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Journal of Chromatography B, 780 (2002) 45–52

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Analysis of *N,N*-diethyl-*m*-toluamide in porcine skin perfusates using solid-phase extraction disks and reversed-phase high-performance liquid chromatography

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Received 26 February 2002; received in revised form 3 June 2002; accepted 11 June 2002

Abstract

N,N-Diethyl-*m*-toluamide (DEET) is frequently used as an insect repellent by military and civilian populations. Because dermal exposure has resulted in several cases of DEET toxicosis, there is a need to rapidly and reliably determine DEET concentrations in biological matrices. An improved method for the analysis of DEET was developed for determining transdermal diffusion of low levels of DEET following application to an in vitro porcine skin flow-through diffusion cell system. The technical improvement involved the use of disk solid-phase extraction (SPE) instead of packed-bed SPE. The disk SPE method required small volumes of preconditioning, wash, and elution solvent (0.5–1 ml) to extract DEET from perfusate samples containing bovine serum albumin (BSA). The limit of quantitation (LOQ) was estimated as 0.08 μg/ml DEET and recoveries from BSA media samples spiked with DEET ranged from 90.1 to 117% with relative standard deviation (RSD) ranging from 2.0 to 13.1%. This method was used to analyze perfusate samples from skin ($n=4$) topically exposed to DEET–ethanol formulations. The data from these analyses determined that DEET permeability in porcine skin was $2.55\times10^{-5}\pm0.54\times10^{-5}$ cm/h.

Keywords: *N,N*-Diethyl-*m*-toluamide

1. Introduction

DEET (*N,N*-diethyl-*m*-toluamide) is commonly used as a repellent for mosquitoes and other insects. It has caused toxicity in humans and in animals, and it is believed to be one of several chemicals associated with the Gulf War Syndrome [1] in veterans who were exposed to high concentrations of DEET. More recent studies have also demonstrated that DEET may potentiate the toxicity of other chemicals and it may also modulate dermal absorption of drugs and pesticides [1,2].

In light of these findings, there is a need to improve the analytical methods used to assay for DEET in biological matrices following human or animal exposure to DEET. Rapid and validated methods are therefore useful to critically evaluate transdermal permeation of DEET formulations following topical exposure. Extracting DEET from

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1570-0232/02/$ – see front matter
PII: S1570-0232(02)00412-9

biological matrices however, can be a challenge as many endogenous compounds may interfere with UV detection at low wavelengths (200–220 nm) where UV absorption is optimum for DEET. Previous analytical methods entailed the use of traditional packed-bed SPE [3–5] and others have used liquid–liquid extraction [6] in separating DEET from the biological matrix. The focus of this research was to evaluate the application of disk SPE extraction technology (Ansys Technologies) instead of the traditional packed-bed columns without compromising chromatographic quality, and to apply this method so as to rapidly assess the permeation of DEET in the porcine skin flow-through diffusion cell system.

Disk SPE technology has been reviewed extensively [7], and it has numerous applications including drug screening [8] and analysis of environmental samples [9]. However, none of the SPE methods have taken advantage of the disk SPE technology for DEET analysis and furthermore applied it to a biological matrix such as BSA. The disks are glass fiber impregnated with bonded silica, which should reduce channeling and clogging sometimes associated with viscous biological matrices such as plasma or bovine serum albumin in traditional packed bed SPE.

This manuscript describes some improvement to the traditional SPE method as it was applied to quantitating diffusion in a porcine skin flow-through diffusion system. Furthermore, this improved method required significantly less solvent for sample extraction/preparation saving time and money when a large number of samples are to be analyzed. This improved method is also capable of analyzing small sample volumes which is the situation often encountered in assessing permeability in skin diffusion studies at early time points. BSA or perfusion media that imitates blood is frequently used to perfuse tissue or whole organs [10]. However most of the previous in vitro transdermal research with DEET [11,12] utilized Franz cells and phosphate buffered solution (PBS) which did not require extraction prior to HPLC analysis. Chemical permeation from skin into PBS does not always reflect absorption into human blood. The BSA used in this study has biochemical properties that are very similar to those of blood.

