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ORIGINAL RESEARCH

A solid phase extraction reversed-phase HPLC method for the simultaneous determination of methoprene, permethrin and selected metabolites in rat plasma and urine

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ABSTRACT: A method was validated and applied for the analysis of the insect growth regulator methoprene [Isopropyl (2E,4E)-11-methoxy-3,7,11-trimethyldodeca-2,4-dienoate], its metabolite methoprene acid, the insecticide permethrin [3-(2,2-dichloro-ethenyl)-2,2-dimethylcyclopropanecarboxylic acid(3-phenoxyphenyl)methylester], and two of its metabolites, *m*-phenoxybenzyl alcohol and *m*-phenoxybenzoic acid, in rat plasma and urine using solid-phase extraction and reversed-phase high performance liquid chromatography. The analytes were separated using gradient of 55–100% acetonitrile in water (pH 4.0) at a flow rate ranging between 0.6 and 1.0 mL/min over a period of 20 min, and UV detection at 210 and 254 nm. The retention times ranged from 7.3 to 18.4 min. The limits of detection ranged between 50 and 100 ng/ml, while limits of quantitation were 100–150 ng/mL. Average percentage recovery of five spiked plasma samples was 83.6 ± 3.9, 80.1 ± 5.4, 82.1 ± 4.4, 83.7 ± 3.9 and 83.1 ± 4.7, and from urine 79.3 ± 4.3, 82.0 ± 5.4, 80.7 ± 4.2, 78.9 ± 5.7 and 83.9 ± 4.5 for methoprene, methoprene acid, permethrin, *m*-phenoxybenzyl alcohol and *m*-phenoxybenzoic acid, respectively. The method was linear and reproducible over the range of 100–1000 ng/mL. This method was applied to analyze the above chemicals and metabolites following their combined administration in rats. Copyright © 2001 John Wiley & Sons, Ltd.

INTRODUCTION

Methoprene [Isopropyl (2E,4E)-11-methoxy-3,7,11-trimethyldodeca-2,4-dienoate] is an insect growth regulator used to control mosquito larva (WHO/FAO data sheets on pesticides, 1984, 47). It interferes with an insect life cycle and prevents it from reaching maturity or reproducing (Dame *et al.*, 1998; Morello *et al.*, 1980). It is used in aquatic areas to control mosquitoes and several types of flies, moths, and fleas (EPA, 1991). Recently, it has been used by several municipalities in the USA for the combat of the West Nile Virus (*Rachel's Environment and Health Biweekly*, 2000, 709; New York City Department of Health, 2000a). When exposed to sunlight, methoprene breaks down into a class of chemicals closely related to retinoids chemicals, which can cause birth defect in humans (Rachel's Environment and Health Biweekly, 2000, 623; Alvarez *et al.*, 2000). According to Oberlander *et al.* (2000), methoprene significantly inhibits cell proliferation after exposure in lepidopteron imaginal cell line. Published studies showed that methoprene was absorbed, metabolized mainly through ester hydrolysis and excreted following oral dose in rats (Hawkins *et al.*, 1977, Wright, 1976), in chickens (Davison, 1976; Quistad *et al.*, 1976), and in guinea pigs and cows (Chamberlain *et al.*, 1975).

Permethrin [3-(2,2-dichloro-ethenyl)-2,2-dimethylcyclopropanecarboxylic acid(3-phenoxyphenyl)methylester] is a widely used insecticide inside homes and in public places to control adult mosquitoes (WHO, 1990; EPA, 2000). It modifies sodium channels to open longer during a depolarization pulse (Narahashi, 1985). Permethrin has been reported to be absorbed into plasma, metabolized and excreted as metabolites in the urine following oral or intravenous dose in rats (Anadon *et al.*, 1991), and in rabbits (Leng *et al.*, 1997). Several analytical methods have been used for identification and quantification of methoprene, permethrin and their metabolites when applied alone in plasma and urine samples. These methods use high performance liquid chromatography (Hunt and Gilbert, 1976; Anadon *et al.*, 1991; Leng *et al.*, 1997), gas chromatography (Miller, 1975; Hunt and Gilbert, 1976; Miller *et al.*, 1975; Sondgrass, 1992), gas chromatography–mass spectrometry (Angerer and Ritter, 1997), and thin-layer chromatography (Hawkins *et al.*, 1977).

