigations showed interindividual differences in pyrethroid metabolism, concerning the activity of carboxylesterases responsible for the detoxification. This is especially important in case of a mixed pesticide exposure, e.g. organophosphates, prolonging the elimination half-life of pyrethroids in plasma [5]. Fig. 1 shows the metabolic pathway of pyrethroids with permethrin as an example. Important pyrethroids with their corresponding metabolites are listed in Table 1.

The biological half-lives for different pyrethroids in blood after uptake via various routes has been determined to be between 2.5 and 12 h in mammals. As a consequence of this rapid metabolism, the concentrations of unchanged pyrethroids in blood and serum are considerably lower than the amount of metabolites excreted in urine. Therefore, the determination of urinary metabolites has been considered to be useful for the estimation of pyrethroid exposures [6].

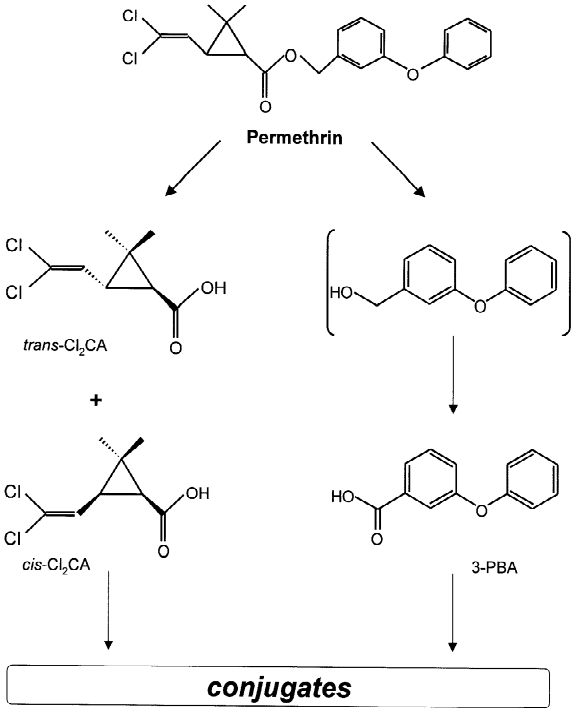

Fig. 1. Main metabolic pathway of permethrin in mammals.

Table 1
Pyrethroids with their corresponding metabolites

Pyrethroid	Metabolites
Permethrin	*cis*- and *trans*-Cl_2CA, 3-PBA
Cypermethrin	*cis*- and *trans*-Cl_2CA, 3-PBA
Deltamethrin	Br_2CA, 3-PBA
β-Cyfluthrin	*cis*- and *trans*-Cl_2CA, F-PBA

Several methods have been published for the determination of pyrethroid metabolites in human urine after occupational or experimental exposure [6–13]. Most methods achieved limits of detection for the various metabolites between 0.5 and 1.0 μg/l, which is not fully satisfactory for the determination of environmental exposure levels.

However, some studies published in recent years have revealed that the general population excretes pyrethroid metabolites [14–16,19]. But most of the values measured in these studies were below the limit of detection (LOD) for the various analytes.

For that reason, the need existed for a sensitive, reliable and practical method for the analysis of pyrethroid metabolites in the urine of the general population, allowing the determination of its background level.

This paper describes a very sensitive method for the quantitative determination of the major metabolites of the most important pyrethroids using a liquid–liquid extraction procedure with *n*-hexane and mass-selective detection after derivatisation with a silylating reagent. The method was applied in the analysis of 24-h urine samples of 46 persons from the general population.

2. Experimental

2.1. Chemicals and materials

Cis-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid (*cis*-Cl_2CA, certified assay:

99.8%), *trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid (*trans*-Cl_2CA, certified assay: 99.8%) and 4-fluoro-3-phenoxybenzoic acid (F-PBA, certified assay: 99.9%) were obtained from Bayer (Leverkusen, Germany); *cis*-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid (Br_2CA) (certified assay: 98.8%) was obtained from Roussel UCLAF (Paris, France).

3-Phenoxybenzoic acid (3-PBA, 99%) and 2-phenoxybenzoic acid (2-PBA, 98%) were obtained from Janssen Chimica (Geel, Belgium).