2. Experimental

2.1. Chemicals

N,N-Diethyl-*m*-toluamide (DEET), 98%, was purchased from Chem Service (West Chester, PA, USA). HPLC grade acetonitrile, reagent grade ammonium acetate, and glacial acetic acid were purchased from Fisher Scientific (Fair Lawn, NJ, USA). All water was purified with an ultra high purity water filtration system (Dracor Water Systems, Durham, NC, USA). All reagents used in preparation of the BSA media were purchased from Fisher Scientific (Fair Lawn, NJ, USA), with the exception of amikacin (GensiaSicor Pharmaceuticals, Irvine, CA, USA), heparin (Elkins-Sinn Pharmaceuticals, Cherry Hill, NJ, USA), and penicillin G sodium (Marsam Pharmaceuticals, Cherry Hill, NJ, USA).

2.2. Apparatus

The HPLC system was equipped with a Waters model 60F solvent pumping system, Waters 600 controller, Waters 717 plus autosampler, Waters temperature control module, and Waters 996 PDA detector (Milford, MA, USA). All data were collected on a Gateway E3110 computer (North Sioux City, SD, USA) utilizing Waters Millennium 32 version 3.05.01 software. A Waters SymmetryShield RP18 (particle size 3.5 μm, 150×4.60 mm I.D.) column was used for the separations.

The 24-port vacuum manifold was obtained from VWR Scientific Products (S. Plainfield, NJ, USA). The solid-phase extraction disks, ANSYS SPEC C_{18} 3 ml columns (sorbent weight 15 mg), were obtained from ANSYS Technologies (Lake Forest, CA, USA).

2.3. HPLC conditions

The mobile phase composition (v/v) was 36% acetonitrile and 64% ammonium acetate buffer (0.03 *M*). The pH of the ammonium acetate buffer was adjusted to pH 4.5 with glacial acetic acid. The guard and analytical column temperature was set at 35±0.5 °C.

The detector wavelength was 220 nm. The flow-rate and injection volume were 1.0 ml/min and 10

μl, respectively. DEET levels in BSA samples were determined by comparing the peak areas of DEET in the samples to an external standard calibration curve.

2.4. Standard solutions

The DEET stock solution (2.740 μg/ml) was prepared by dissolving approximately 0.1370 g of DEET in 50 ml of acetonitrile. Two DEET working solutions were prepared by diluting the DEET stock solution with acetonitrile. The 100-μg/ml DEET working solution was prepared by diluting approximately 1.825 ml of the DEET stock solution to 50 ml with acetonitrile. The 50-μg/ml DEET working solution was prepared by diluting approximately 0.912 ml of the DEET stock solution to 50 ml with acetonitrile. The standards were prepared by spiking the same volumes of the elution solvent with the same amounts of DEET used for spiking the BSA media. For example, approximately 4 ml of the solid-phase extraction (SPE) elution solvent was added to each of the five 16×125 mm borosilicate glass tubes. The SPE elution solvent consisted of 40% acetonitrile and 60% ammonium acetate buffer (pH 4.5, 0.03 *M*). An 8-μl volume of the 50-μg/ml DEET working solution was added to the first tube. A 40-μl sample of the 50-μg/ml DEET working solution was added to the second tube. Forty (40) μl of the 100-μg/ml DEET working solution was added to the third tube. A 7.3-μl sample of the 2.740 μg/ml DEET stock solution was added to the fourth tube. The fifth tube was used as a blank. The final DEET standard concentrations were 0.1, 0.5, 1.0, and 5.0 μg/ml DEET. These DEET concentrations covered the range of DEET levels extracted from BSA samples observed in previous studies [2]. All solutions were vortexed briefly. The spiked samples were prepared in the same manner using BSA media instead of SPE elution solvent. The preparation of the BSA (4.5%) media was previously described [13]. In brief, 2 liters of BSA media contained the following: NaCl, 13.78 g; KCl, 0.71 g; $CaCl_2$, 0.56 g; KH_2PO_4, 0.32 g; $MgSO_4$–$7H_2O$, O.58 g; $NaHCO_3$, 5.50 g; Dextrose, 2.40 g; BSA Fraction V, 90.0 g. The media pH was adjusted to approximately 7.4, and 0.25 ml of amikacin (250 mg/ml) was added to control bacterial growth, and the BSA media was frozen until needed. Prior to use in the flow-through diffusion cell system, the BSA media was thawed and 10 ml of sodium heparin (1000 USP units/ml) and 0.1 ml of penicillin G sodium (250 000 Units/ml) were added to the BSA media.

