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**13. ABSTRACT** ***(Maximum 200 Words)***

The focus of this research was to quantitate the dermal absorption and cutaneous toxicity of chemical mixtures relevant to the Gulf War Illness. These studies employed in vitro porcine skin models, as well as inert membrane and human epidermal keratinocyte cell cultures. Percutaneous absorption of topically dosed *N,N*-Diethyl-*m*-toluamide (DEET) and $^{14}$C-permethrin was assessed after co-exposure to infused pyridostigmine bromide, diisopropylfluorphosphate (DFP); and/or topical exposure to ethanol/water vehicles, low-level sulfur mustard (HD), DFP, JP-8 jet fuel or occlusive and fabric dressing. Cutaneous irritation was assessed by monitoring pro-inflammatory cytokines (IL-8, TNFα) or prostaglandin $E_2$ release from skin. Pyridostigmine infusion consistently suppressed IL-8 release in perfused skin and keratinocyte cultures. In perfused skin, infusion of pyridostigmine and DFP enhanced $^{14}$C-permethrin transdermal flux five-fold and DEET flux two-fold. HD slightly increased $^{14}$C-permethrin, but decreased DEET absorption. JP-8 increased absorption of both compounds. Occlusion dramatically increased DEET, and slightly increased $^{14}$C-permethrin absorption. Diffusion cell studies showed a number of effects, some of which projected into the perfused skin results. Silastic uniformly overestimated absorption of both compounds. These data are among the first to clearly demonstrate an effect of systemic drugs on dermal absorption, and underscore the complexity of risk assessments of complex chemical mixtures.

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## INTRODUCTION

The purpose of this research is to quantitate the dermal absorption and cutaneous toxicity of chemical mixtures that veterans may have been exposed to during the Persian Gulf War. Chemicals studied include topical permethrin, *N,N*-diethly-*m*-toluamide (DEET), sulfur mustard (HD), jet fuel JP-8, and diisopropylfluorophosphate (DFP) as a chemical warfare nerve agent simulant; as well as systemic pyridostigmine bromide and DFP. A number of these compounds have been implicated as being potential neurotoxicants involved in Gulf War Illness symptomology. In addition, vehicle effects as well as the effect of occlusion were investigated. For topically exposed chemicals to have a role in a systemic disease syndrome, sufficient percutaneous absorption must occur to achieve toxic systemic blood concentrations. This research is thus focused on determining whether exposure to combinations of these agents enhances or retards systemic absorption of permethrin and DEET compared to single chemical exposure. These complex interaction studies were conducted in three in vitro model systems; silastic and porcine skin flow-through diffusion cells, and the isolated perfused porcine skin flap (IPPSF). The potential for dermal irritation was studied by assaying IPPSF perfusate for pro-inflammatory cytokines (IL-8, TNF$\alpha$, $PGE_2$) that have been used as biomarkers of direct toxic effects to the skin. Finally, the effects of systemic exposure to DFP and/or pyridostigmine will assess whether percutaneous absorption of a topically applied chemical can be modified by compounds present in the systemic circulation. This variable has not been experimentally addressed in the past.

## BODY

The presentation of these research results on the percutaneous absorption of permethrin and DEET after exposure in complex chemical mixtures will primarily be presented in the form of complete manuscripts attached in the Appendix. The Statement of Work is presented in the box below.

**Approved Statement of Work**

**I. Adapt analytical methods (GC and/or HPLC) for compounds and biomarkers that are applicable in all three systems.**

**II. Assess the role of chemical mixture components including DEET, DFP, HD, permethrin, and jet fuels across skin and silastic membrane diffusion cells.**

**III. Determine the influence of systemic pyridostigmine bromide and/or DFP exposure on the percutaneous absorption of simultaneous dermal exposure to DEET and permethrin in IPPSFs.**

**IV. Assess effects of occlusion and impregnation of fabric with permethrin on the bioavailability of DEET and permethrin.**

**V. Evaluation of biomarkers of cutaneous toxicity.**

### Results Relative to Statement of Work

The method development aims (SOW I and V) are described in the research manuscripts. Details of the permethrin methods were presented in the first and second annual reports. The analytical method for DEET has been presented separately in manuscript #5 in the Appendix due to a unique extraction technique that warrants publication.