N-*tert.*-butyldimethylsilyl-*N*-methyltrifluoroacetatamid (MTBSTFA, 98%) was purchased from Merck-Schuchardt (Hohenbrunn, Germany). Toluene, *n*-hexane and hydrochloric acid (37%) were all of the highest analytical grade available and supplied by Merck (Darmstadt, Germany).

To make 0.1 N NaOH, 4 g of sodium hydroxide were dissolved in 1000 ml of bidistilled water.

2.2. The internal standard solution

The stock solution for the internal standard (IS) was prepared by dissolving 10 mg 2-PBA in 10 ml methanol (1 g/l). A 50-μl volume of this stock solution was diluted to the mark in a 50-ml glass volumetric flask with bidistilled water (1 mg/l). The resulting 2-PBA standard solution is used for the sample preparation (see Section 2.3.).

2.3. Sample preparation

An aliquot of 10 ml of the urine sample was transferred to a 20-ml glass vial with screw top and spiked with 100 μl of the internal standard solution. For the hydrolysis of conjugated carboxylic acids, 1 ml of concentrated hydrochloric acid (37%) was added and the samples were heated for 1 h at 90°C in an oven. Then the samples were cooled to room temperature.

The acidic urine samples were then extracted two times with 5 ml of *n*-hexane by mechanically shaking for 10 min. The samples were centrifuged for 5 min at 1500 *g* and the organic layers were taken up and combined in a 20-ml glass vial with screw top.

For further clean-up, 2 ml of 0.1 N NaOH were added to the organic phase and the carboxylic acids were re-extracted into the aqueous phase by mechanically shaking for 10 min. After centrifugation for 5 min at 1500 *g*, the organic phase was discarded.

The remaining aqueous phase was acidified by adding 100 μl of concentrated hydrochloric acid (37%) and once again extracted with 2 ml *n*-hexane. Following centrifugation at 1500 *g* for 5 min, 1.8 ml of the hexane extract was transferred to a 2-ml autosampler vial.

The extract was evaporated to dryness in a vacuum centrifuge (Speed Vac SC 110, Bachofer GmbH). The residue was dissolved in 50 μl of toluene and 10 μl of *N*-*tert.*-butyldimethylsilyl-*N*-methyltrifluoroacetamid (MTBSTFA) were added. The solution was then transferred to microvials and sealed with vial caps. For derivatisation, the vials were heated at 70°C for 45 min in an oven. A 1-μl volume of this sample was then analysed by GC–MS.

2.4. Calibration procedure and quality control

Five separate starting solutions were prepared by dissolving 10 mg of the metabolites *cis*-Cl_2CA, *trans*-Cl_2CA, Br_2CA, F-PBA and 3-PBA with toluene in five separate 10-ml glass volumetric flasks (1 g/l). From these starting solutions, a multi-component stock solution was prepared by diluting 100 μl of each starting solution to the mark with methanol in a 20-ml glass volumetric flask (5 mg/l). From this stock solution, two working solutions were prepared in methanol.

Working solution A: 1 ml of the stock solution was diluted to the mark in a 10-ml glass volumetric flask (500 μg/l).

Working solution B: 100 μl of the stock solution were diluted to the mark in a 10-ml glass volumetric flask (50 μg/l).

Five calibration standards with concentrations ranging from 0.25 to 5 μg/l were prepared from these working solutions by diluting with pooled urine (creatinine content: 0.37 g/l) or water. The calibration standards were stable for more than 12 months at −20°C.

Linear calibration curves were obtained by plotting the quotients of the peak areas of the pyrethroid metabolites and the internal standard as a function of the spiked concentration. These graphs were used to

ascertain the unknown concentrations of pyrethroid metabolites in urine samples.

For quality control purposes, we used pooled urine from laboratory personnel. Two pooled urines with creatinine contents of 0.55 and 1.06 g/l were spiked with metabolite concentrations of 1 and 0.4 μg/l, respectively. The pooled urines were divided into aliquots and stored at −18°C. For quality assurance, both control samples were included in each analytical series.

Recovery was calculated by analysing the spiked and unspiked pooled urine as described and comparing the results with the spiked amount of pyrethroid metabolites.

Within-series imprecision was determined by analysing the quality control sample (0.4 μg/l) eight times in one analytical run. Between-day imprecision was determined by analysing both quality control samples on 12 different days.

