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Simultaneous determination of pyridostigmine bromide, *N*,*N*-diethyl-*m*-toluamide, permethrin, and their metabolites in rat plasma and urine by high-performance liquid chromatography

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Abstract

A rapid and simple method was developed for the separation and quantification of the anti nerve agent drug pyridostignmine bromide (PB; 3-dimethylaminocarbonyloxy-*N*-methyl pyridinium bromide) its metabolite *N*-methyl-3-hydroxypyridinium bromide, the insect repellent DEET (*N*,*N*-diethyl-*m*-toluamide), its metabolites *m*-toluamide and *m*-toluic acid, the insecticide permethrin (3-(2,2-dichloro-ethenyl)-2,2-dimethylcyclopropanecarboxylic acid(3-phenoxyphenyl)methy-lester), and two of its metabolites *m*-phenoxybenzyl alcohol, and *m*-phenoxybenzoic acid in rat plasma and urine. The method is based on using C_{18} Sep-Pak® cartridges for solid-phase extraction (SPE) and high-performance liquid chromatography (HPLC) with reversed-phase C_{18} column, and gradient UV detection ranging between 208 and 230 nm. The compounds were separated using gradient of 1 to 99% acetonitrile in water (pH 3.20) at a flow-rate ranging between 0.5 and 1.7 ml/min in a period of 17 min. The retention times ranged from 5.7 to 14.5 min. The limits of detection were ranged between 20 and 100 ng/ml, while limits of quantitation were 150–200 ng/ml. Average percentage recovery of five spiked plasma samples were 51.4±10.6, 71.1±11.0, 82.3±6.7, 60.4±11.8, 63.6±10.1, 69.3±8.5, 68.3±12.0, 82.6±8.1, and from urine 55.9±9.8, 60.3±7.4, 77.9±9.1, 61.7±13.5, 68.6±8.9, 62.0±9.5, 72.9±9.1, and 72.1±8.0, for pyridostigmine bromide, DEET, permethrin, *N*-methyl-3-hydroxypyridinium bromide, *m*-toluamide, *m*-toluic acid, *m*-phenoxybenzyl alcohol and *m*-phenoxybenzoic acid, respectively. The relationship between peak areas and concentration was linear over the range between 100 and 5000 ng/ml. This method was applied to analyze the above chemicals and metabolites following their administration in rats.

Keywords: Pyridostigmine bromide; *N*,*N*-Diethyl-*m*-toluamide; Permethrin

1. Introduction

Simultaneous exposure to pyridostigmine bromide, DEET and permethrin has resulted in enhanced of neurotoxicity in hens [1], and caused significant increase in lethality in rats [2]. Also acute interaction resulted in seizures and death following combined application of pyridostigmine bromide and DEET in mice [5]. Based on these reports, combined chemical exposure has been proposed as a possible cause of Gulf War veterans illness [1,3–8]. Pyridostigmine bromide was used as an antidotal drug against possible attack by organophosphate nerve agents, DEET was applied as insect repellent on the skin of

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veterans, and permethrin was sprayed against orthropod vectors on the battle dress uniforms [35]. Pyridostigmine bromide has been reported to be absorbed into plasma and excreted in urine following oral or intravenous dose in rat [9,10,17,18,27,29,36], in man [16,24], and in dog [15,28]. Absorption and excretion of DEET and metabolites were rapid after dermal application in human [11,13], in rats [19], and in dogs [14]. Permethrin was also reported to be absorbed into plasma, metabolized and excreted as metabolites in the urine following oral or intravenous dose in rats [20], and in rabbits [23].

