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Simultaneous analysis of insect repellent DEET, sunscreen oxybenzone and five relevant metabolites by reversed-phase HPLC with UV detection: Application to an in vivo study in a piglet model

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Abstract

N,*N*-Diethyl-*m*-toluamide (DEET) and oxybenzone are two essential active ingredients in insect repellent and sunscreen preparations. We developed and validated a simple, sensitive, and selective HPLC assay to simultaneously measure DEET, oxybenzone and five primary metabolites of DEET and oxybenzone in biological samples including plasma, urine and skin strips. The compounds were separated on a reversed-phase C_{18} column using three-stage gradient steps with methanol and water. DEET and two relevant metabolites were detected at 254 nm, while oxybenzone and three relevant metabolites were detected at 289 nm. The limit of detection was 0.6 ng for DEET and 0.5 ng for oxybenzone, respectively. The developed method was further applied to analyze various biological samples from an in vivo animal study that evaluated concurrent use of commercially available insect repellent and sunscreen preparations.
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Keywords: Reversed-phase HPLC with UV detection; Concurrent application; Repellent DEET; Sunscreen oxybenzone; Relevant metabolites

1. Introduction

Concurrent use of insect repellent and sunscreen preparations has become a commonly-practiced summer routine for the general public in North America since 1999, due mainly to imminent health threats from West Nile virus and awareness of skin cancers and sun safety. *N*,*N*-Diethyl-*m*-toluamide (DEET, Fig. 1a) has been used as one of the most effective insect repellents for decades [1,2]. Oxybenzone (OBZ, Fig. 1b) is an essential UVA/UVB blocking agent that is extensively used in commercially available sunscreen products [3,4]. Systemic absorption of both compounds after topical applications has been separately studied [5–7]. Recently we reported a synergistic percutaneous permeation between DEET and oxybenzone after the two active ingredients were used simultaneously in vitro [8,9].

DEET is subject to prompt metabolism in vivo after percutaneous absorption [10]. Its primary metabolites include *N*,*N*-diethyl-*m*-hydroxymethylbenzamide (DHMB, Fig. 1c) and *N*-ethyl-*m*-toluamide (ET, Fig. 1d). High performance liquid chromatography (HPLC) with ultraviolet (UV) or mass spectrometry (MS) detection has been used to measure DEET and its metabolites in biological samples [10–12]. Major metabolites of oxybenzone include 2,4-dihydroxybenzophenone (DHB, Fig. 1e), 2,2′-dihydroxy-4-methoxybenzophenone (DMB, Fig. 1f), and 2,3,4-trihydroxybenzophenone (THB, Fig. 1g) [7]. HPLC assays with UV detection have also been developed to measure concentrations of these compounds in biological samples [13,14]. Nevertheless, no study has been attempted to simultaneously measure concentrations of DEET, oxybenzone and their primary metabolites in biological samples after a concurrent topical application. We have previously developed and validated a simple isocratic reversed-phase HPLC-UV method to measure concentrations of DEET and oxybenzone in samples collected from

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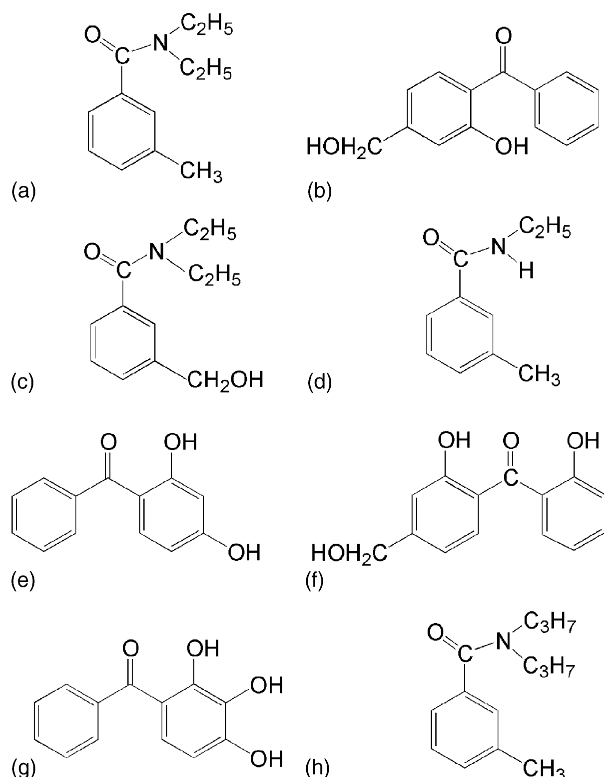