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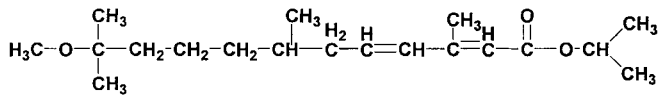
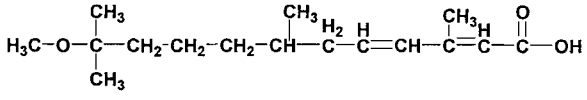
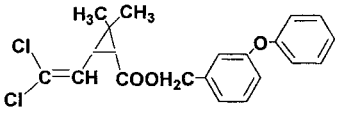
Abbreviations used: LOD, limit of detection; LOQ, limit of quantitation.



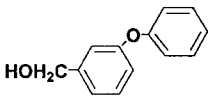
Methoprene



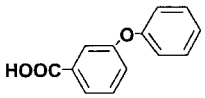
Methoprene acid



Permethrin



m-Phenoxybenzyl alcohol



m-Phenoxybenzoic acid

Figure 1. Structures of methoprene, methoprene acid, permethrin, *m*-phenoxybenzyl alcohol and *m*-phenoxybenzoic acid.

In their efforts to combat the deadly West Nile Virus, local authorities in the USA are using methoprene to kill mosquito larvae in catch basins and other standing water, concurrently applying pyrethroide insecticides such as permethrin to control adult mosquitoes (*Rachel's Environment and Health Biweekly*, 2000, 709; New York City Department of Health, 2000b). Also, both methoprene and permethrin are concurrently sprayed in animal shelters against mosquitoes (Benchaoui *et al.*, 2000). As a result, people could be exposed to a combination of methoprene and permethrin, causing potential toxic interactions. We plan to study the pharmacokinetic profile of each compound alone and in combination. To carry out that, a method is needed for simultaneous analysis of the two compounds and their metabolites in plasma and urine samples. In this study we present a reliable method for simultaneous analysis of methoprene, permethrin and their metabolites in rat plasma and urine using solid-phase extraction coupled with reversed-phase high performance liquid chromatography.

EXPERIMENTAL

Chemicals and materials. Methoprene acid [Fig. 1; 99% isopropyl (2E,4E)-11-methoxy-3,7,11-trimethyldodeca-2,4-dienoate acid], *m*-phenoxybenzoic acid, and *m*-phenoxybenzyl alcohol were obtained from Sigma Chemical Co. (St Louis, MO, USA). Methoprene [99% isopropyl (2E,4E)-11-methoxy-3,7,11-trimethyldodeca-2,4-dienoate], and permethrin [3-(2,2-dichloroethenyl)2,2-dimethylcyclopropanecarboxylicacid(3-phenoxyphenyl) methylester] were obtained from Chem Service, Inc. (West Chester, PA, USA). C_{18} Sep-Pak cartridges were obtained from Waters Corporation (Waters Corporation, Milford, MA, USA). Water (HPLC grade) and acetonitrile were obtained from Mallinckrodt Baker Inc. (Paris, Kentucky, USA).

Animals. Rats (Sprague–Dawley) were purchased from Zivic Miller (Zelienople, PA, USA). The animals were kept in plastic metabolic cages. Five rats were treated with a single oral dose of 10 mg/kg of methoprene in corn oil, and a single dermal dose of 1.3 mg/kg of permethrin applied in ethanol on a 1 cm^2 shaved area on the back of the neck of each rat. Five untreated control rats were treated with dermal dose of ethanol. The animals were held in metabolic cages to allow collection of urine samples. Urine samples were collected from treated and control rats 12 h after dosing. The animals were anesthetized with halothane and sacrificed by heart exsanguinations at 12 h. Blood was collected via heart puncture with a heparinized syringe and centrifuged at 2400 rpm for 15 min at 5°C to separate plasma. Urine and plasma samples were stored at −20°C prior to analysis.

Instrumentation. The liquid chromatographic system (Waters 2690 Separation Module) consisted of a Waters 600E Multisolvent delivery system pump, a Waters Ultra WISP 715 auto injector, and a Waters 2487 Dual λ absorbance detector (Waters Corporation, Milford, MA). A guard column (Supelco, 2 cm × 4.0 mm, 5 μm; Supelco Park, Bellefonte, PA), and a reversed-phase C_{18} column (μBondapak™ C_{18} 125A° 10 μm, 3.9 × 300 mm; Waters Corporation, Milford, MA) were used.