Several analytical methods have been used for identification and quantification of the above chemicals and their metabolites, when applied alone in plasma and urine samples. These methods used high-performance liquid chromatography (HPLC) [9,11–14,25,34], HPLC–mass spectrometry [19], gas chromatography [21–24,39–41], gas chromatography–mass spectrometry [26,32,38,42], and thin layer chromatography [22,30,31]. Other techniques were also used, e.g. Micellar electrokinetic chromatography (MEKC) [33], radiochromatoelectrophoresis [34], electrophoresis with paper chromatography [36], and radioaminoassay [36]. Limits of detection of the chemicals and metabolites in plasma or urine samples when analyzed using HPLC–UV, following individual application were ranged between 10 and 100 ng/ml [11,20,25], while their recoveries were between 65 and 95% [20,25,37].

In this study we present a reliable method for simultaneous analysis of the above chemicals and their metabolites in rat plasma and urine using solid-phase extraction (SPE) coupled with reversed-phase HPLC (RP-HPLC).

2. Experimental

2.1. Chemicals and materials

DEET (*N,N*-Diethyl-*m*-toluamide) (Fig. 1) was obtained from Aldrich Chem Co., Inc. (Milwankee, WI, USA), Pyridostigmine bromide (PB; 3-dimethylaminocarbonyloxy-*N*-methyl pyridinium bromide), *m*-phenoxybenzoic acid, and *m*-phenoxybenzyl alcohol (Fig. 1) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Permethrin(3-(2,2-dichloro-ethenyl)-2,2-dimethylcyclopropanecarboxylicacid(3-phenoxyphenyl) methylester) was obtained from Chem Service, Inc. (West Chester, PA, USA), *m*-Toluamide, and *m*-toluic acid were purchased from Fisher Scientific (Pittsburgh, PA, USA), *N*-methyl-3-hydoxypyridinium bromide was prepared following the method by Somani et al. [16]. Water (HPLC grade) and acetonitrile were obtained from Mallinckrodt Baker, Inc. (Paris, Kentucky, USA). C_{18} Sep-Pak^{R} cartridges were obtained from Waters Corporation (Waters Corporation, Milford, MA, USA).

2.2. Animals

Rats (Sprague–Dawley) were purchased from Zivic Miller (Zelienople, PA, USA). The animals were kept in plastic metabolic cages. Three groups, each of five rats were treated with a single oral dose of 13 mg/kg of pyridostigmine bromide, a single dermal dose 400 mg/kg of DEET, and a single dermal dose of 1.3 mg/kg of permethrin. Another group of five rats were treated with a combination of the above chemicals. Five untreated control rats were treated with oral dose of water, or dermal dose of ethanol. The animals were held in metabolic cages allow collection of urine samples. Urine samples were collected from treated and control rats after 8 h of dosing. The animals were anesthetized with halothane and scarified by heart exsanguinations at 8 h. Blood was collected via heart puncture with a heparinized syringe and centrifuged at 2400 rev./min for 15 min at 5°C to separate plasma. Urine and plasma samples were stored at −20°C prior to analysis.

2.3. Instrumentation

The liquid chromatographic system (Waters 2690 Separation Module), consisted of a Waters 600E Multisolvent delivery system pumps, a Waters Ultra WISP 715 autoinjector, 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 were used, (Waters Corporation, Milford, MA).

