Rapid Determination of N,N-Diethyl-m-Toluamide and Permethrin in Human Plasma by Gas Chromatography-Mass Spectrometry and Pyridostigmine Bromide by High-Performance Liquid Chromatography

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Abstract

A rapid and highly sensitive gas chromatography–mass spectrometry (GC–MS) method for simultaneous determination of N,N-diethyl-m-toluamide (DEET) and permethrin with 2H10-phenanthrene (98 atom %) as an internal standard and a separate external standard high-performance liquid chromatography (HPLC) method for pyridostigmine bromide (PB) determination in human plasma were developed and validated. The GC–MS method for DEET and permethrin quantification utilizes a one-step extraction with tert-butylmethyl ether. The HPLC method for PB quantification involves a solid-phase extraction and UV detection. The range of the analytical method for DEET and permethrin was 1 ng/mL to 100 ng/mL and for PB was 5 ng/mL to 100 ng/mL. Recovery from plasma proved to be more than 80%. The intraday precision ranged from 1.3% to 8% for DEET, from 2.1% to 11.4% for permethrin, and from 3.0% to 4.8% for PB. The interday precision was 3% for DEET, ranged from 5% to 9% for permethrin, and from 5% to 9% for PB. The accuracy for the limit of quantification was 92% ± 8% relative standard deviation (RSD) for DEET, 112% ± 11% RSD for permethrin, and 109% ± 5% RSD for PB. All 3 compounds were stable in human plasma at -80°C for at least 12 months and after 2 freeze-thaw cycles with RSD values ranging from 7.1% (DEET, 80 ng/mL) to 8.1% (DEET, 8 ng/mL), from 2.3% (permethrin, 80 ng/mL) to 11.6% (permethrin, 8 ng/mL), and from 0.2% (PB, 80 ng/mL) to 3.6% (PB, 8 ng/mL). Both methods were successfully applied to pharmacokinetic/pharmacodynamic studies of combined exposure of DEET (skin application), permethrin (treated uniforms), and PB (30 mg orally three times/day for four doses) in healthy volunteers (n = 81).

Introduction

N,N-Diethyl-m-toluamide (DEET) is the principal active ingredient in most personal insect repellents and is highly effective against a broad spectrum of insect pests (1,2). DEET was first developed by the U.S. Army for use by military personnel and later registered for general public use (3). Although DEET is generally considered a safe chemical, and it is estimated that DEET-containing products are used by approximately one-third of the United States population annually, there have been reports of possible neurotoxic and skin irritant effects after excessive exposure (4–7).

Permethrin is a widely used insecticide to control biting insects, and it works by interfering with sodium channels, receptor-ionophore complexes, neurotransmitters, and ATPases (8–10).

Pyridostigmine bromide (PB) is an inhibitor of acetylcholinesterase used in anesthesiology to reverse non-depolarizing neuromuscular blockade, and PB was approved by Food and Drug Administration for myasthenia gravis therapy in 1955. PB was employed prophylactically by American and British soldiers against organophosphate nerve agents in the 1990–1991 Gulf War (11).

Recent reports have suggested that high-level exposure to DEET in combination with PB and permethrin can lead to neurotoxicity and significant neurobehavioral deficits associated with inhibition of brainstem acetylcholinesterase activities in animal models (12–17).

DEET, permethrin, and PB were widely used during the Persian Gulf War, and it is estimated that more than 250,000 Gulf War veterans were exposed to these chemicals. The need for more studies to determine the impact of co-exposure of...
humans to these agents during stressful conditions on physical and neurocognitive performance has become more important as a result of evidence that wartime stress may facilitate PB penetration of the blood-brain barrier, and simultaneous exposure to DEET, permethrin, and PB may be a possible cause of Gulf War Syndrome (18-20). The availability of sensitive analytical methods to quantify DEET, permethrin, and PB in human biological fluids could provide important information when investigating the possible associations of these compounds with biological effects related to the Gulf War Syndrome.

Several separate analytical methods have been developed for quantification of these compounds in human plasma or urine using high-performance liquid chromatography (HPLC) (21–34), gas chromatography with nitrogen-phosphorus detection (GC-NPD) (35–37), HPLC–thermospray mass spectrometer (TS-MS) (38), GC–MS (15,39–42), and liquid chromatography–MS–MS (43).