Sample preparation. Plasma and urine samples (0.5 mL) from untreated rats were spiked with concentrations ranging between 100 and 000 ng/mL of each of methoprene, methoprene acid, permethrin, *m*-phenoxybenzyl alcohol and *m*-phenoxybenzoic acid. Spiked and treated samples were acidified with 100 μL of 1 N acetic acid (pH 5.0). Disposable C_{18} Sep-Pak Vac 3 cm^3 (500 mg) cartridges were conditioned with 3 mL of acetonitrile, and then equilibrated using 3 mL of water prior use. The spiked urine and plasma samples were vortexed for 30 s, centrifuged for 5 min at 1000 ***g***, and the supernatant was loaded into the disposable cartridges, then washed with 3 mL of water, and eluted by 2 mL of methanol, then by 2 mL of acetonitrile and reduced in a marked test tube to 500 μL using a gentle stream of nitrogen, prior to analysis by HPLC.

Chromatographic conditions. A 10 μL solution of plasma or urine residues was injected into HPLC. The mobile phase was a water (adjusted to pH 4.0 using 1 N acetic acid):acetonitrile gradient at flow rate of 0.6 mL/min starting from zero time to 9 min, increased to 1 mL/min by 10 min, then returned to 0.6 mL/min at 18 min. The gradient started at 55% acetonitrile, increased