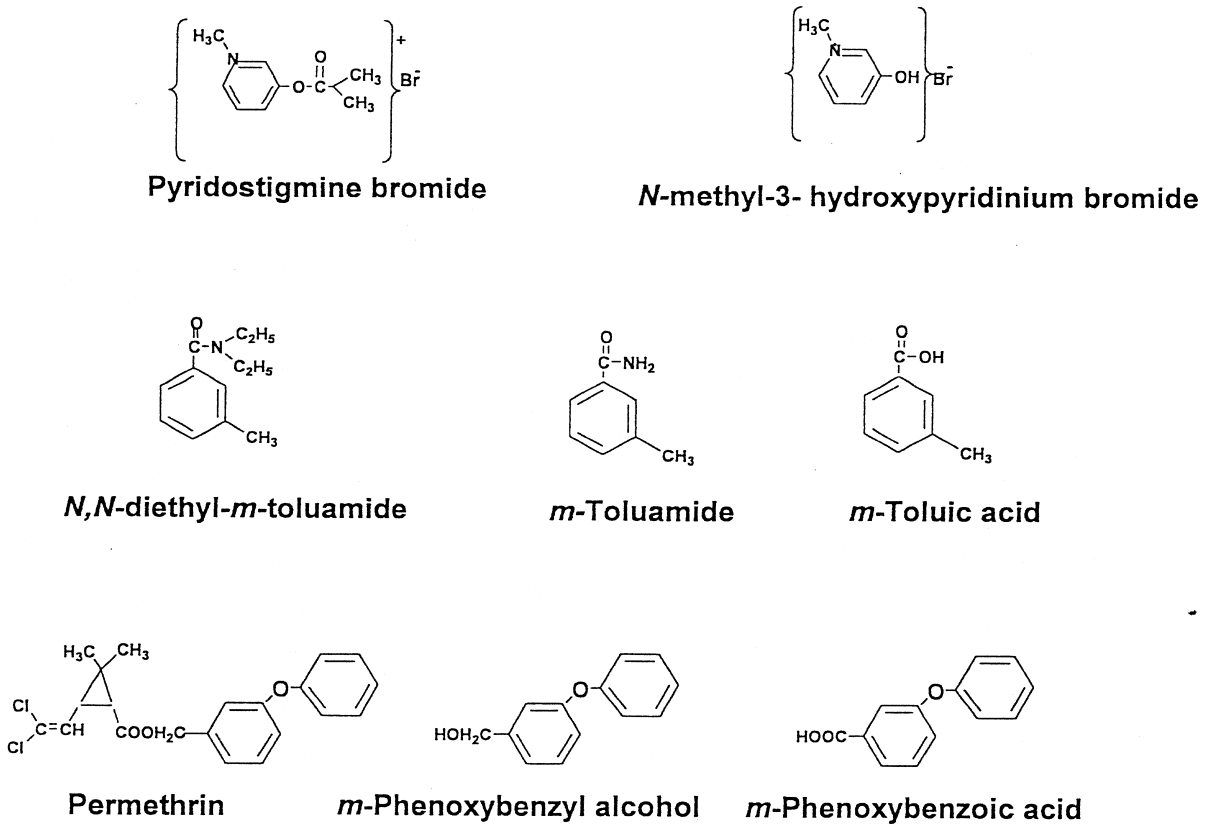

Fig. 1. Chemical structures of pyridostigmine bromide, DEET, permethrin, *N*-methyl-3-hydroxypyridinium bromide, *m*-toluamide, *m*-toluic acid, *m*-phenoxybenzyl alcohol, and *m*-phenoxybenzoic acid.

2.4. Sample preparation

A 0.2 ml plasma and urine samples from untreated rats were spiked with concentrations ranging between 100 and 5000 ng/ml of each of pyridostigmine bromide, DEET, permethrin, *N*-methyl-3-hydroxypyridinium bromide, *m*-toluamide, *m*-toluic acid, *m*-phenoxybenzoic acid, and *m*-phenoxybenzyl alcohol. Spiked and treated samples were acidified with 1 N acetic acid (pH 5.0). Disposable C_{18} Sep-Pak Vac 3cc (500 mg) cartridges (Waters Corporation, Milford, MA) were conditioned with 3 ml of acetonitrile, then equilibrated using 3 ml of water prior to 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 2 ml of water, and eluted two times by 1 ml of methanol, then twice by 1 ml of acetonitrile, and reduced to 500 μl using a stream of nitrogen, prior to analysis by HPLC.