2.5. Sample preparation

In order to determine the amount of DEET absorbed through the porcine skin, it was necessary to extract DEET from the perfusate prior to analysis by HPLC. The perfusate from the porcine skin flow-through experiments consisted of BSA media. The perfusate was extracted by placing 1 ml of each sample into 16×125-mm glass tubes. Each sample was then diluted with 3 ml of HPLC grade water. The solid-phase extraction disks housed inside 3 ml plastic syringe barrels, were placed onto the vacuum manifold. Glass tubes (16×125 mm) were placed inside the vacuum manifold to collect the filtrates. The conditioning step involved adding 500 μl of acetonitrile to the disks which was allowed to flow by gravity into the glass collection tubes, and this was followed by 500 μl of ultra-pure water. Care was taken not to allow the disks to become dry in order to insure that the C_{18} ligands bonded to the surface of the embedded silica particles would not collapse. The samples were loaded onto the disks using glass disposable Pasteur pipettes. The samples were allowed to flow through the disk by gravity or low vacuum (2 in. Hg) at a flow rate of approximately 1 ml/min in order to optimize recovery of the analyte, DEET. Preliminary analyses with higher flow-rates resulted in variable recoveries. When each sample meniscus reached the top of the disks, 500 μl of the wash solvent (10:90 Acetonitrile: pH 4.5 ammonium acetate buffer, 0.03 *M*) was added to the disks. Once the sample wash flowed through the disks, a strong vacuum (15 in. Hg) was applied for 5 min in order to dry out the disks before the final elution step. New clean glass collection tubes were placed in the vacuum manifold. The samples were then eluted at approximately 1 ml/min with 1 ml of the elution solvent (40:60 acetonitrile: pH 4.5 ammonium acetate buffer, 0.03 *M*) and placed directly into HPLC vials for analysis.

2.6. Method validation

Recovery samples and blanks were run with every batch of samples as part of the quality control. Intra-day precision and accuracy was determined by extracting all replicates of four different spiked concentrations on the same day. Inter-day precision and accuracy was determined by extracting one replicate of four different spiked concentrations on six different days. Since some of the early perfusion samples were taken at smaller time intervals, it was necessary to use 0.5 ml samples with 0.5 ml elution volumes for samples taken up to 1 h post exposure. The elution volumes were always kept the same as the sample volumes so that there was no dilution factor.

2.7. Flux and permeability of DEET in porcine skin

Fresh skin sections 0.050 cm thick were dermatomed from the back of 25–30 kg Yorkshire cross pigs ($n=4$), and then carefully mounted into a 14-cell flow-through diffusion cell system (Crown Glass Company, Somerville, NJ, USA) and perfused with BSA [2,13]. Skin membrane thickness was confirmed with a micrometer prior to placing the skin sections into the flow-through diffusion cells. The diffusion cell temperatures were kept constant at 37 ± 1 °C throughout the course of the experiments to mimic average body temperatures in humans. The temperature of dermal side of the skin is usually close to the body temperature, while the epidermal or surface side is slightly less than 37 °C. These four diffusion cells which have a cell receptor volume of 0.13 ml were dosed with 10 μl dose volume of a 75% DEET and 25% ethanol solution. The BSA media was perfused through the lower receiver compartments of each cell at a flow rate of approximately 4 ml per hour. The BSA effluent from each receiver compartment was sampled at 0, 10, 20, 30, 45, and 60 min after dosing. After the initial 60-min exposure, samples were obtained every 30 min until 8 h. Samples were immediately frozen at −80 °C until extraction and analysis by disk SPE and HPLC–UV. The following equation was used to calculate the apparent permeability of DEET through porcine skin:

$$K_p = J/\Delta C$$

where K_p is the apparent permeability (cm/h), J is the flux at steady state, and ΔC is assumed to be the concentration of DEET in the dosing solution. For these experiments, ΔC was equal to 7.470 μg DEET in the 10 μl of dosing solution and applied to a surface area of 0.32 cm^2. The steady state flux, J ($\mu g/cm^2/h$), was estimated by calculating the slope of the plot of cumulative amount of DEET per unit surface area ($\mu g/cm^2$) versus time (h). The following equation was used to calculate DEET diffusivity (D, cm^2/h) in porcine skin:

$$D = L^2/6\tau$$

where L is the thickness of skin membrane (0.050 cm), and τ is the lag time which is obtained by extrapolating the slope from the cumulative plot to the time axis.

3. Results and discussion

3.1. Chromatography

Fig. 1A shows a typical chromatogram for a sample containing 1.0 μg/ml of DEET extracted from BSA media. The blank (Fig. 1B) did not contain any peaks eluting at or near the same retention time as DEET. This is unique as the BSA media contains several antibiotics such as penicillin and amikacin and endogenous compounds associated with skin. DEET typically eluted at 9.5 min under the conditions specified. Any variation in the retention time of DEET was monitored with external standards in every daily sample run. The four external standards were injected at the beginning, in the middle and at the end of each set of samples, and the peak areas did not vary for these external standards. Fig. 1 also further demonstrates that the clean chromatography often associated with packed-bed SPE was not compromised with the disk SPE method. Setting the column temperature to 35 °C allowed for more control of experimental conditions since the ambient temperature varied. Furthermore, at the higher column temperature, the viscosity of the mobile phase was decreased, thereby reducing the back-pressure on the column, and providing sharper