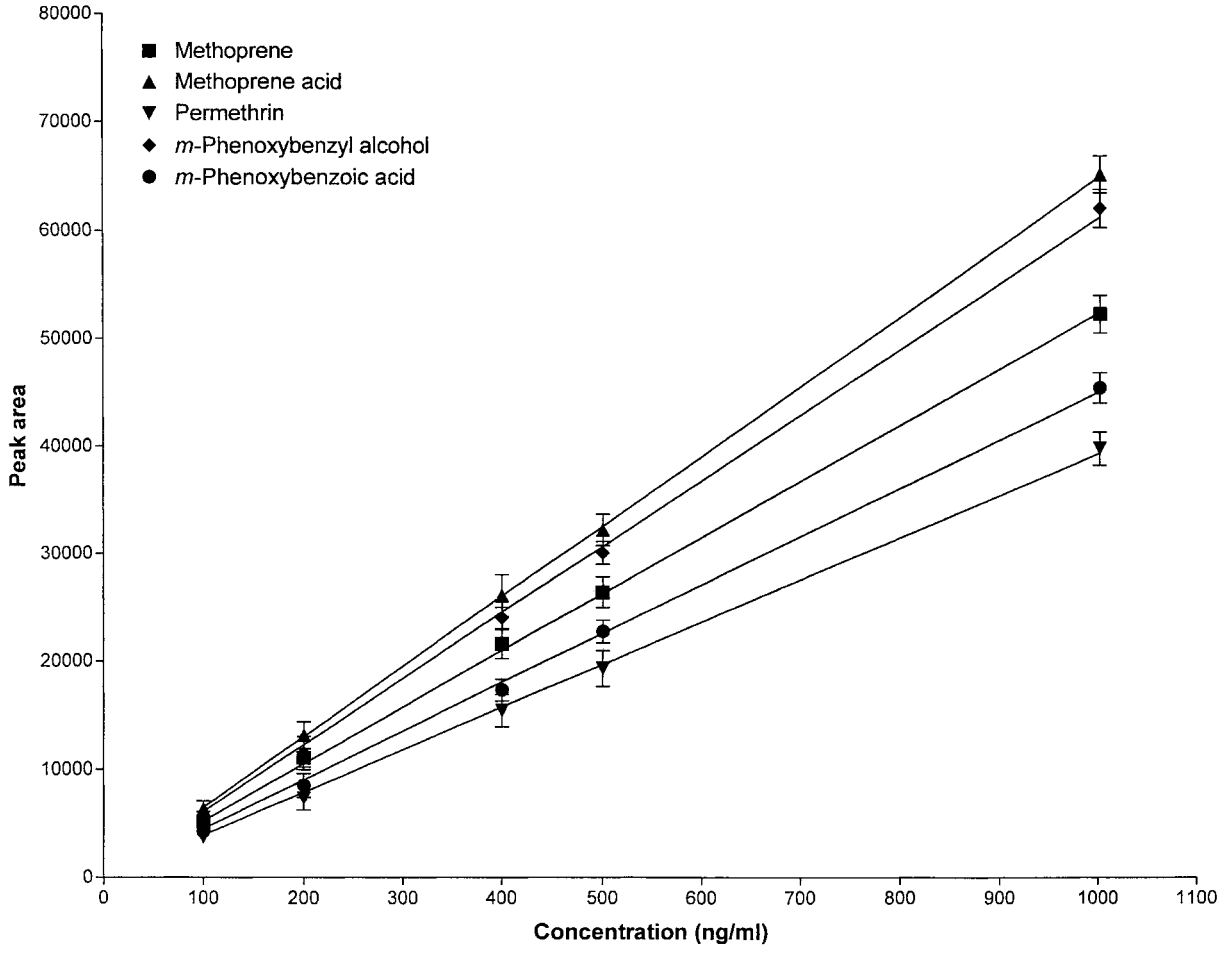


Figure 2. Standard calibration curves of methoprene, methoprene acid, permethrin, *m*-phenoxybenzyl alcohol and *m*-phenoxybenzoic acid.

to 60% acetonitrile at 10 min, then increased to 80% acetonitrile by 13 min. Then the system returned to 55% acetonitrile at 18 min, and was kept under this condition for 2 min to re-equilibrate. The eluents were monitored by UV detection of wavelength of 254 nm for methoprene and methoprene acid and at 210 nm for permethrin, *m*-phenoxybenzyl alcohol and *m*-phenoxybenzoic acid. The total run time was 20 min. The chromatographic analysis was performed at ambient temperature. Compound standards were injected alone and in mixture into the HPLC.

Calibration procedures. Five different calibration standards of a mixture of methoprene, methoprene acid, permethrin, *m*-phenoxybenzyl alcohol and *m*-phenoxybenzoic acid were prepared in acetonitrile. Their concentrations ranged from 100 to 1000 ng/mL. Linear calibration curves were obtained by plotting the peak areas of the individual chemicals as a function of the concentration. Then r^2 was determined for each compound using GraphPad Prism program for windows (GraphPad Software, Inc., San Diego, CA, USA).

Accuracy and precision. Intra-day precision and accuracy of the method were determined in plasma and urine samples spiked with the analytes. Plasma and urine samples ($n = 5$) were spiked with concentrations of 100, 200, 400, 500 and 1000 ng/mL. The samples were analyzed on the same day. The relative error percentage accuracy was determined as mean of detected concentration/added concentration × 100. For determination of precision, the coefficient of variation (CV) was calculated.

Limits of detection (LOD) and limits of quantitation (LOQ). Limits of detection and limits of quantitation were detected or quantified, taking into consideration a 1:3 and 1:10 noise level:calibration point ratio, respectively. The LOQ was repeated five times for confirmation.

RESULTS

Standard calibration curves

The standard calibration curves of peak area against concentration of methoprene, methoprene acid, permethrin, *m*-phenoxybenzyl alcohol and *m*-phenoxybenzoic acid are shown in Fig. 2. Linearity of the calibration