For the estimation of potential matrix influences, relative recoveries were also determined using five individual urine samples from different people without known exposure to pyrethroids, covering a wide range of creatinine contents (0.26–2.0 g/l). The urine samples were analysed unspiked and with a spiked concentration of 1 μg/l for each metabolite. Recoveries were calculated as described above.

As the *tert*.-butyl-dimethylsilyl derivatives of the pyrethroid metabolites are not commercially available, the absolute recovery of the analytes could only be calculated up to the derivatisation step. For this reason, a solution of the metabolites in 50 μl toluene (200 μg/l) was derivatised directly and analysed by GC–MS. The results obtained were compared with those of a processed urinary standard (1 μg/l).

2.5. Gas chromatography

Analysis was carried out on a Hewlett-Packard HP 5890 Series II plus gas chromatograph equipped with a Hewlett-Packard HP 7673 autosampler and a split/splitless injector operating in splitless mode. The inlet purge off time was 1 min. The operating temperature of the injector was 280°C.

Chromatographic separation was performed using a HP-35 capillary column (crosslinked 35% diphenyl-dimethylpolysiloxane, 60 m×0.25 mm I.D., 0.25 μm film thickness) purchased from Hewlett-Packard (Waldbronn, Germany).

Helium 5.0 was used as the carrier gas at a constant flow of 1.07 ml/min. The initial column temperature of 90°C was held for 1 min, then raised at a rate of 25°C/min to 120°C. It was then raised at a rate of 2.1°C/min to 240°C, held at this temperature for 1.5 min and finally raised at 25°C/min to 310°C, remaining at this temperature for 7 min. The injection volume was 1 μl. The retention times for the derivatised analytes and the internal standard under the described conditions are summarised in Table 2.

2.6. Mass spectrometry

A Hewlett-Packard HP MSD 5972 Series mass-spectrometer fitted with a quadrupole mass filter was used in electron impact (EI) mode. EI mass spectra of the derivatised analytes were obtained at an energy level of 70 eV and the electron multiplier voltage was 2300 V (+300 rel.). The MSD transfer line temperature was maintained at 300°C.

For the quantitative analysis of the pyrethroid metabolites, selected ion monitoring (SIM) was used and ions with the masses listed in Table 2 were monitored.

2.7. Human studies

In the present study, we investigated a group of 46 persons (26 females, 20 males) without known exposure to pyrethroids. The age of the individuals ranged from 17 to 61 with a median age of 34 years.

Twenty-four-hour urine samples were collected by these persons in sealable plastic bottles and stored in

Table 2
Retention times and detected masses of the pyrethroid metabolites

tert.-Butyl-dimethylsilyl derivatives of	Retention time (min)	Detected masses
cis-Cl_2CA	27.36	265, 267, 128, 307
trans-Cl_2CA	28.40	265, 267, 128, 307
Br_2CA	36.72	355, 353, 357, 172
2-PBA (IS)	50.36	271, 227, 197
F-PBA	51.30	289, 245, 215
3-PBA	53.14	271, 227, 197

Quantifier ions are underlined.

the freezer at −18°C until sample preparation. The creatinine content of these urine samples was between 0.36 and 2.82 g/l with a median value of 0.91 g/l.

3. Results and discussion

In order to achieve a limit of detection below 0.1 μg/l, we used a liquid–liquid extraction procedure under acidic conditions with *n*-hexane and temporary re-extraction of the carboxylic acids into the basic aqueous phase [16]. Additionally, we introduced a new derivatisation reagent (MTBSTFA). Fig. 2 shows a survey of the whole clean-up procedure.

Our method can be performed quickly and routinely with satisfactory limits of detection, does not require high quantities of organic solvents or expensive chemicals and does not need special devices like high resolution mass spectrometry. Thus, it has the requirements of a routine method for the determination of pyrethroid metabolites in the environmental concentration range.