Fig. 1. Structure of test compounds: (a) *N*,*N*-diethyl-*m*-toluamide (DEET); (b) oxybenzone (OBZ); (c) *N*,*N*-diethyl-*m*-hydroxymethylbenzamide (DHBM); (d) *N*-ethyl-*m*-toluamide (ET); (e) 2,4-dihydroxybenzophenone (DHB); (f) 2,2′-dihydroxy-4-methoxybenzophenone (DMB); (g) 2,3,4-trihydroxybenzophenone (THB) and (h) *N*,*N*-dipropyl-*m*-toluamide (DPT).

in vitro diffusion studies [8,9]. However, it was not feasible to use this assay for the measurement of biological samples from in vivo pharmacokinetic studies that will likely contain DEET, oxybenzone and their primary metabolites.

In this study, we developed a simple, selective and sensitive HPLC assay that was able to quantify DEET, oxybenzone and five relevant metabolites in biological samples. The chromatographic conditions were optimized to achieve satisfactory separation of the compounds, high sensitivity of the detection, reasonable runtime and simple pretreatment of the samples. The assay was further used to measure various biological samples including plasma, urine and skin strips that were collected from a preliminary in vivo animal study in which three commercially available repellent and sunscreen products were tested in piglets.

2. Experimental

2.1. Materials and reagents

DEET and oxybenzone were purchased from Fluka Chemika GmbH (Buchs, Switzerland) and Riedel-de Haën GmbH (Seelze, Germany), respectively. Metabolites of DEET, DHMB and ET, and the internal standard, *N*,*N*-dipropyl-*m*-toluamide (DPT, Fig. 1h), were provided as gifts by Dr. W.G. Taylor, Agriculture and Agri-Food Canada, Saskatoon, Canada. Metabolites of oxybenzone, DHB, DMB and THB, were purchased from Sigma–Aldrich Co. (Oakville, Ontario, Canada). Glacial acetic acid was purchased from Mallinckrodt Chemicals (Paris, Kentucky, USA). HPLC-grade acetonitrile, isopropanol and methanol were obtained from Fisher Scientific (Fair Lawn, New Jersey, USA). Deionized water was obtained from a Milli-Q® Pure Water System (Nepean, Ontario, Canada).

2.2. HPLC conditions

Test compounds were simultaneously separated and analyzed using a Waters® 2690 Alliance Solvent Delivery System, a 996 Photodiode Array Detector and a Symmetry® C_{18} column (3.9 mm × 150 mm, 4 μm) (Milford, Massachusetts, USA) at ambient temperature. The HPLC system was operated by Millennium® 32 Software.

The mobile phase was composed of methanol and water (pH 2.83, adjusted with glacial acetic acid). Three-stage gradient steps were used, 0–3 min, 50% methanol/50% water (v/v); 3–9.5 min, 70% methanol/30% water; 9.5–14 min, 90% methanol/10% water. A 2 min interval was allowed between two injections to condition the column. The flow rate was maintained at 0.8 ml/min throughout the sample run. DEET, its two metabolites and the internal standard were detected at 254 nm, while oxybenzone and its three metabolites were detected at 289 nm.

2.3. Sample preparations

The standard stock solutions were prepared by accurately weighing each compound and then dissolving in individual volumetric flasks with methanol at 1.0 mg/ml. The working solutions of each compound were prepared by further dilution of the stock solutions with methanol before used for spiking blank plasma and urine samples in assay development and validation.

Calibration curves were prepared by spiking 1.0 ml of blank plasma or urine specimen with the working solutions to concentrations of 0.1, 0.5, 1.0, 2.0, 4.0 and 5.0 μg/ml. The samples were stirred for 30 min at 500 rpm on a Variomag® multi-station magnetic stirrer (Daytona Beach, Florida, USA) and vigorously vortexed for 1 min. Three hundred microliters of a mixture of methanol and acetonitrile (1:2, v/v) were then added to the samples, and the solutions were further vortexed for 1 min, following by centrifugation at 10,000 × *g* for 10 min. The supernatants were transferred into sample vials, and 10 μl was injected to the HPLC system for drug analysis.