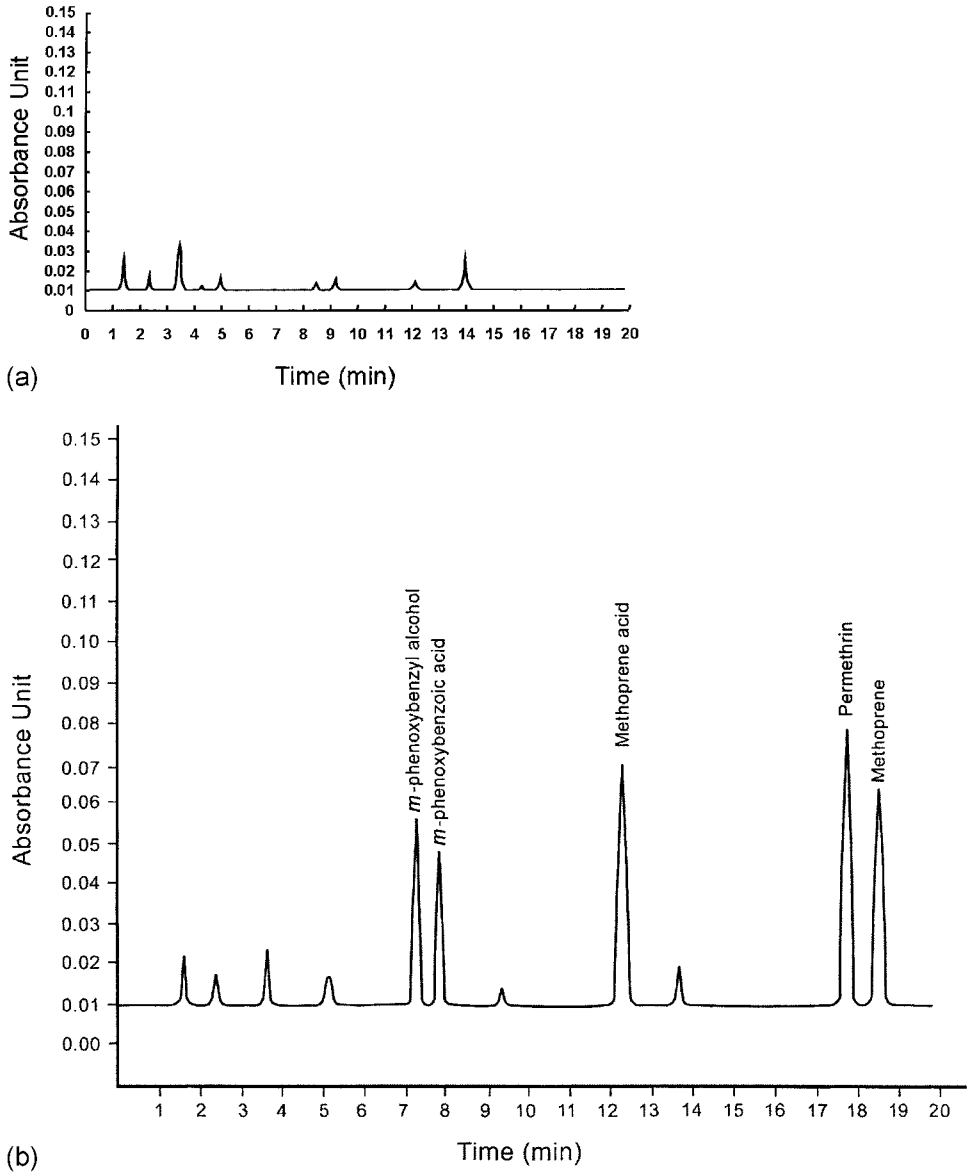


Figure 3. (a) Chromatogram of blank plasma sample and (b) spiked plasma sample with 500 ng/mL of *m*-phenoxybenzyl alcohol, *m*-phenoxybenzoic acid, methoprene acid, permethrin and methoprene under established HPLC conditions.

curves for the three compounds was achieved at concentrations ranging from 100 to 1000 ng/mL. The determined r^2 value was 0.9997, 0.9999, 0.9997, 0.9999 and 0.9996 for methoprene, methoprene acid, permethrin, *m*-phenoxybenzyl alcohol and *m*-phenoxybenzoic acid, respectively. Serial dilution of the samples of higher concentration was carried out. Linear curves were generated after dilution of the analytes.

Chromatogram

Chromatographic profiles were obtained for blank [Figures 3(a) and 4(a)] and spiked [Figures 3(b) and 4(b)] rat plasma and urine samples after solid phase extraction using C_{18} Sep Pak cartridges under HPLC conditions as described above. Retention times were 7.3, 7.9, 12.3, 17.6 and 18.4 min for *m*-phenoxybenzyl alcohol, *m*-phenoxybenzoic acid, methoprene acid, permethrin and methoprene, respectively. The total run time was 20 min. There was no interference from endogenous substances in plasma and urine matrices. Plasma samples were spiked with methoprene at room temperature (without incubation). The samples were analyzed following sample preparation as described under the Experimental section. The chromatogram was monitored for the presence of methoprene acid. No traces of this compound were detected.