2.5. Chromatographic conditions

A 10 μl solution of plasma or urine residues was injected into HPLC. The mobile phase was water (adjusted to pH 3.20 using 1 N acetic acid):acetonitrile gradient at flow-rate programmed from 0.5 to 1.7 ml/min. The gradient started at 1% acetonitrile, increased to 75% acetonitrile at 6 min, then increased to 99% acetonitrile by 11 min. Then the system returned to 1% acetonitrile at 15 min where it was kept under this condition for 2 min to re-equilibrate. The eluents were monitored by UV detection of wavelength of 208 nm for pyridostigmine bromide and *N*-methyl-3-hydroxypyridinium bromide, 210 nm for DEET, *m*-toluamide, and *m*-

toluic acid and at 230 nm for permethrin, *m*-phenoxybenzyl alcohol, and *m*-phenoxybenzoic acid. The chromatographic analysis was performed at ambient temperature.

2.6. Calibration procedures

Five different calibration standards of a mixture of pyridostigmine bromide, DEET, permethrin, *N*-methyl-3-hydroxypyridinium bromide, *m*-toluamide, *m*-toluic acid, *m*-phenoxybenzyl alcohol, and *m*-phenoxybenzoic acid were prepared in acetonitrile. Their concentrations ranged from 100 to 5000 ng/ml. Linear calibration curves were obtained by plotting the peak areas of the individual chemicals as a function of the concentration using GraphPad Prism program for windows (GraphPad Software, Inc., San Diego, CA, USA). The standard curves were used to determine recovery of the chemicals from plasma and urine samples.

2.7. Limits of detection and limits of quantitation

Limits of detection (LOD) were determined at the lowest concentration to be detected, taking into consideration a 1:3 baseline noise: calibration point ratio. A reproducible lowest possible concentration was considered as the limit of quantitation (LOQ). The LOQ was repeated five times for confirmation.

3. Results

3.1. Standard calibration curves

The standard calibration curves of peak area against concentration of pyridostigmine bromide, DEET, and permethrin, *N*-methyl-3-hydroxypyridinium bromide, *m*-toluamide, *m*-toluic acid, *m*-phenoxybenzyl alcohol, and *m*-phenoxybenzoic acid are shown in Fig. 2. Linearity of the calibration curves for the three compounds was achieved at concentrations ranging from 100 to 5000 ng/ml.

3.2. Chromatogram

Chromatographic profiles were obtained for rat plasma and urine samples after solid-phase extraction using C_{18} Sep Pak® cartridges under HPLC conditions, described above (Figs. 3 and 4). Retention times were 6.8, 9.5, 14.4, 5.7, 7.5, 8.6, 10.7 and 11.3 min for pyridostigmine bromide, DEET, and permethrin, *N*-methyl-3-hydroxypyridinium bromide, *m*-toluamide, *m*-toluic acid, *m*-phenoxybenzyl alcohol, and *m*-phenoxybenzoic acid, respectively. The total run time was 17 min. Clean chromatogram shows no interference from endogenous substances in plasma and urine samples. This suggests an efficient sample preparation and clean up method.

3.3. Extraction efficiency and recovery

The average extraction recoveries of pyridostigmine bromide, DEET, permethrin, *N*-methyl-3-hydroxypyridinium bromide, *m*-toluamide, *m*-toluic acid, *m*-phenoxybenzoic acid, and *m*-phenoxybenzyl alcohol were determined at concentrations ranged between 100 and 5000 μg/ml (Tables 1 and 2).

Spiked plasma and urine samples were extracted and analyzed for each concentration in five replicates. Average percentage recoveries were 51.4±10.6, 71.1±11.2, 82.3.±6.7, 60.4±11.8, 63.6±10.1, 69.3±8.5, 68.3±12.0 and 82.6±8.1 from plasma, and 55.9±9.8, 60.3±7.4, 77.9±9.1, 53.9±9.7, 64.2±6.5, 71.7±4.2, 86.5±6.1 and 89.7±4.1 from urine for pyridostigmine bromide, DEET, and permethrin, *N*-methyl-3-hydroxypyridinium bromide, *m*-toluamide, *m*-toluic acid, *m*-phenoxybenzyl alcohol, and *m*-phenoxybenzoic acid, respectively.