2.4. Assay optimization and validation

Assay optimization was carried out based on previous isocratic reversed-phase HPLC methods that were developed

and used in our laboratory for in vitro diffusion of DEET and oxybenzone. Since six new compounds were added to the samples and there were also numerous biological components present, three-stage solvent gradient steps were used to improve separation and detection of each individual compound.

Method validation was performed by injecting six replicates of spiked plasma samples at 0.1 and 5.0 μg/ml of each compound during the same working day (intra-day variation) and on six different days (inter-day variation). The peak area ratios and variations were calculated. In addition, extraction recovery was determined by checking spiked plasma and urine samples at known concentrations of 0.5 and 5.0 μg/ml. Six replicates were also used in extraction recovery determination.

2.5. Preliminary in vivo animal study

Transdermal penetration and systemic absorption of DEET and oxybenzone were tested in nine 3-week-old piglets (approximately 5 kg body weight). The animal use protocol was approved by the University of Manitoba Fort Garry Campus Animal Use Protocol Management and Review Committee and conducted according to current guidelines published by the Canadian Council for Animal Care (CCAC). Piglet was selected as the test model because of its anatomical skin similarity to humans [15,16]. In addition, the piglet provided sufficient back surface for topical application and blood volume for pharmacokinetic studies, while its size was easily manageable for animal handling and blood sampling.

A venous catheter was surgically inserted into the jugular vein of the animals to facilitate blood collection, using established standard techniques [17]. The animals were allowed 3 days for full recovery from the surgery before the study commenced. Before dermal application of repellent and sunscreen preparations, an area of 150 cm^2 (10 cm × 15 cm) on the back was shaven with an electric clipper to clean the hair. One gram of the three commercially available products (a repellent lotion that contained 9% DEET, a sunscreen lotion that contained 6% oxybenzone, and a combined repellent/sunscreen lotion that contained 9% DEET and 5% oxybenzone) was evenly applied to the shaven back area.

To evaluate dermal penetration of DEET and oxybenzone, skin tape strips were collected at 2, 12 and 48 h after the application. Before skin stripping, a small area of the application site on the back (2 cm × 4 cm) was cleaned twice with alcohol swipes to remove the applied dose. After complete drying of the skin surface, 12 strips of Scotch® tape (3 M, St. Paul, Minnesota, USA) were evenly applied to the skin and pressed for 10 s, and then removed. Each tape strip was placed individually in labeled polyester tube, and stored at −20 °C until drug analysis. Different application areas were used for each skin stripping in order to realistically represent the dermal penetration of DEET and oxybenzone from the applied preparations.

Two ml of blood samples were collected from the inserted jugular vein catheter to heparinized Vacutainer® tubes (BD, Franklin Lakes, New Jersey, USA) at 0, 2, 4, 6, 8, 12, 24 and 48 h after the dermal application. Plasma was separated by centrifugation at 5000 × *g* for 10 min, and immediately transferred to labeled polyester tubes and stored at −20 °C until drug analysis. Urine samples were collected directly from the bladder at 48 h after euthanasia of the animals. The volume of urine samples was measured and a portion of the urine was immediately transferred to labeled polyester tubes and stored at −20 °C until drug analysis.

Plasma and urine samples were extracted as previously described before the HPLC analysis. For skin tape strips, each tape was first placed in 1.0 ml isopropanol to dissolve the adhesive materials, followed by extraction of the test compounds with 2.0 ml of acetonitrile. The mixtures were centrifuged at 10,000 × *g* for 10 min, and the supernatants were transferred to sample vials and injected into the HPLC system.

2.6. Data analysis

The concentrations of DEET and oxybenzone in plasma, urine and tape strips were calculated based on the developed assay. The presence of the five metabolites in plasma, urine and skin strip samples was also checked, and quantified where detected. Plasma concentrations of DEET and oxybenzone were subject to pharmacokinetic simulation using WinNonlin® program (Pharsight Corporation, Mountain View, California, USA). Transdermal penetration of DEET and oxybenzone after topical application was calculated based on the recovered amounts found in tape strips. Statistical analysis was performed using two-way ANOVA and Tukey's test (PC-SAS® 8.02, SAS Institute Inc., Cary, North Carolina, USA). The following statistical analyses of the data were conducted: (a) the area under the plasma concentration curve (AUC) of DEET and oxybenzone among the three test preparations; (b) the pharmacokinetic parameters of DEET and oxybenzone among the three test preparations; (c) the percutaneous permeation of DEET and oxybenzone among the three application methods. Differences were considered statistically significant at $p \leq 0.05$.