To support a clinical trial exposing healthy human subjects to permethrin-impregnated uniforms, DEET-containing skin cream, and oral PB, in a manner consistent with current US military doctrine, sensitive and reliable analytical methods are required to measure these three compounds in plasma at low nanograms-per-milliliter levels. A report by Abu-Qare et al. (24) included DEET, permethrin, and PB rat plasma levels, however, and the limits of quantification (LOQ) for their HPLC method ranged from 100 to 150 ng/mL for these three compounds. We developed a simple, reliable, and sensitive GC–MS internal standard analytical method for the determination of DEET and permethrin with LOQs of 1 ng/mL and an external standard method for quantification of PB by HPLC with an LOQ of 5 ng/mL in human plasma.

**Experimental Procedures**

**Reagents and materials**

DEET and the internal standard, 2H10-phenanthrene (98 atom %), were purchased from Aldrich Chem. Co. (Milwaukee, WI). PB (3-dimethylaminocarboxyloxy-N-methyl pyridinium bromide) and acetic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Permethrin (3-(2,2-dichloro-ethenyl)-2,2-dimethylcyclopropanecarboxylic acid (3-phenoxyphenyl)methyl ester) was purchased from Chem. Service, Inc. (West Chester, PA). Water, acetonitrile, methanol, methyl-tert butyl ether (MTBE), and toluene (all HPLC grade) were purchased from Fisher Scientific (Pittsburgh, PA). Waters Oasis HLB extraction cartridges were purchased from Waters Corporation (Milford, MA).

**Preparation of DEET and permethrin standards**

A stock solution of DEET and permethrin was prepared by adding 5 μg/mL solutions of both compounds prepared in methanol to a glass volumetric flask and diluting with drug-free heparinized human plasma to yield a final concentration of 400 ng/mL. The stock solution was stored at -20°C until used. Working standard solutions were prepared from the stock solution by serial dilution with blank human plasma. Plasma calibration standards of DEET and permethrin were 1, 10, 25, 50, and 100 ng/mL. An independent stock solution of DEET and permethrin was used for preparation of quality control (QC) samples. The QC solutions at 8 and 80 ng/mL were prepared as described for the standard solutions.

**Preparation of PB standards**

A stock solution (10 μg/mL) of PB prepared in water in a glass volumetric flask was used for preparation five different calibration standards. Standard water solutions of PB at levels 50, 100, 250, 500, and 1000 ng/mL were stored at -4°C. To prepare plasma standard solutions, water PB solutions were diluted 10 times with drug-free heparinized human plasma. An independent stock solution of PB was used for preparation of QC samples at 8 and 80 ng/mL.

**Sample preparation for quantification of DEET and permethrin by GC–MS**

Aliquots (1 mL) of plasma standard and quality control samples were placed in disposable glass screw-cap tubes with Teflon-lined caps. Following the addition of 0.1 mL of internal standard methanol solution (2 μg/mL), 5 mL of MTBE was added. The samples were mixed with a multi-tube vortex mixer and centrifuged at 3500 rev/min (Sorvall RT. 6000D, Dupont) at 0°C for 10 min. After freezing the lower aqueous layer for 15 min at -80°C, the upper organic layer was decanted into a glass tube and evaporated to dryness with a Heto Speed-Vac apparatus (Heto Lab. Equipment A/S, Copenhagen, Denmark) for approximately 30 min at 60°C. The residue was reconstituted with 40 μL of toluene, mixed with a vortex mixer for 5 min, and centrifuged at 12,000 rpm (Sorvall MC 12V, Dupont) for 10 min. The resulting solution was then transferred to glass insert tubes (Kimble Chromatography) and sealed with blue screw caps (Agilent, 5182-0717) for GC–MS analysis. A 1-μL aliquot of the final solution was injected into the GC–MS for quantification of DEET and permethrin.