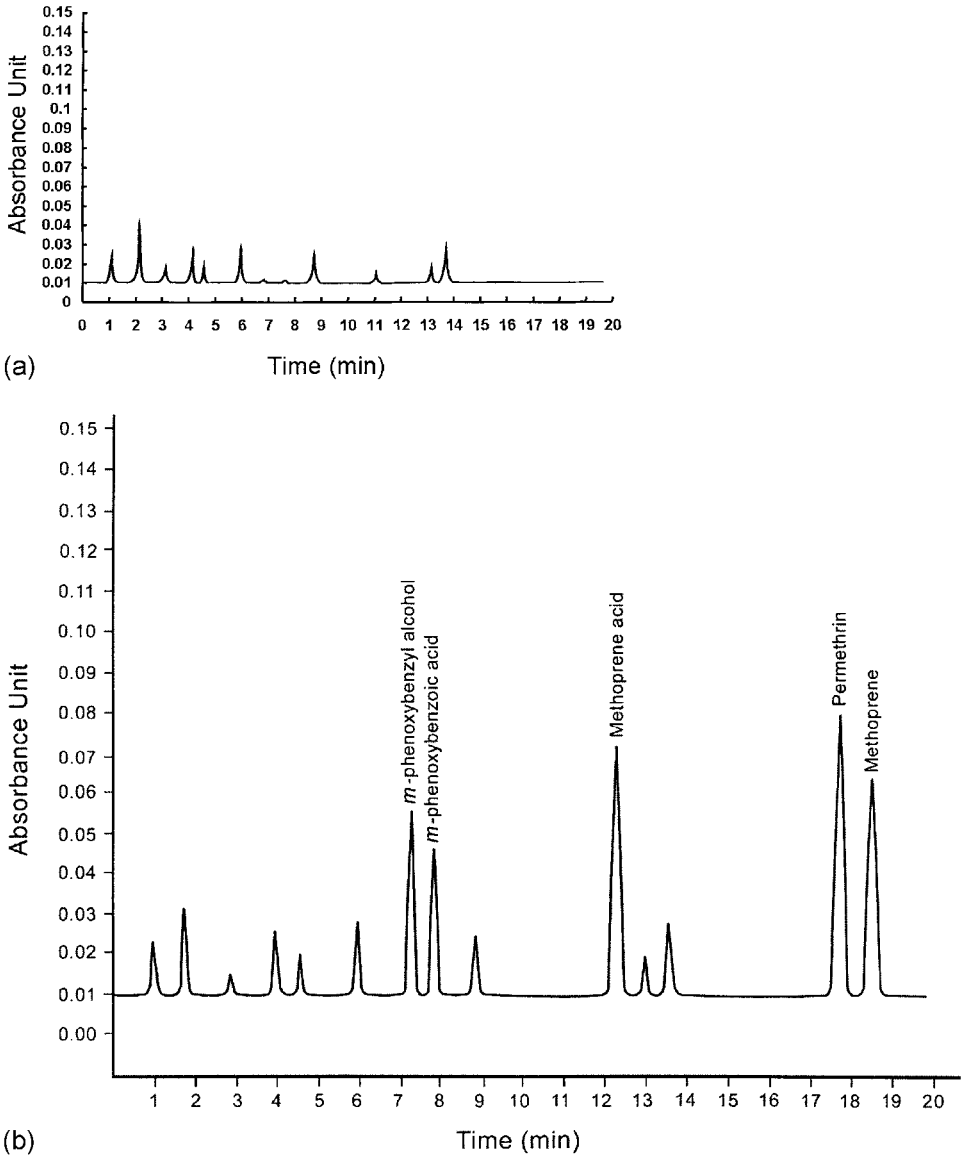


Figure 4. (a) Chromatogram of blank urine sample and (b) spiked urine sample with 500 ng/mL of *m*-phenoxybenzyl alcohol, *m*-phenoxybenzoic acid, methoprene acid, permethrin and methoprene under established HPLC conditions.