3. Results and discussion

3.1. Chromatography

The use of insect repellents and sunscreens to prevent vector-borne diseases and skin cancers has dramatically increased over the years. They are able to provide convenient, economic and practical approaches for both the field workers to conduct their duties and the general public to enjoy outdoor recreational activities while minimizing potential health threats from the environment. Designed as topical skin preparations, active ingredients from repellents and sunscreens

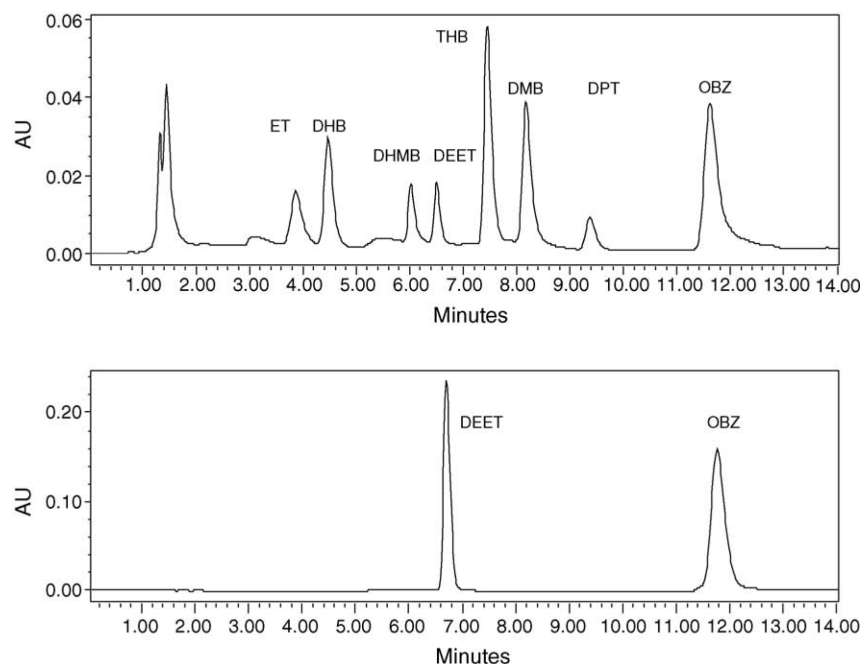

Fig. 2. Representative chromatograms of the test compounds at 289 nm in spiked plasma (5.0 μg/ml, top) and in tape strip (bottom).

such as DEET and oxybenzone should possess minimal percutaneous penetration and systemic absorption. Analytical methods available to date have dealt with either insect repellents or sunscreens separately because there had been no need to apply both the preparations concurrently. This situation has however changed due to extensive healthcare awareness of West Nile virus and skin cancers. In addition, we have reported the synergistic percutaneous penetration between DEET and oxybenzone when applied simultaneously in vitro.

Fig. 2 shows the representative chromatograms of spiked plasma samples and tape strips, respectively. DEET, oxybenzone and their metabolites in plasma were well separated with the help of three-stage gradient steps. Components present in tape strips and commercially available products did not interfere with the analysis of DEET and oxybenzone, as sufficient amount of the applied dose was recovered in the tape strips and the assay was highly sensitive and selective for the test compounds. Measurements of biological samples generally require highly robust and sensitive assays to compensate for drug loss due to extraction procedures and to eliminate potential interferences from components other than the test targets. This developed assay not only offered simultaneous detection of major compounds that would likely be found from concurrent use of insect repellent and sunscreen products, but also provided rapid and selective detection of multiple compounds. In addition, assay sensitivity was further improved in this method compared to our previous method [8,9]. The sample runtime of the assay was also shortened compared to other earlier reports that analyzed the components separately [10,11]. The method was capable of measuring drug contents from different samples including plasma, urine and skin strips with minimal extraction modifications, which could significantly reduce time and resource required to provide an analytical method for pharmacokinetic studies.