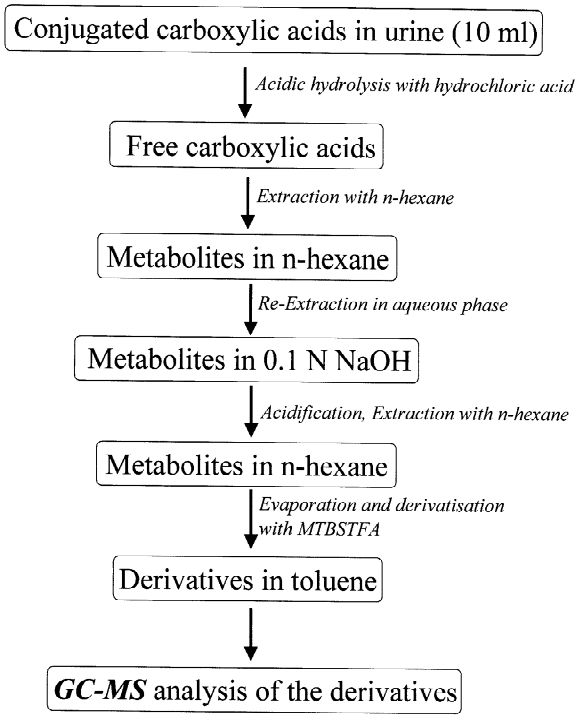

Fig. 2. Scheme for sample preparation.

3.1. Clean-up and derivatisation

For an effective clean-up from the urinary matrix, we used liquid–liquid extraction with *n*-hexane under acidic conditions. The use of this totally non-polar solvent diminishes the interfering analytical background very efficiently.

Furthermore, the re-extraction of the acidic metabolites into the aqueous phase with 0.1 N NaOH additionally separates the analytes from more non-polar components in the matrix, resulting in very clean extracts.

The dried extract was derivatised with MTBSTFA in toluene. The derivatisation procedure is quick and easy to perform and produces good and reproducible results for GC–MS. In fact, the fragmentation of the derivatives in the EI-mode of the mass spectrometer is favourable, because the loss of the *tert.*-butyl-moiety results in very stable ions with rather high *m*/*z*-ratios, increasing the sensitivity of the method. So, we could use the mass spectrometer in the more stable and robust EI-mode, which is important for routine analysis. Fig. 3 shows the mass spectrum of derivatised *trans*-Cl_2CA.

A further advantage of this procedure is that the derivatisation step leading to the more volatile *tert.*-butyl-dimethylsilylesters is carried out in sealed vials as the last step, so that no losses of analyte can occur during concentration of the extracts.

3.2. GC–MS analysis

The *tert.*-butyl-dimethylsilyl derivatives of the cyclopropanecarboxylic acids (*cis*- and *trans*-Cl_2CA, Br_2CA) were registered with four masses each. The phenoxybenzoic acids, including the internal standard (F-PBA, 3-PBA, 2-PBA) were registered with three masses. At least two fragment ions are still detectable at the limit of detection. The mass ratios of the quantifier ions of the analytes and the internal standard were used for quantification. The metabolites were identified both by their retention times and the mass ratio of the detected fragment ions, resulting in high specifity.

The analytes were very effectively separated from the interfering analytical background on the HP-35 column. Other columns, for example a DB 35-MS column, surprisingly seemed less suitable for this

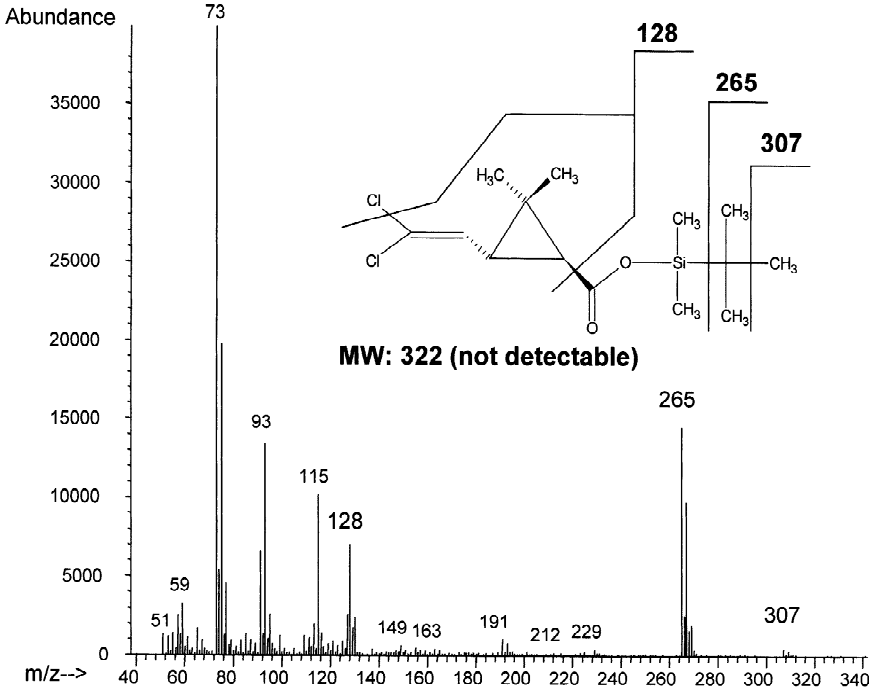

Fig. 3. Mass spectrum of derivatised *trans*-Cl_2CA.

separation, producing fronting peaks and more interference in the recorded ion traces.