**Sample preparation for quantification of PB by HPLC**

Solid-phase extraction (SPE) of PB from plasma was accomplished using an HLB 30 mg/1 mL SPE Oasis cartridge (WAT 094225, Waters) which was placed on Waters Extraction Manifold (WAT 200677) with vacuum set to 5 in. Hg. The column was preconditioned with 1 mL of methanol and 1 mL of water. Aliquots (0.4 mL) of plasma standard samples were applied to SPE Oasis cartridges. The SPE cartridges were washed with 1 mL of 0.01% NH4OH in water and then 1 mL of 10/90% (v/v) methanol/water. PB was eluted with 0.9 mL of 93/5/2% (v/v/v) water/methanol/acetic acid mixture. The samples were evaporated to dryness with a Heto Speed-Vac apparatus (Heto Lab. Equipment A/S) at 25°C. The residue was reconstituted with 40 μL of water, mixed with a vortex mixer for 5 min, and centrifuged at 12,000 rpm (Sorvall MC 12V, Dupont) for 10 min. The supernatant was transferred to glass insert tubes (Kimble Chromatography) and analyzed by HPLC. The injection volume for each sample was 20 μL of the final solution.
Instrumental Methods

Quantification of DEET and permethrin by GC-MS

DEET and permethrin analyses were performed on a GC coupled with a quadrupole MS (Agilent 6890 GC system with a 5973 mass selective detector). Separation of compounds was achieved on a 30-m HP-5MS 5% phenyl methyl siloxane capillary column (250-μm i.d., 0.25-μm film thickness). Helium at a flow rate of 1.2 mL/min was used as the carrier gas. The inlet temperature was set at 275°C. The column temperature was initially held at 90°C for 2 min and then programmed to 250°C at a rate of 10°C/min. Ionization of the compounds was accomplished using electron impact at 70 eV. The quadrupole was operated in the single ion monitoring mode. Electron impact fragment ions at m/z 190 for DEET, m/z 183 for permethrin, and the molecular ion at m/z 188 for 2H10-phenanthrene was used for quantification of the three compounds, respectively. Data reduction was achieved with the HP Chemstation data acquisition system, and peak areas were used for quantification.

Quantification of PB by HPLC

Quantification of PB by HPLC analysis was performed using a Hewlett-Packard HP 1100 equipped with a UV detector set at 200 nm. Separation of compounds was achieved on a Poly LC cation exchange column, PolyCAT A 100 × 2.1 mm, 5 μm, 300 Å (Nest Group) with a PolyCAT guard column Javelin 2.1 × 10 mm, 5 μm, 300 Å (Nest Group). The column temperature was maintained at ambient temperature. The mobile phase consisted of 0.447 g Na sulfate anhydrous dissolved in 140 mL of water, 3 mL of 5 mM sulfuric acid (pH is 4.7–5), and 110 mL of acetonitrile at a flow rate of 0.2 mL/min. The analysis run time was 8 min under isocratic conditions. Peak heights were used for quantification of PB.

Recovery and Stability Studies

Recovery of DEET, permethrin, and PB from plasma

Plasma calibration standards of DEET (1 and 100 ng/mL), permethrin (1 and 100 ng/mL), and PB (5 and 100 ng/mL) were analyzed by GC–MS or HPLC as described. Similar non-extracted reference standards were prepared for DEET, permethrin, and PB in their respective injection solutions and analyzed by GC–MS or HPLC as described.

DEET, permethrin, and PB stability studies

For long-term stability studies, DEET, permethrin, and PB QC samples at 8 ng/mL and 80 ng/mL levels for each compound were placed into sterile cryogenic vials (5 mL) (Corning, Canada) and stored at −80°C. Individual frozen samples of each compound were thawed and analyzed at 3, 6, and 12 months following freezing. The stability of DEET, permethrin, and PB was assessed by comparing the results of these analyses to those determined for freshly prepared QC samples. Similarly, freeze-thaw stability was determined by analyzing the plasma samples immediately after preparation and after repeated freeze-thaw cycles on two consecutive days.