3.2. Assay precision and accuracy

Table 1 lists calibration linearity and detection limit of the developed assay. Excellent linearity of each test compound was achieved between the concentrations of 0.1–5.0 μg/ml. While limit of detection (LOD) was calculated as 3 times greater than the baseline noise, limit of quantification (LOQ) was calculated as 10 times greater than the baseline noise. Under the current chromatographic conditions, LOD and LOQ ranged 0.5–0.7 ng and 1.7–2.3 ng for the test compounds, respectively. The total runtime for each injection was 14 min, which was considered satisfactory for biological samples. In order to maximize assay sensitivity, DEET and its metabolites were detected at 254 nm, while oxybenzone and its metabolites were detected at 289 nm. The selection of two different wavelengths for drug analysis by photodiode array

Table 1
Calibration of the test compounds in spiked plasma samples ($n = 6$)

Compound	Detection wavelength (nm)	Linear range (μg/ml)	Correlation coefficient (r^2)	Detection limit (ng)
DEET	254	0.1–5.0	0.9999	0.6
DHMB	254	0.1–5.0	0.9999	0.6
DHB	289	0.1–5.0	0.9999	0.7
DMB	289	0.1–5.0	0.9999	0.6
ET	254	0.1–5.0	0.9998	0.5
OBZ	289	0.1–5.0	0.9998	0.5
THB	289	0.1–5.0	0.9999	0.5

Table 2
Inter- and intra-day variation of the test compounds in spiked plasma samples ($n = 6$, mean ± S.D.)

Compound	Inter-day variation				Intra-day variation			
	0.1 μg/ml	R.S.D. (%)	5.0 μg/ml	R.S.D. (%)	0.1 μg/ml	R.S.D. (%)	5.0 μg/ml	R.S.D. (%)
DEET	0.09 ± 0.003	3.26	4.96 ± 0.03	0.60	0.10 ± 0.004	4.08	4.92 ± 0.04	0.73
DHMB	0.10 ± 0.003	3.01	5.03 ± 0.40	7.85	0.10 ± 0.26	5.05	4.99 ± 0.02	0.44
DHB	0.10 ± 0.003	3.03	4.97 ± 0.62	1.25	0.10 ± 0.05	4.99	5.05 ± 0.39	7.70
DMB	0.10 ± 0.01	9.28	4.99 ± 0.40	7.96	0.10 ± 0.01	6.25	4.98 ± 0.02	0.46
ET	0.10 ± 0.04	3.91	4.96 ± 0.03	0.67	0.10 ± 0.20	7.88	4.93 ± 0.30	6.11
OBZ	0.10 ± 0.004	4.17	4.97 ± 0.27	5.33	0.10 ± 0.001	1.04	4.99 ± 0.30	6.05
THB	0.10 ± 0.01	4.90	5.01 ± 0.03	0.58	0.09 ± 0.001	1.09	4.97 ± 0.31	6.20

detector provided further improvement in method sensitivity and selectivity.

Assay validation was conducted at two concentrations in plasma, and Table 2 lists the inter- and intra-day assay variation. The analytical variation ranged 0.44–9.28%, indicating satisfactory accuracy and reproducibility of the instrumentation. In addition, sample pretreatment was relatively easy and straightforward, which involved simple extraction and dilution with various organic solvents. The extraction recovery for all test compounds from plasma and urine samples was greater than 78% (ranging 78–99%, Table 3). The achieved extraction recovery enabled accurate quantification of all test compounds, particularly those metabolites that would normally be found in biological specimens in trace amount from a topical skin application.

3.3. Method application

Fig. 3 shows the plasma concentration of DEET and oxybenzone versus time plots. Compared to their single-component preparation, simultaneous use of repellent and sunscreen produced higher plasma concentrations of DEET and oxybenzone after topical application, indicating enhanced percutaneous penetration and systemic absorption of DEET and oxybenzone. This in vivo percutaneous profile between DEET and oxybenzone was identical to what had been previously found in diffusion studies in vitro [8,9]. The AUC (0–48 h) of DEET and oxybenzone from the combined repellent/sunscreen product was significantly higher than that of its individual counterparts, 446.21 ± 18.30 and 286.59 ± 11.65 μg h/ml (mean ± S.D.) for DEET and oxybenzone, respectively, versus 335.95 ± 13.83 and 178.24 ± 21.31 μg h/ml for DEET and oxybenzone. Pharmacokinetic simulation using one-compartment model indicated a very close percutaneous absorption lag-time for both DEET and oxybenzone (0.97 ± 0.04 h). However, DEET showed a significantly shorter elimination half-life (5.25 ± 0.95 h) than oxybenzone (7.14 ± 1.29 h). In addition, the calculated time to reach the maximal concentration (T_{max}) for oxybenzone (1.81 ± 0.43 h) was significantly shorter than that for DEET (2.65 ± 0.27 h), which might indicate the role of oxybenzone as a percutaneous penetration enhancer [8,9,18].