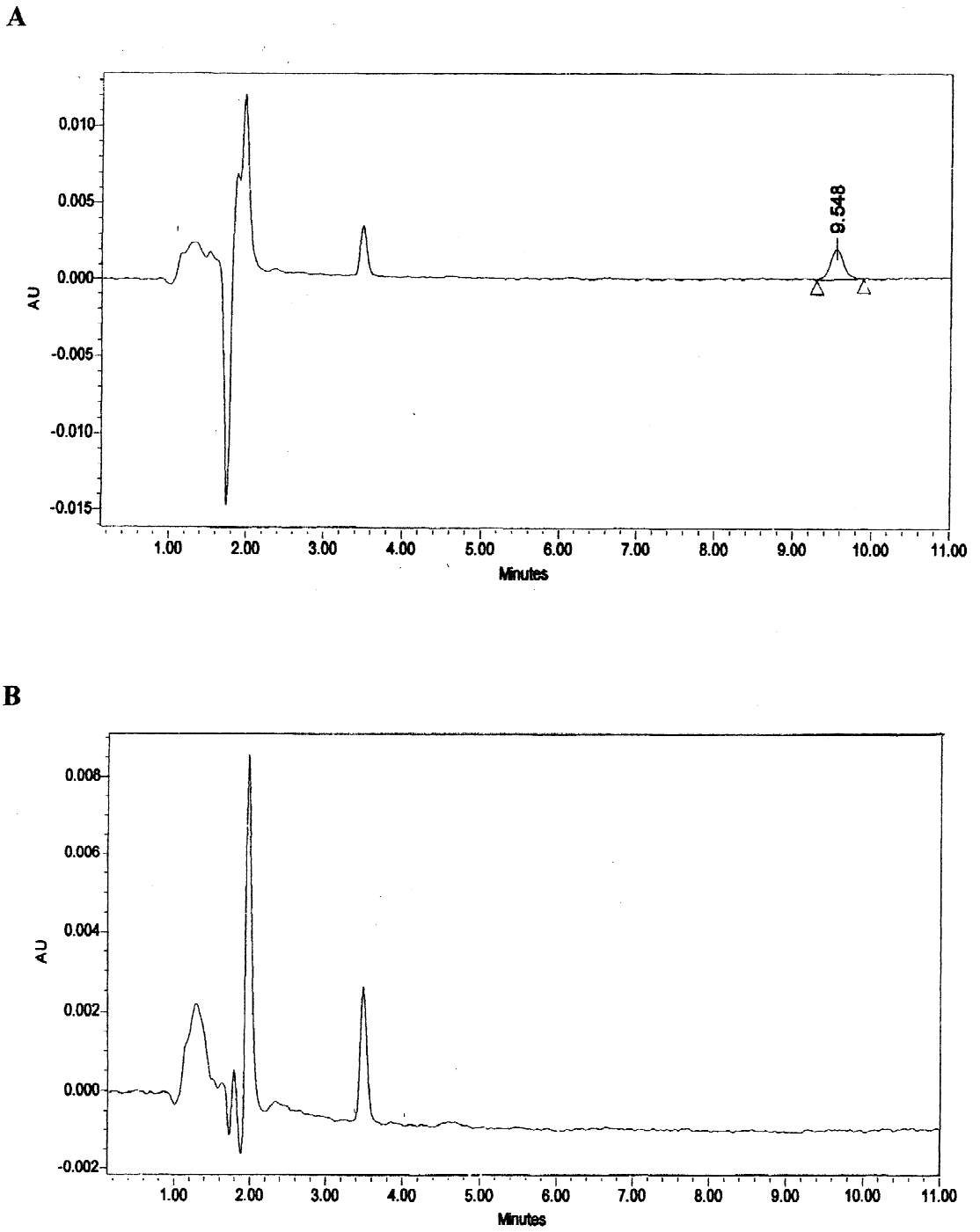

Fig. 1. Representative chromatograms of bovine serum albumin (BSA) media spiked with 1.0 μg/ml of DEET (A) and not spiked with DEET (B).

Table 1
Inter-day precision and accuracy of DEET in BSA media for 1.0 ml and 0.5 ml sample and elution volumes

Spiked concentration (μg/ml)	Calculated concentration (mean±SD, $n=6$) (μg/ml)		RSD (%)		Percent recovery (mean±SD, $n=6$)	
	1.0 ml	0.5 ml	1.0 ml	0.5 ml	1.0 ml	0.5 ml
0.1	0.09±0.01	0.12±0.01	11.0	9.2	93.3±10.2	117.2±10.8
0.5	0.50±0.03	0.45±0.05	6.4	2.0	100.6±6.4	90.1±10.8
1.0	0.98±0.03	0.94±0.12	3.1	13.1	98.1±3.0	93.6±12.3
5.0	5.14±0.23	4.97±0.26	4.5	5.3	102.9±4.7	99.3±5.2

peaks and more reproducible retention time of the analyte, DEET.

3.2. Linearity

The calibration line ($y = 24698x$) was obtained from injecting the external standards described above. The slope of the external standard plot, covering a DEET concentration range of 0.1 to 5.0 μg/ml, was 24698±362 (slope±SD, $n=6$) with the y-intercept assigned a value of zero. The external standard curve was linear in the range of concentrations that were detected in the samples with a correlation coefficient of 0.9998±0.0001 (R^2±SD, $n=6$).

3.3. Precision and accuracy

The results for the inter-day and intra-day precision and accuracy are listed in Tables 1 and 2, respectively. The precision and accuracy for the 1.0 and 0.5 ml sample and elution volumes are listed in separate columns in both tables. The sample and elution volumes were always kept the same. For example, if 1.0 ml of sample was extracted, then 1.0 ml of elution solvent was used for the final elution step, thus keeping the dilution factor equal to one.