3.4. LOD

Blank plasma and urine samples from untreated rats were used as references for plasma and urine collections. LOD were calculated from a peak signal-to-noise ratio of 3:1. The resulting detection limits range were 100, 50, 50, 100, 100, 80, 20 and 30 for pyridostigmine, DEET, permethrin, *N*-methyl-3-hydroxypyridine, *m*-toluamide, *m*-toluic acid, *m*-phenoxy benzylalcohol, and *m*-phenoxy benzoic acid, respectively.

3.5. LOQ

LOQ were determined to be 150 ng/ml for pyridostigmine bromide and DEET and 100 ng/ml

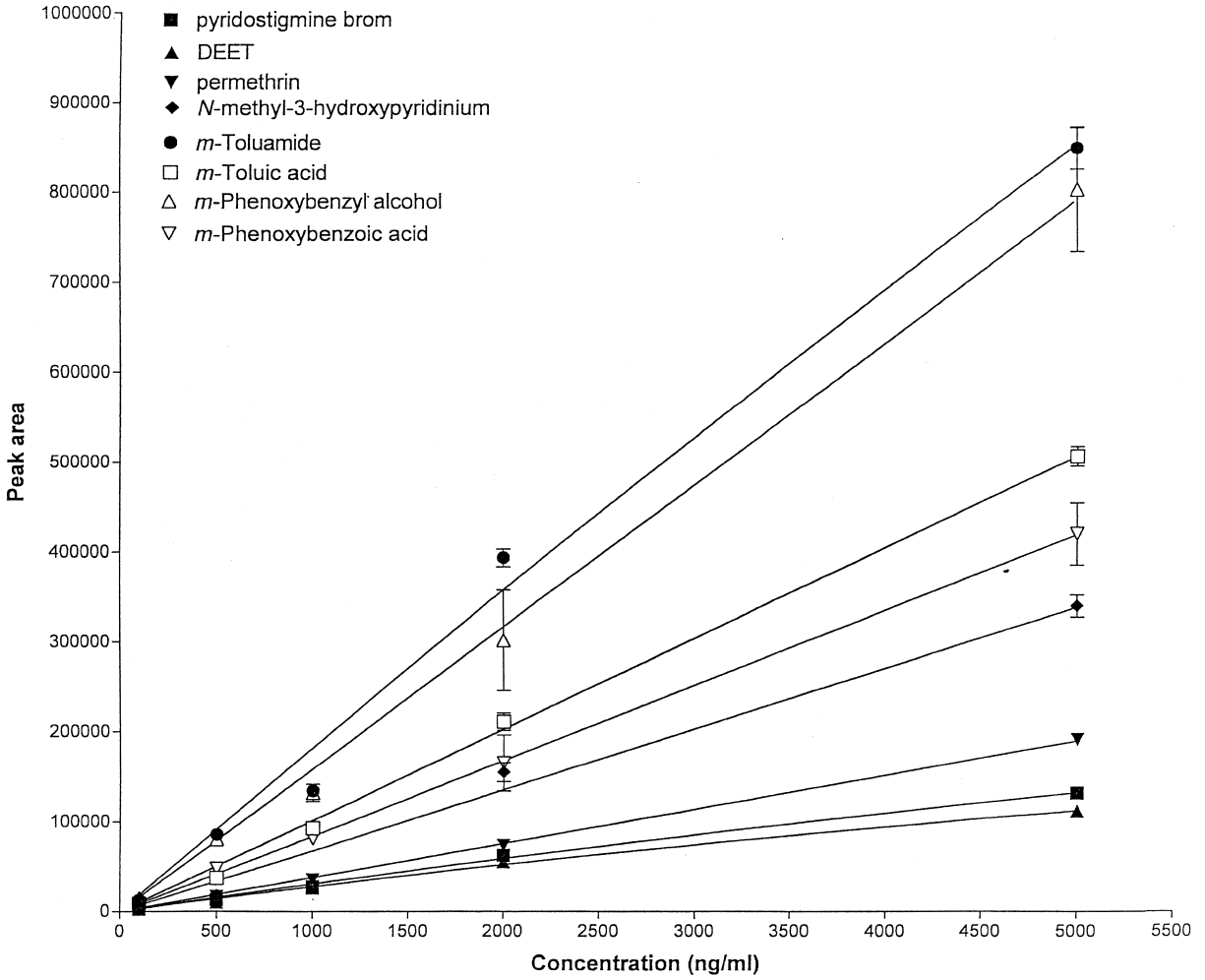

Fig. 2. Standard calibration curves of pyridostigmine bromide, DEET, permethrin, *N*-methyl-3-hydroxypyridinium bromide, *m*-toluamide, *m*-toluic acid, *m*-phenoxybenzyl alcohol, and *m*-phenoxybenzoic acid.

for permethrin in plasma. In urine limits of quantitation were 200, 150, and 100, 150, 150, 100, 100, 100 ng/ml for pyridostigmine bromide, DEET, and permethrin, *N*-methyl-3-hydroxypyridine, *m*-toluamide, *m*-toluic acid, *m*-phenoxybenzyl alcohol, and *m*-phenoxybenzoic acid, respectively.

3.6. Application of the method to biological samples