3.3. Reliability of the method

3.3.1. Calibration graphs

The calibration graphs were linear for all five metabolites in the range of 0.25–5.0 μg/l. In each case, the correlation coefficients of the calibration graphs were higher than 0.998.

No pyrethroid metabolites were detectable in the unspiked pooled urine used for the calibration curves. Calibration curves generated from urinary standards yielded slightly higher slopes (about 5%) than aqueous standards. This is in the range of the standard deviation of the method and indicates that there are no significant matrix effects as a result of the very efficient clean-up procedure.

3.3.2. Precision and accuracy

The RSD of the within-series imprecision was determined to be between 2.0 and 5.4% at a spiked concentration of 0.4 μg/l. The relative recoveries for the different pyrethroid metabolites were found to be between 79.3 and 93.4% at the spiked concentration, taking into account the determined background level for this pooled urine.

The analysis of the unspiked quality control urine revealed concentrations of 0.08, 0.15 and 0.14 μg/l for *cis*-Cl_2CA, *trans*-Cl_2CA and 3-PBA, respectively. Br_2CA and F-PBA could not be detected in the unspiked urine.

Between-day imprecision was determined by analysing two quality control samples on 12 different days. The RSD of the between-day imprecision was in the range of 11.4–19.5% at a spiked concentration of 0.4 μg/l and 9.1–15.8% at a spiked concentration of 1 μg/l.

To provide even more information, we determined the accuracy of our method using individual urine samples from five different people. The urine samples covered a wide range of creatinine contents (0.26–2.0 g/l) and therefore serve very well for an estimation of matrix influences. The calculated re-

coveries were between 95 and 108.5% for the different metabolites. The creatinine content did not show any significant influence on these parameters. As a result, matrix effects could not be observed for our method.

The absolute recoveries up to the derivatisation step during the analytical procedure were calculated to be in the range of 76.6–125.1%, depending on the analyte. The precision and accuracy data are presented in Table 3.

The validation data for the method presented are very good, taking into account the low concentration range. At higher concentrations, Arrebola et al. reported recoveries between 89.9 and 121.1% with RSD of about 14% at a concentration of 10 μg/l [18]. Angerer and Ritter achieved recoveries in the range of 90–98% with between-run coefficients of variation of 6.3–8.8% at a concentration of about 10 μg/l [7].

3.3.3. External quality control

The results obtained by the new method were assured by participation at an interlaboratory comparison program organised in Germany for the determination of pyrethroid metabolites in human urine. Our results corresponded very well with the theoretical values of the spiked urine samples and the results of other participating laboratories ($n=7$). This is another hint for the good accuracy of the method presented.

3.3.4. Detection limit

No pyrethroid metabolites were detectable in bidistilled water as blank samples. The pooled urine used for calibration purposes was checked for the concentration of analytes. This urine turned out to be very suitable for the calibration, as no metabolites could be detected. The limit of detection, defined as a signal-to-noise ratio of three, for the registered quantifier ions listed in Table 2 was considered to be 0.05 μg/l. At this concentration, at least two of the recorded mass fragments of each analyte can be detected.

3.3.5. Sources of error

The solution for the silylating procedure must be completely dry. As the method presented does not include a drying step with Na_2SO_4 or molecular sieves, the hexane phase in the final extraction step should be taken up carefully, avoiding contamination with water.

Occasionally, we observed an interfering peak in the region of the internal standard. Although a baseline separation could not be achieved under the conditions described, it does not influence the quantification of the analytes, as both peaks are adequately separated.