Mean recoveries for the inter-day assay ranged from 90.1 to 117.2% and RSD ranged from 2.0 to 11%. It should be noted that the lowest spiked concentration of 0.10 μg/ml DEET gave a mean recovered concentration of 0.09±0.01 μg/ml and 0.12±0.01 μg/ml for the 1.0 and 0.5 ml samples, respectively. Mean recoveries for the intra-day assay were also excellent, and ranged from 95.1 to 104.3% and the RSD ranged from 2.3 to 9.9% for concentrations ranging from 0.1 to 5.0 μg/ml. The Waters system with autosampler used in this study was very reliable and based on the reproducibility of our data presented above, the external standard method was preferred over an internal standard method.

3.4. Limits of detection (LOD) and quantitation (LOQ)

Using the definition of the limit of detection (LOD) and limit of quantitation (LOQ) as being a peak having a signal-to-noise ratio of at least 3:1 and 10:1 respectively, LOD was 0.02 μg/ml DEET and

Table 2
Intra-day precision and accuracy of DEET in BSA media for 1.0 and 0.5 ml sample and elution volumes

Spiked concentration (μg/ml)	Calculated concentration (mean±SD, $n=6$) (μg/ml)		RSD (%)		Percent recovery (mean±SD, $n=6$)	
	1.0 ml	0.5 ml	1.0 ml	0.5 ml	1.0 ml	0.5 ml
0.1	0.10±0.01	0.10±0.01	8.8	9.9	95.1±8.3	103.6±10.2
0.5	0.52±0.04	0.52±0.03	8.0	5.0	104.3±8.3	103.3±5.2
1.0	0.98±0.04	1.01±0.10	3.8	9.7	98.1±3.8	100.5±9.7
5.0	4.92±0.11	4.81±0.21	2.3	4.4	98.5±2.2	96.2±4.3

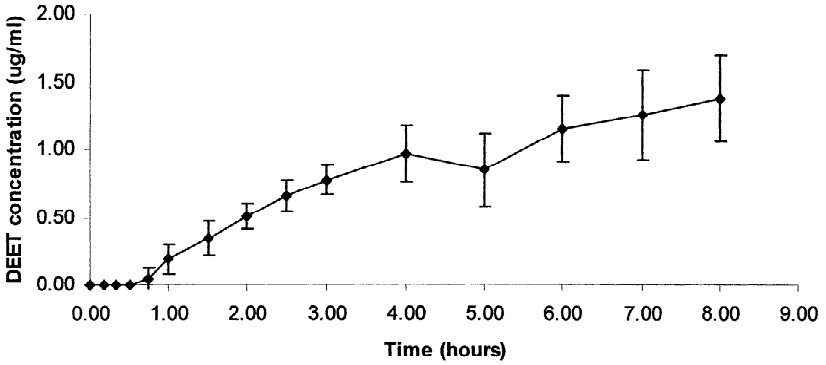

Fig. 2. DEET concentration versus time plot with standard error following topical administration of 75% DEET in ethanol to porcine skin in vitro ($n=4$). Samples for each perfused skin section at each time point were analyzed in duplicate.

LOQ was 0.08 μg/ml DEET under the conditions used.

3.5. Transdermal permeability of DEET in porcine skin flow-through cell

The lowest DEET concentration detected in the BSA samples was 0.33 μg/ml at 45 min. Fig. 2 shows the plot of DEET concentration versus time following a topical application of DEET to porcine skin. Fig. 3 depicts a plot of a cumulative amount of DEET permeated during the 8-h perfusion, and this information was used to calculate flux and permeability. The average flux was 19.07±4.03 (μg/cm^2/h), the lag time was 3.02±0.32 h, the apparent permeability was $2.55\times10^{-5}\pm0.54\times10^{-5}$cm/h, and the diffusivity was $1.39\times10^{-4}\pm0.15\times10^{-4}$ cm^2/h.