Results

Calibration curves for DEET, permethrin, and PB

Figure 1 shows selected ion chromatograms of extracts from a control plasma sample and a calibration standard containing DEET, the internal standard, and the cis-trans permethrin isomers. The earlier eluting isomer of permethrin was used for quantification of this compound. Replicate calibration standards (n = 5) for each level were analyzed and the peak-area ratios, for DEET (m/z 190) and permethrin (m/z 183) to the internal standard (m/z 188), respectively, were used to construct

![Figure 1. Selected ion chromatograms of an extract from a plasma blank sample (A) and a 100 ng/mL plasma calibration standard (B). Compounds monitored are DEET ion at m/z 190 (peak 1); internal standard ion at m/z 188 (peak 2); and permethrin ion at m/z 183 (peak 3). Gas chromatography elution times were 9.6 min for DEET, 11.9 min for the internal standard, and 20.3 min and 20.5 min for the two permethrin isomers. The non-specific peak at 26 min represents products resulted from chromatographic column cleaning.](image)

Figure 2. HPLC chromatograms of plasma extracts from a plasma blank sample (A) and a 100 ng/mL PB calibration standard (B).
calibration curves. Linear regression analysis using the JMP software (SAS Institute Inc., Cary, NC) was performed for each calibration curve. The calibration curves were linear (correlation coefficient $r^2 \geq 0.998$) for both DEET and permethrin over a concentration range of 1 ng/L to 100 ng/mL.

Table I. Intra- and Interday Precision and Accuracy of Assays ($n = 5$)

<table>
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<th>Added (ng/mL)</th>
<th>Mean (ng/mL)</th>
<th>SD</th>
<th>%RSD</th>
<th>Accuracy</th>
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<td>Intraday precision</td>
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Figure 2 shows HPLC chromatograms of extracts from a control plasma sample and a PB calibration standard. An external standard method was developed for quantifying PB in human plasma over a level of 5 to 100 ng/mL. Replicate calibration standards ($n = 5$) for each level were analyzed, and peak heights were used for quantification.

Precision and accuracy

Table I lists the validation results for both intra- and interday reproducibility of the methods for quantifying DEET and permethrin. The LOQ was defined as the lowest plasma standard level whose expected mean value was within 15% of the actual value and did not deviate by more than 20%. As shown in Table I, the expected mean value determined for DEET was 92% ± 8% relative standard deviation (RSD) ($n = 5$), for permethrin was 112% ± 11% RSD ($n = 5$), and for PB was 109% ± 5% RSD. Based on these criteria, the LOQs for DEET, permethrin, and PB were acceptable.

The intraday precision and accuracy were determined after analyzing five replicates of each calibration standard. As listed in Table I, the precision (RSD) determined for each calibration standard level ranged from 1.3% to 8.0% for DEET, from 2.1% to 11.4% for permethrin, and from 3.0% to 4.8% for PB. The interday precision of the GC-MS method developed for quantifying DEET and permethrin and the HPLC method developed for quantifying PB were assessed on three subsequent days after analysis of five replicate 8 ng/mL and 80 ng/mL DEET, permethrin, and PB QC samples. The results listed in Table I for the QC standards show that the method is reproducible and accurate for both DEET and permethrin. The precision (RSD) of determination was 3% for DEET, ranged from 5% to 9% for permethrin, and from 5% to 9% for PB.

Recovery of DEET, permethrin, and PB from human plasma

The recoveries of DEET and permethrin at 1 and 100 ng/mL and for PB at 5 and 100 ng/mL from human plasma QC samples were determined for five replicate samples at each level. Mean recoveries at the low and high levels were 90% and 107% for DEET and 79% and 82% for permethrin, respectively. The mean recoveries of PB were 79% at 5 ng/mL and 86% at 100 ng/mL.

Stability of DEET, permethrin, and PB in human plasma

The stability of DEET, permethrin, and PB in plasma was investigated using QC samples at 8 and 80 ng/mL. The initial plasma levels determined for DEET, permethrin, and PB were compared with results obtained from the same samples stored at -80°C for 3, 6, or 12 months. As shown in Table II, the RSD for triplicate determinations of each QC sample at each time point was less than 13%, and the expected values determined for all QC samples ranged from 88 to 110% of their true value. The results demonstrated that DEET, permethrin, and PB were stable in