Extraction efficiency and recovery

The average extraction recoveries of methoprene, methoprene acid, permethrin, *m*-phenoxybenzyl alcohol and *m*-phenoxybenzoic acid were determined at concentrations ranging between 100 and 1000 ng/mL (Tables 1 and 2). Spiked plasma and urine samples were extracted and analyzed for each concentration in five replicates. Average percentage recoveries were, from plasma, 83.6 ± 3.9, 80.1 ± 5.4, 82.1 ± 4.4, 83.7 ± 3.9 and 83.1 ± 4.7, and, from urine, 79.3 ± 4.3, 82.0 ± 5.4, 80.7 ± 4.2, 78.9 ± 5.7 and 83.9 ± 4.5 for methoprene, methoprene acid, permethrin, *m*-phenoxybenzyl alcohol and *m*-phenoxybenzoic acid, respectively. Spiking of blank plasma samples with methoprene. The sample preparation procedure (1 h) did not show the formation of methoprene acid through hydrolysis.

Accuracy and precision

Average percentage relative errors of accuracy for all added concentrations to plasma samples were 3.0 ± 0.8, 2.8 ± 1.0, 2.6 ± 1.2, 3.3 ± 1.4 and $4.0 \pm 1.2\%$, and in urine samples were 3.2 ± 0.6, 2.4 ± 0.9, 3.0 ± 1.1, 2.9 ± 0.8 and $3.1 \pm 0.8\%$ for methoprene, methoprene acid, permethrin, *m*-phenoxybenzyl alcohol, and *m*-phenoxybenzoic acid, respectively. Intra-day precision was determined as percentage coefficient of variation

Table 1. Percentage recovery[a] of methoprene, permethrin and their metabolites from rat plasma

Concentration (ng/mL)	Methoprene	Methoprene acid	Permethrin	*m*-Phenoxybenzyl alcohol	*m*-Phenoxybenzoic acid
1000	88.9 ± 2.9	85.3 ± 4.0	85.6 ± 3.2	90.4 ± 5.2	88.1 ± 6.2
500	86.1 ± 5.7	81.2 ± 6.9	82.3 ± 5.1	86.1 ± 3.1	82.3 ± 4.0
400	82.5 ± 3.9	80.6 ± 5.8	85.1 ± 4.6	83.2 ± 4.5	84.3 ± 4.7
200	80.3 ± 4.2	78.1 ± 4.1	80.4 ± 2.1	79.1 ± 2.7	81.0 ± 2.8
100	80.0 ± 2.9	75.4 ± 6.1	77.2 ± 6.9	79.5 ± 4.1	80.0 ± 3.1

[a] Values are expressed as mean ± SD of five replicates.

Table 2. Percentage recovery[a] of methoprene, permethrin and their metabolites from rat urine

Concentration (ng/mL)	Methoprene	Methoprene acid	Permethrin	*m*-Phenoxybenzyl alcohol	*m*-Phenoxybenzoic acid
1000	86.8 ± 3.2	88.2 ± 5.6	82.1 ± 2.9	85.0 ± 6.1	88.5 ± 3.7
500	81.2 ± 4.9	83.2 ± 6.1	80.6 ± 3.8	81.3 ± 4.9	86.2 ± 5.2
400	76.4 ± 6.1	82.4 ± 4.9	83.4 ± 5.1	75.6 ± 6.2	83.1 ± 5.0
200	78.2 ± 5.2	77.1 ± 5.9	80.2 ± 4.8	76.4 ± 5.2	81.6 ± 3.9
100	74.2 ± 3.9	79.1 ± 4.7	77.3 ± 4.5	76.1 ± 6.0	80.3 ± 4.8

[a] Values are expressed as mean ± SD of five replicates.

(%CV) for plasma and urine samples and ranged between 1.9 and 2.8%.

Limits of detection

Blank plasma and urine samples from untreated rats were used as references for plasma and urine collections. Limits of detection were calculated from a peak signal to noise ratio of 3:1. The resulting detection limits range was 100, 100, 50, 50 and 50 ng/mL for methoprene, methoprene acid, permethrin, *m*-phenoxybenzyl alcohol, and *m*-phenoxybenzoic acid, respectively.

Limits of quantitation

Limits of quantitation were determined to be 150, 150, 100, 100 and 100 ng/mL from plasma, and 150, 150, 100, 100 and 100 ng/mL from urine for methoprene, methoprene acid, permethrin, *m*-phenoxybenzyl alcohol and *m*-phenoxybenzoic acid, respectively.