In order to validate the method, the method was applied for analysis of the chemicals in treated rats, when applied alone or in combination. The rats were sacrified at 8 h following dosing. In plasma, their levels were 224±123, 1320±346 and 182±76 ng/ml for pyridostigmine bromide, DEET, and permethrin, while concentration of metabolites in plasma were. 107.3±21.5, 98.4±14.8, 107.5±5.7 and 142.7±27.1 ng/ml for *m*-toluamide, *m*-toluic acid, *m*-phenoxybenzyl alcohol, and *m*-phenoxybenzoic acid, respectively. *N*-methyl-3-hydroxypyridinium bromide a metabolite of pyridostigmine bromide was not detected in rat plasma. Levels of pyridostigmine bromide and DEET in rat urine were 712±186 ng/ml and 3.2±0.82 μg/ml, respectively. DEET metabolites *m*-toluamide and *m*-toluic acid, and

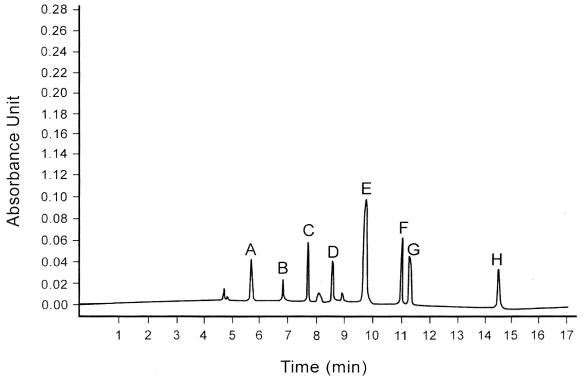

Fig. 3. Chromatogram of plasma sample of (A) *N*-methyl-3-hydoxypyridinium bromide; (B) PB (pyridostigmine bromide); (C) *m*-toluamide; (D) *m*-toluic acid; (E) DEET; (F) *m*-phenoxybenzyl alcohol; (G) *m*-phenoxybenzoic acid; and (H) permethrin under established HPLC conditions.

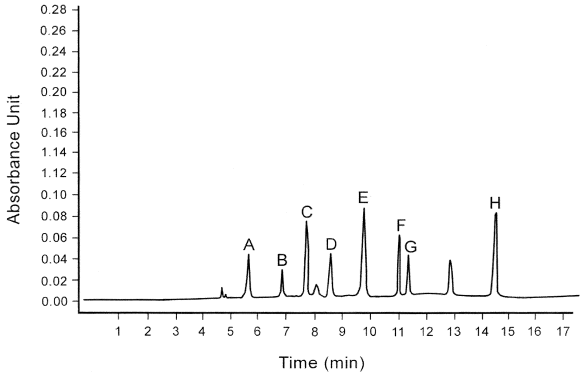

Fig. 4. Chromatogram of spiked urine sample with (A) *N*-methyl-3-hydoxypyridinium bromide; (B) PB (pyridostigmine bromide); (C) *m*-toluamide; (D) *m*-toluic acid; (E) DEET; (F) *m*-phenoxybenzyl alcohol; (G) *m*-phenoxybenzoic acid; and (H) permethrin under established HPLC conditions.