Measurement of skin penetration rate and extent of a topical preparation by tape stripping has been widely used to estimate percutaneous absorption in different skin layers in dermatology [19–21]. Once a permeant penetrates across stratum corneum, it is likely to be absorbed systemically into the blood circulation. Understanding the skin penetration profiles of a compound is beneficial in evaluating transdermal drug delivery and absorption. Fig. 4 shows the total recovery amount of DEET and oxybenzone from 12 tape strips at different time intervals after the topical dermal applications. Compared to its single-component counterparts, total recovery of both DEET and oxybenzone from combined repellent/sunscreen preparation was significantly higher at all sampling intervals after the topical application; the increments of percutaneous penetration of DEET were 61% at 2 h, 52% at 12 h and 106% at 48 h, respectively, while the increments of percutaneous penetration of oxybenzone were 69% at 2 h, 76% at 12 h and 37% at 48 h, respectively. Recovery of DEET was significantly higher than that of oxybenzone,

Table 3
Extraction recovery of the test compounds from spiked plasma and urine samples ($n = 6$, mean ± S.D.)

Compound	Plasma extraction recovery (%)		Urine extraction recovery (%)	
	0.5 μg/ml	5.0 μg/ml	0.5 μg/ml	5.0 μg/ml
DEET	90.36 ± 2.78	86.74 ± 4.08	85.65 ± 1.44	88.05 ± 3.18
DHMB	83.19 ± 2.27	88.02 ± 2.99	83.19 ± 2.27	83.83 ± 4.60
DHB	94.13 ± 3.32	97.60 ± 1.68	92.76 ± 2.76	95.12 ± 2.39
DMB	78.97 ± 2.34	91.46 ± 4.47	91.04 ± 4.28	96.96 ± 2.60
ET	83.31 ± 4.97	85.19 ± 2.97	77.71 ± 2.10	81.55 ± 2.53
OBZ	99.18 ± 1.20	98.60 ± 1.01	99.28 ± 1.67	99.02 ± 1.09
THB	89.99 ± 2.67	95.19 ± 3.22	89.83 ± 3.21	89.10 ± 1.35

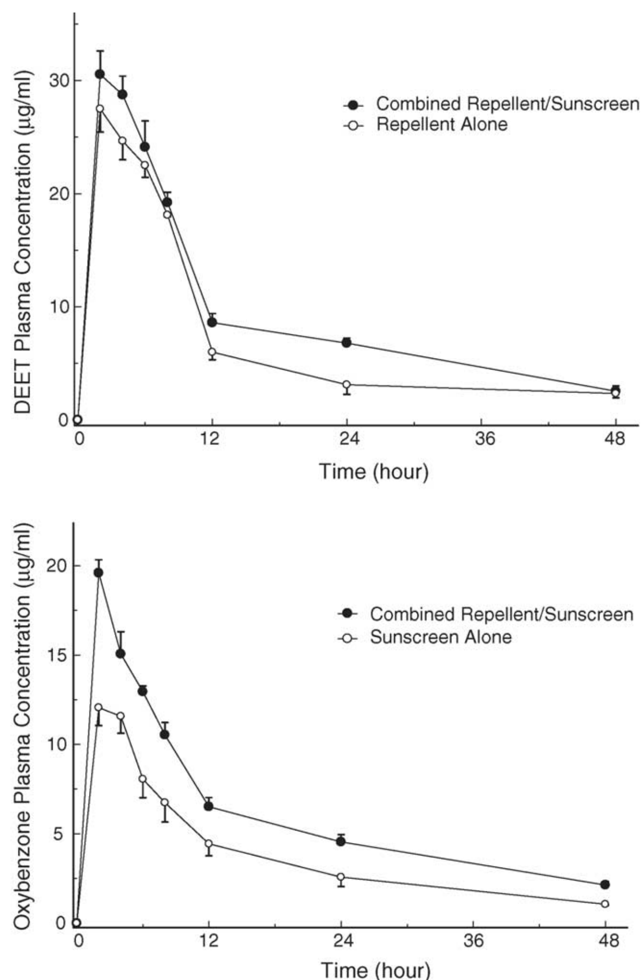

Fig. 3. Plasma concentration–time curves of DEET (top) and oxybenzone (bottom) ($n = 3$, mean ± S.D.).