3.4. Results of biological monitoring

Our method has been applied to detect and quantify pyrethroid metabolites in the urine of non-occupationally exposed subjects. The results of the biomonitoring of 46 persons (26 females, 20 males) are summarised in Table 4. Fig. 4 shows the chromatogram of a processed urinary standard and

Table 3
Precision and accuracy data for the analytes

Metabolite	Within-series: Pooled urine $n=8$ (creatinine: 1.06 g/l); spiked concentration 0.4 μg/l		Between-day: Pooled urine $n=12$ (creatinine: 1.06 g/l); spiked concentration 0.4 μg/l		Pooled urine $n=12$ (creatinine: 0.55 g/l); spiked concentration 1.0 μg/l		Individual urines ($n=5$); spiked concentration 1.0 μg/l	Absolute recovery (%) up to derivatisation step; spiked concentration 1.0 μg/l
	Imprecision (%)	Accuracy (%)	Imprecision (%)	Accuracy (%)	Imprecision (%)	Accuracy (%)	Accuracy (%)	
cis-Cl_2CA	2.0	88.5	12.3	85	12.4	102	103	76.6
trans-Cl_2CA	5.4	93.4	11.4	100	11.8	94	108	77.7
Br_2CA	2.1	79.3	17.1	85	9.1	81	95	84.4
F-PBA	3.7	90.0	19.5	98	15.5	98	102	117.1
3-PBA	2.8	84.7	17.9	99	15.8	106	99	125.1

Accuracy given as the average relative recovery.

Table 4
Results of biological monitoring for pyrethroid metabolites in urine of the general population ($n=46$)

	cis-Cl_2CA	*trans*-Cl_2CA	Br_2CA	F-PBA	3-PBA
$n>$LOD (%)	52	72	13	4	70
Median (μg/l)	0.06	0.11	<0.05	<0.05	0.16
95th percentile (μg/l)	0.29	0.64	0.17	<0.05	0.67
Max. value (μg/l)	1.5	3.5	0.4	0.2	1.7

The limit of detection (LOD) was 0.05 μg/l for each analyte.

the urine sample of a person of the examined collective.

Our results confirm a baseline excretion of pyrethroid metabolites in the general population already published by other authors [14–16]. According to these authors, the most probable reason for this excretion may be the uptake of pyrethroids with the daily diet.

Using this method, it was possible to detect the urinary metabolites in up to 72% of the urine samples of the general population. As a result of this study, the method presented here has proved its suitability for the routine analysis of urinary pyrethroid metabolites in environmental medicine.

3.5. Comparison with other methods

So far, only a few methods suitable for the determination of the background level of urinary pyrethroid metabolites in the general population have been published [15–19]. Liquid–liquid extraction is the most commonly used sample preparation technique [16,17,19], a few methods involve extraction of urine on RP-18-solid-phase cartridges [15,18].

Gas chromatography with mass-selective detection is mostly used for the detection and quantification of the metabolites. Recently, a HPLC–MS–MS method has been published, allowing the determination of 3-PBA with a LOD of 0.5 μg/l [19].

In some cases, methylation of the metabolites was carried out for derivatisation in GC–MS, either using diazomethane [16] or a mixture of concentrated sulphuric acid and methanol [15].

The most sensitive methods published so far used a carbodiimide-coupled esterification with hexafluoroisopropanol as derivatisation procedure. Using capillary gas chromatography with high resolution mass spectrometry in negative chemical ionisation mode, Leng et al. achieved a limit of detection of 0.03 μg/l for all analytes [17]. Arrebola et al. stated a limit of detection for the pyrethroid metabolites between 2 and 19 ng/l using GC-ion trap in EI-mode [18].

Both methods either require devices that are not easily available in every laboratory or have a rather time-consuming clean-up procedure. Consequently, the applicability of these methods could not be demonstrated in the general population. In fact, Leng et al. investigated only one occupationally not exposed person, whereas Arrebola et al. analysed urines of just two control persons.

4. Conclusions

Summarising, we have developed and validated a method for the trace analysis of pyrethroid metabolites in urine of the general population. The method presented here is precise, accurate and very sensitive. The data for accuracy and imprecision can be described as excellent, even at concentrations well below 1 μg/l. Matrix influences or severe interferences in chromatography were not observed during our study.

The method proved to be practicable and appropriate for routine analysis. The limit of detection of 0.05 μg/l for all analytes is sufficiently low for the determination of the background level in the general population.

Acknowledgements

This work was financially supported by the Verum Foundation. Furthermore, we would like to thank Dr Gerda Philippeit for providing the urine samples of the collective.