permethrin and its metabolites *m*-phenoxybenzyl alcohol and *m*-phenoxybenzoic acid, have not been detected in urine after 8 h of dosing.

4. Discussion

The present study reports the development of an HPLC method for quantitative and qualitative analysis of pyridostigmine bromide, DEET, permethrin and their metabolites in plasma and urine of treated rats.

Linearity of standard calibration curves for the chemicals in the present method is in consistent with previous reports. Eilln et al. [9] reported linear range between 40 and 500 ng/ml for DEET in plasma using HPLC, while Yaylor et al. [14] reported a linearity over a range between 19 and 1910 ng/ml for DEET using gas chromatography (GC). Also, Chan et al. [38] reported a linear range for pyridostigmine bromide in human plasma over concentrations between 50 and 1000 ng/ml.

The chromatogram obtained following SPE and HPLC analysis shows no interference from plasma and urine subjects, indicating an efficient clean up method used. Also simultaneous and rapid analyses of the parent compounds and metabolites are cost efficient and save time for sample preparation.

Recoveries of the chemicals and metabolites were suitable for application of the method for analysis of treated samples for parent compounds and their metabolites. Low recovery for pyridostigmine bromide might have resulted from the use of solvent system that was not quite suitable for extracting pyridostigmine bromide, and at the same time it was needed for extracting and analyzing the other two

Table 1
Percentage recovery of pyridostigmine bromide, DEET permethrin, and metabolites in rat plasma[a]

Concentration (ng/ml)	Pyridostigmine bromide	DEET	Permethrin	*N*-methyl-3-hydroxypyriodinium bromide	*m*-Toluic acid	*m*-Toluamide	*m*-Phenoxybenzyl alcohol	*m*-Phenoxybenzoic acid
5000	61.8±5.4	81.4±4.3	81.4±7.6	53.4±13.6	61.5±12.1	73.4±13.6	65.7±18.2	89.2±9.7
2000	63.8±7.1	84.1±9.5	87.1±9.3	64.7±18.3	65.3±7.2	64.7±9.8	61.5±13.8	80.1±10.2
1000	55.5±10.7	73.6±8.7	82.1±8.3	57.1±9.2	68.4±17.6	70.2±4.1	68.7±10.2	73.6±8.3
200	58.4±13.6	71.2±4.8	72.1±6.7	59.8±7.4	60.5±13.8	71.8±8.6	75.3±8.2	86.7±6.4
100	44.1±10.9	60.7±16.7	71.5±8.9	66.2±10.4	62.1±4.6	66.2±6.5	70.4±9.7	83.4±5.8

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

Table 2
Percentage recovery of pyridostigmine bromide, DEET permethrin, and metabolites from rat urine[a]

Concentration (ng/ml)	Pyridostigmine bromide	DEET	Permethrin	*N*-methyl-3-hydroxypyridinium bromide	*m*-Toluamide	*m*-Toluic acid	*m*-Phenoxybenzyl alcohol	*m*-Phenoxybenzoic acid
5000	54.1±7.3	62.8±10.9	90.7±3.9	62.2±12.8	69.2±10.6	59.8±12.3	66.8±6.5	75.6±4.1
2000	53.8±8.4	58.1±7.5	83.1±4.2	52.7±10.6	61.9±9.7	63.4±8.7	73.8±12.3	71.9±12.1
1000	49.6±8.3	52.7±7.4	70.8±8.7	61.8±9.3	75.3±7.6	69.7±5.2	82.6±7.8	77.8±3.6
200	48.0±13.2	60.9±8.2	71.7±10.1	64.7±8.4	71.9±5.6	61.2±14.7	71.9±5.8	65.4±9.5
100	52.3±8.5	57.6±10.7	68.4±4.0	67.2±13.2	64.5±11.2	58.4±6.5	69.8±13.1	69.8±10.9