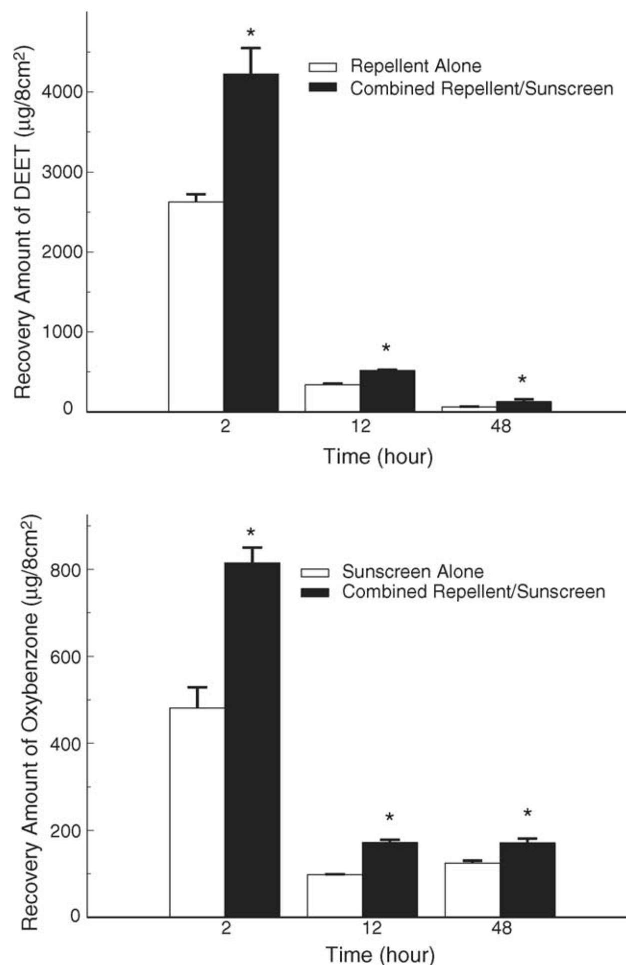

Fig. 4. Total recovery of DEET (top) and oxybenzone (bottom) from 12 tape strips ($n = 3$, mean ± S.D., *significant difference from single-component preparation, $p \leq 0.05$).

which was consistent with what was found in plasma concentrations. This might indicate that concurrent use of repellent and sunscreen preparations impacted DEET more than oxybenzone in term of percutaneous penetration and systemic absorption. Similar profiles were also found in our laboratory with in vitro diffusion studies with various membrane models [7,8].

No quantitative concentrations of major metabolites of DEET and oxybenzone were found in tape strips, indicating minimal metabolism of DEET and oxybenzone in stratum corneum. Both DEET and oxybenzone have relatively short elimination half-lives, so no quantitative concentrations of major metabolites were detected in plasma samples. However, urine samples collected 48 h after the topical application showed various amounts of metabolites (Table 4). When blood samples are not available for drug monitoring, the developed assay could still be used to analyze metabolites in urine or skin samples for pharmacokinetic studies, which showed satisfactory versatility and adaptability of the assay.

Table 4
Concentrations (μg/ml) of the test compounds in urine samples 48 h after the topical application ($n = 3$, mean ± S.D.)

Compound	Repellent	Sunscreen	Repellent + sunscreen
DEET	No detection	–	No detection
DHMB	0.99 ± 0.04	–	1.33 ± 0.07
DHB	–	0.48 ± 0.03	0.48 ± 0.06
DMB	–	No detection	No detection
ET	0.55 ± 0.05	–	0.55 ± 0.05
OBZ	–	0.10 ± 0.01	0.13 ± 0.01
THB	–	0.06 ± 0.01	0.06 ± 0.03

4. Conclusion

The reversed-phase HPLC-UV assay described above for the simultaneous analysis of insect repellent DEET, sunscreen oxybenzone and five relevant metabolites involved simple pretreatment and extraction of the biological samples including plasma, urine and skin tape strips. Eight related test compounds were well separated with the help of three-stage solvent gradient steps, and the chromatographic runtime was 14 min. This method was capable of providing sufficient

accuracy, specificity, selectivity, and versatility, which would be suitable for simultaneous quantification of DEET, oxybenzone and their metabolites in pharmacokinetic and pharmacodynamic studies in vivo. The method application to an in vivo animal study confirmed the enhanced percutaneous penetration and systemic absorption of DEET and oxybenzone when repellent and sunscreen preparations were used concurrently.

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