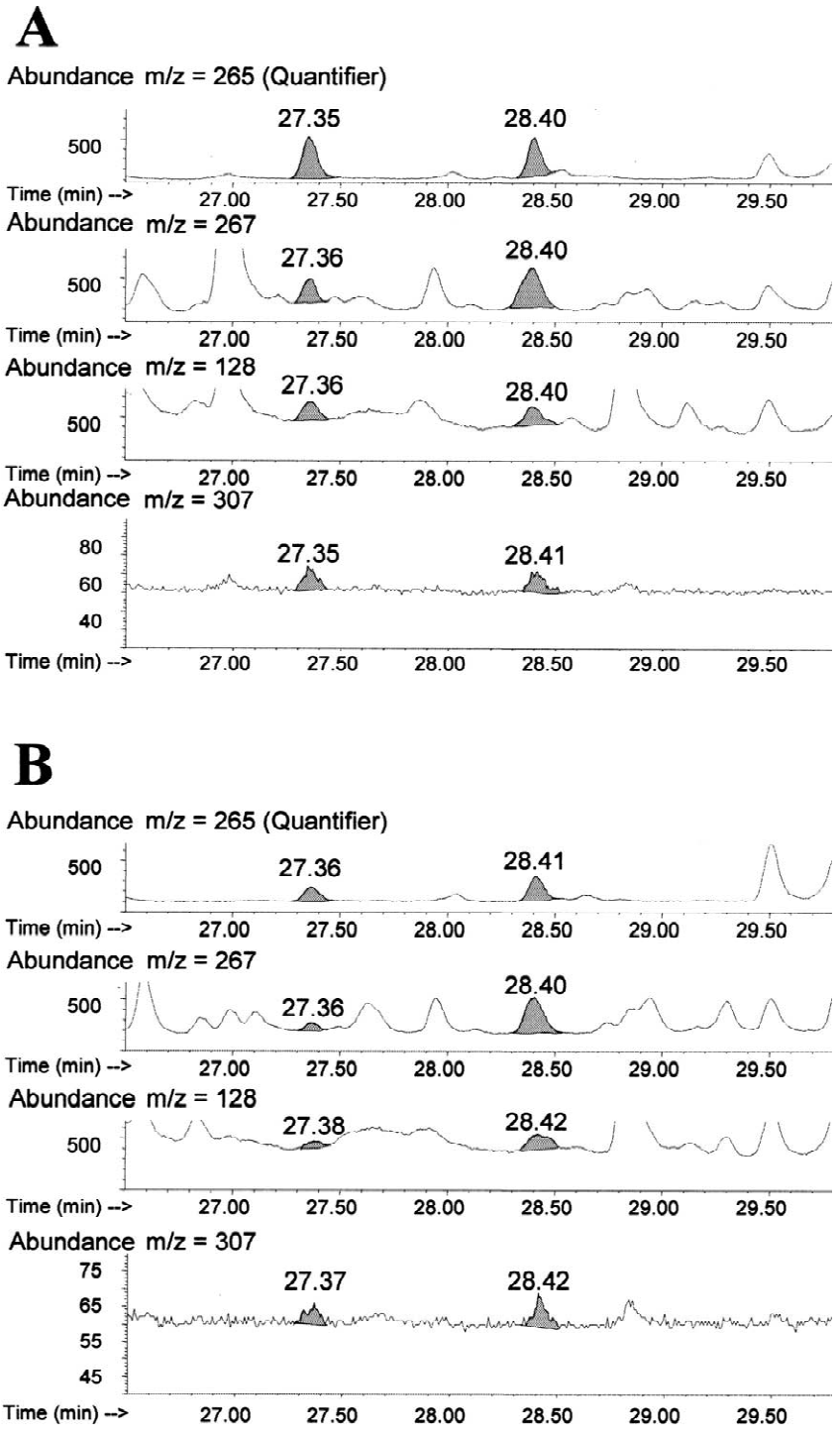

Fig. 4. GC–EI-MS-SIM chromatogram of a processed urinary standard (0.25 μg/l) (A) and a urine sample of a person without known exposure to pyrethroids (B) ($R(t)$=27.35 min: *cis*-Cl_2CA: 0.10 μg/l; $R(t)$=28.40 min: *trans*-Cl_2CA: 0.16 μg/l).

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