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

chemicals and metabolites under similar conditions. Also hydrolysis of pyridostigmine bromide during the extraction is possible, in a previous study Aquilonius and Hartvig [43] reported that extraction and analysis of pyrdostigmine bromide was a challenge to the analytical chemists, because of its in vitro hydrolysis could take place in buffer solutions, plasma and blood. Percentage recoveries depends on the matrix, extracting solvent, method of analysis, and the amount to be analyzed. Recoveries of DEET from serum and urine were reported to be 93–95%, and 65–70%, respectively using GC–MS as an analytical technique [38], while recovery of DEET from water samples was 45.6% using Micllar kinetic chromatography method [33]. Hennis et al. [15] reported a recovery of 50% of *N*-methyl-3-hydroxypyridinium from dog plasma and urine, while Chan et al. [37] reported a recovery of 82% of pyridostigmine bromide from plasma at low concentration of 50 ng/ml, while its recovery was 92% when a concentration of 400 ng/ml was used. In previous studies, recovery of pyrethroides and metabolites from rat urine ranged between 90 and 98% using GC–MS [26], while the recovery was 92% at high concentration of 400 ng/ml using GC. In our method, recoveries differed with individual chemicals. Recoveries of the chemicals analyzed in our method was between 55 and 83%. This range lies within the reported values in the literature, taking into consideration simultaneous analysis of the parent chemicals and their metabolites.

The LOD reported in our method allow to analyzing samples from treated animals following doses resemble real exposure. Our ability to detect the three compounds and metabolites in plasma after 8 h of dosing is an evidence of the method suitability. LOD and LOQ depended upon the nature of the matrix, rate of application, and method of analysis [10,38–40]. Pyridostigmine bromide and DEET were also detected in urine samples at 8 h, while failure to detect permethrin and metabolites in urine might be 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. Hennis et al. [15] reported a 50 ng/ml as a limit of detection of *N*-methyl-3-hydroxypyridinium in dog plasma using ion-exchange liquid chromatography, while Miller and Verma [36] reported a 2.5 ng/ml as detection limit of pyridostigmine bromide in tissues using radioimmunoassay method, while using HPLC technique, limits of detection of pyridostigmine bromide in plasma was 10 ng/ml [25], and ranged between 2.7 and 18.6 ng/ml in plasma using GC [41]. The detection limit of DEET was 90 and 90 ng/g from urine and serum, respectively, using HPLC–UV method [11], and 15 ng/ml for DEET in human and dog plasma using HPLC [34], while it was 25 ng in cosmetic products using high-performance thin-layer chromatography (HPTLC) method [30]. Detection limits of permethrin in urine samples were 0.3–0.5 μg/l using GC–MS technique [26], and 5 μg/l in plasma using GC method [39]. The reported LOD in the literature are consistent with our results for the simultaneous analysis of the combined chemicals and their metabolites, which ranged between 20 and 100 ng/ml.

A rapid and simple HPLC method was developed for separation and residual determination of pyridostigmine bromide, DEET, permethrin and selected metabolites in rat spiked and treated plasma and urine samples. SPE 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. This method could also be used in pharmacokinetics studies to assess distribution of the parent compounds and metabolites in body tissues and fluids. The use of SPE is advantageous compared to liquid–liquid extraction which is a time consuming and requires large amounts of organic solvents. The main advantage of the method is the ability to analyze simultaneously the three chemicals and their metabolites under similar conditions, saving time and expenses for sample preparation.

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