Table II. Long-Term Stability of DEET, Permethrin, and PB

<table>
<thead>
<tr>
<th>Time (months)</th>
<th>QC Sample (ng/mL)</th>
<th>DEET (ng/mL)</th>
<th>%RSD</th>
<th>Permethrin (ng/mL)</th>
<th>%RSD</th>
<th>PB (ng/mL)</th>
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human plasma at -80°C for at least 12 months. The freeze-thaw stability of DEET, permethrin, and PB was determined by analyzing the plasma samples in triplicate immediately after preparation and after repeated freeze-thaw cycles on two consecutive days. All three compounds were stable in human plasma after two freeze-thaw cycles with RSD values ranging from 7.1% (DEET, 80 ng/mL) to 8.1% (DEET, 8 ng/mL), from 2.3% (permethrin, 80 ng/mL) to 11.6% (permethrin, 8 ng/mL), and from 0.2% (PB, 80 ng/mL) to 3.6% (PB, 8 ng/mL).

Discussion

The development of a sensitive GC–MS procedure using a simple one step solvent extraction for simultaneous determination of DEET and permethrin in plasma ranging from 1 to 100 ng/mL provides a useful alternative to the existing published methods. LOQs reported for HPLC methods developed by Abu-Qare and Abou-Donia (24,25) for quantification of DEET in plasma and by Smallwood et al. (26) in human urine and plasma ranged from 90 to 200 ng/mL. An HPLC method developed by Qiu and Jun (34) for quantification of DEET in human plasma reported an LOQ of 15 ng/mL. Although their method was only 15 times less sensitive than our GC–MS method, their sample preparation required a time-consuming SPE rather than a simple MTBE extraction. To our knowledge only Abu-Qare and Abou-Donia (24,31–33) have reported HPLC methods for quantification of permethrin in rat plasma; however, their method (LOQ = 100 ng/mL) was 100-fold less sensitive than our method.

A method to quantify plasma DEET levels ranging from 19 to 1910 ng/mL by GC method using a nitrogen-phosphorus detector was reported by Taylor et al. (35). In addition, a GC–MS method was developed (36) to quantify DEET in the plasma of a woman following intentional oral ingestion of DEET; however, the method described was not optimized to quantify the low levels seen following dermal application. Hoy et al. (15) used a GC–MS method they developed to quantify both DEET and permethrin in rat plasma, but no validation data or LOQs for their analyses were reported.

We developed and validated an HPLC method for quantifying PB in human plasma over a range of 5 to 100 ng/mL using a 0.4-mL sample and SPE. Numerous methods have been reported for quantifying PB in biological fluids using solid-phase or cation exchange extraction sample preparation procedures. Matsunaga et al. (22) and Marino et al. (23) described HPLC methods for quantifying PB in human plasma with LOQs of 10 and 1.53 ng/mL, respectively; however, no validation data were presented. Other HPLC methods that reported LOQs ranging from 40 to 200 ng/mL for quantification of plasma PB concentrations (27–30) are less sensitive than the method we describe.

GC using an NPD detector and GC–MS methods have been developed to quantify PB in biological fluids (15,39,41,42). Because PB is a quaternary amine, which is thermally degraded in the GC injector to a tertiary amine, the choice of internal standard is critical to the success of this method. Unless a stable isotope analogue is used, it is our opinion that this method should not be used for routine monitoring of PB in human plasma. An HPLC–thermospray MS (38) and LC–MS method (43) have been reported capable of quantifying PB in plasma at 0.5 and 0.1 ng/mL levels, respectively, which are 2 to 10 times more sensitive than the method we describe. The instrumentation used to perform the former analysis is rather specialized and not commonly used for quantifying drugs in human plasma. Our method offers an alternative to the latter method using LC–MS for quantifying PB plasma levels ≥ 1 ng/mL.

The GC–MS and HPLC methods we developed were applied to pharmacokinetic/pharmacodynamic studies of combined exposure to DEET (skin application), permethrin (treated uniforms), and PB (30 mg orally three times/day for four doses) in healthy volunteers. The results of these studies will be published elsewhere.

In summary, the present report is the first that demonstrates a simple and rapid GC–MS procedure with a single-step liquid–liquid extraction for simultaneous determination of DEET and permethrin in human plasma. The validated methods we developed proved to be useful and reliable for the determination of DEET, permethrin, and PB in human plasma at low nanograms-per-milliliter levels.

Acknowledgments

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