Porcine skin is similar anatomically and physiologically to human skin, and dermal absorption of many chemicals in pigs skin is often times comparable to that in human skin [14]. However our in vitro study suggests that DEET absorption kinetics in pig skin was significantly different from that in human skin in vitro utilizing Franz static cells [11]. In these earlier studies, 70% DEET in 30% ethanol was applied to the human skin in vitro system, and permeability and diffusivity were calculated to be 1.21×10^{-3} cm/h and 1.61×10^{-8} cm^2/hr, respec-

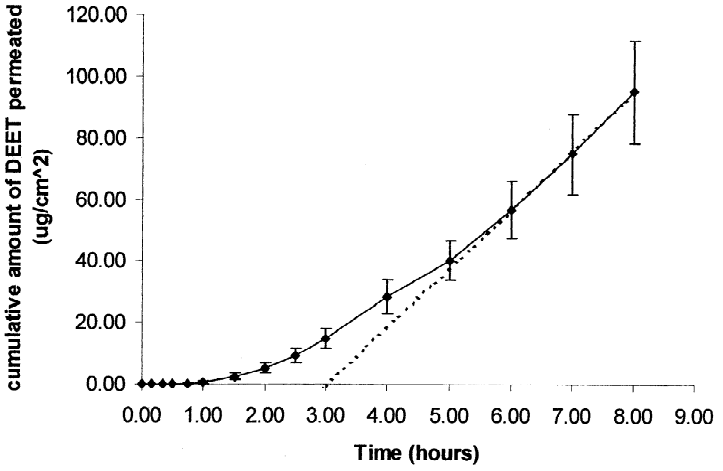

Fig. 3. Cumulative amount of DEET versus time with standard error following topical administration of 75% DEET in ethanol to porcine skin in vitro ($n=4$). DEET flux and permeability were determined from the best estimated straight line (dotted line) at steady state, and lag time was determined as the point where this straight line crossed the time-axis.

tively. Although these are comparisons across different in vitro systems, it does suggest that DEET is more permeable in human skin than pig skin. In the vertical Franz static cell system, the membrane is fully hydrated during the entire exposure, and this can result in an overestimation of chemical diffusion in human skin in vivo. Again, these like many other skin in vitro diffusion studies utilized phosphate buffer solution, and not one containing BSA which was used in the present study. In an effort to assess whether this disk SPE method could be applicable to other biological matrices after an in vivo exposure, we evaluated extraction efficiency of DEET from plasma and urine. Our preliminary studies demonstrated that recoveries ranged from 95.6 to 108.7% ($n=4$) for both pig plamsa and urine spiked with 0.1, 0.5, 1.0, and 5.0 ppm DEET. The RSD ranged from 0.8 to 3.5% for these intra-day assays. This work therefore has resulted in the development of a validated disk SPE method for rapidly assessing DEET levels in a biological matrix, and it can be used in numerous applications such as assessing the in vitro diffusion of DEET in skin. The reader should also be aware that most studies employ buffer solution as the receiving media, and not BSA, as the former simplifies chemical analysis. This manuscript has therefore demonstrated that BSA, which in some ways is analogous to blood, can be used as a receiving media, and DEET can be analyzed with the described disk SPE method.

4. Conclusions

We have reported a minor improvement in the solid-phase extraction of DEET coupled with HPLC–UV detection utilizing disks SPE rather than packed-bed SPE. This improved method uses half of the solvents required for packed-bed solid-phase extraction and therefore the time required to perform the separation of DEET from a complex biological matrix. Larger solvent volumes will require a longer time in the vacuum manifold to pull the solvent through the disks, and this may also require frequent changing of the sample collecting tubes. We were also able to validate this method using low volumes (0.5 ml) of the biological matrices. This application may be useful in the screening of small biological sample volumes at DEET concentrations as low as 0.1 ppm from human in vivo studies as well as testing small sample volumes from in vitro experimentation. This method was applied to the porcine-skin flow-through cell to determine the apparent permeability of DEET. The BSA perfusate used in this system consists of proteins (bovine albumin) as well as certain antibiotics which could have potentially interfered with the separation and chromatography. However, the disk SPE provided quality chromatograms and reproducible results in the validation and its application in assessing in vitro DEET skin permeation.

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