Application of the method in rats

People could be exposed to methoprene dermal or oral. Both routs are applicable. The main objective in this manuscript is to develop and validate the method for future pharmacokinetics studies using both routes of administration. Permethrin is often sprayed on cloths, so we used the dermal rout. The method was applied for the determination of the analytes in rats after oral and dermal administration of methoprene and permethrin, respectively. The rats were sacrificed 12 h after dosing. In plasma, methoprene, methoprene acid, permethrin and *m*-phenoxybenzyl alcohol were identified. Their levels were 216 ± 51, 172 ± 26, 159 ± 41 and 195 ± 62 ng/ml, respectively. In urine, only methoprene acid was detected. Its level after 12 h of dosing was 279 ± 82 ng/mL.

DISCUSSION

The present study reports on the development of a method for separation and quantitative analysis of methoprene, permethrin and their metabolites in rat plasma and urine.

Linearity of standard calibration curves for the chemicals in this method was obtained over a range between 100 and 1000 ng/mL. Simultaneous analyses of the parent compounds and metabolites are cost efficient and save time for sample preparation. The advantage of using solid phase extraction compared to liquid–liquid extraction is saved time and solvent. In this method, individual compound standards were injected to confirm the absence of metabolites. In this method, we simultaneously determined the six analytes. This is another advantage compared to previously published methods.

Recoveries of the chemicals and metabolites were suitable for application of the method in analysis of treated samples for parent compounds and their metabolites that resemble real-life situations. Percentage recoveries depend on the matrix, extracting solvent, method of analysis, and the amount to be analyzed. In this study recoveries ranged between 74 and 90%. This range lies

within the reported values in the literature, taking into consideration simultaneous analysis of the parent chemicals and their metabolites. In previous studies, recovery of pyrethroids and metabolites from rat urine ranged between 90 and 98% using GC-MS (Angerer and Ritter, 1997). Bissacot and Vassilieff (1997) reported recoveries between 78 and 91% of four pyrethroids from milk and blood of lactating dairy cows using HPLC. Recovery of methoprene from bovine fat samples was between 84 and 96% using the liquid extraction method and GC technique (Hunt and Gilbert, 1976).

The ability to detect the parent compounds and some metabolites in rat plasma after 12 h of combined dosing is evidence of the method's suitability. The failure to detect methoprene in urine samples following oral dose in rats showed that methoprene is rapidly metabolized in rat. Also the inability to identified permethrin and its selected metabolites in urine might due to the low dermal dose of permethrin that used (1.3 mg/kg), its low absorption through skin, and to rapid hydrolysis and conjugation of permethrin and the targeted metabolites. Detection limits of permethrin in urine samples were 0.3–0.5 μg/L using GC-MS technique (Angerer and Ritter, 1997), and 5 μg/L in plasma using GC method (Sondgrass, 1992). Bissacot and Vassilieff (1997) reported detection limit of 1 μg/g of four pyrethroids in milk and blood of lactating dairy cows using HPLC. The reported limits of detection in the literature are consistent with the limits of detection for the simultaneous analysis of the combined chemicals and their metabolites in this method, which ranged between 50 and 100 ng/mL. The LOD of methoprene in rat urine was 0.001 ppm and in tissues was 0.01 ppm using GC technique (Miller *et al.*, 1975), while its LOQ was 0.008 ppm from bovine rat (Hunt and Gilbert, 1976).

The results showed that methoprene acid has not been formed *in vitro* through ester hydrolysis. Plasma samples were spiked with methoprene at room temperature (without incubation). The samples were analyzed following sample preparation as described under methods. Sample preparation takes less than 1 h. Detection of methoprene acid in plasma (*in vivo*) following administration of methoprene in rats was after 12 h of dosing. The samples were collected, plasma was separated and immediately frozen prior to the analysis. The presence of methophrene acid in these samples resulted from *in vivo* hydrolysis.

A reliable and simple HPLC method was developed for separation and quantification of methoprene, permethrin and selected metabolites in rat spiked and treated plasma and urine samples. Solid-phase extraction was used which selectively extracted the above chemicals from plasma and urine samples without interference of an expected mixture of metabolites and endogenous compounds. The method could be applied routinely for monitoring of the above chemicals in human plasma and urine samples of persons exposed to the combined chemicals. Also this method could be used in the pharmacokinetic studies to assess distribution of the parent compounds and metabolites in body tissues and fluids following combined exposure. The main advantage of the method is the ability to analyze simultaneously the two chemicals and their metabolites under similar conditions, saving time and expenses for sample preparation.

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