The major research findings, and their relationship to the Statement of Work are as follows: the data on the effects of topical and infused agents on chemical absorption in the IPPSF, as well as in both vitro models (SOW II, III and IV), are presented for $^{14}C$-permethrin in manuscripts #1 and #3, and for DEET in manuscript #4 of the Appendix. The work on biomarkers as indicators of cutaneous toxicity (SOW V) is presented in

Appendix Manuscript #2. We did not complete additional studies on the stereochemistry of permethrin absorption beyond that reported in the Second Annual Report due to the overall low level of permethrin absorption detected, which required all interaction studies to utilize radiolabelled permethrin and reporting of all results as $^{14}$C-permethrin activity.

**General Findings**

The research has been presented in these manuscripts (see Appendix) on the basis of the major findings of this work. This defined the nature of the statistical analyses employed that are reported in these manuscripts. These findings include the effects of systemic infusions of pyridostigmine bromide and DFP on modulating $^{14}$C-permethrin absorption and skin deposition; on the differential effects of sulfur mustard on increasing $^{14}$C-permethrin, but decreasing DEET absorption; on the unexpected finding of blunted cytokine release from perfused skin when pyridostigmine is present; and finally on some miscellaneous effects detected when multiple chemical exposures occur. All of the specific aims of this research project have been addressed in the five full-length publications attached.

Relevance to Human Absorption: The primary model that was used to explore these interactions was the IPPSF, which was shown to closely approximate human absorption for these study compounds, consistent with its published validation for other chemicals (Wester et al., 1999). The data generated for single chemical penetration (vehicle controls), of 1.5 to 1.6% of applied dose over 8 hrs was close to the 2 % reported in humans (Bartlett and Hubbel, 1987). DEET absorption appeared constant across doses in our single chemical exposures, with a rate of approximately 1-3 $\mu g/cm^2/hr$. This constant rate is the same as that reported in humans (Selim et al., 1995). $^{14}$C-permethrin and DEET

absorption could maximally be enhanced five-fold. It is problematic whether this level of permethrin absorption would be consistent with systemic effects in the Gulf War Illness of the nature originally presented by Abou-Donia and co-workers (1996). In contrast, a five-fold increase in DEET flux, over a level that already results in significant systemic absorption, could play a potentiating factor.

**Major Interactions**

The major scientific finding of this research was that systemic infusions of pyridostigmine bromide and DFP significantly enhanced the percutaneous absorption and skin deposition of $^{14}$C-permethrin in the IPPSF and to some extent in perfused porcine skin (Appendix Manuscript #1). Similarly, absorption of DEET was also enhanced with systemic exposure to this combination, albeit to a lesser degree (Appendix Manuscript #3). *This represents the first report that systemic exposure to a drug or chemical may enhance dermal absorption of a topically applied compound.* The mechanism of this is not clear as its effect is seen for such chemically diverse chemicals as DEET and permethrin. In a separate experiment not funded by this grant, pyridostigmine bromide infused into the IPPSF did not increase the absorption of topically applied pentachlorophenol (PCP). Thus, this is not a universal action, but potentially could be a factor modulating chemical absorption in a military environment.

A second important finding from this research was the *different effect that sulfur mustard had on increasing DEET (Appendix Manuscript # 3) but decreasing $^{14}$C-permethrin absorption* (Appendix Manuscript # 4). The most likely explanation for this divergence is the mechanism of rate-limiting skin permeability for permethrin (diffusional and metabolic barriers) versus DEET (diffusional barrier). This is fully

described in Appendix Manuscript # 3. Sulfur mustard, by inducing inflammation and disrupting the skin function, would increase permeability to DEET. In contrast, it appears that a significant fraction of the little permethrin that is absorbed may be a metabolite. This does not necessarily alter its toxicologic significance since either the parent drug or metabolite could possess toxicologic activity. Sulfur mustard effects on reducing cutaneous enzyme activity would decrease metabolism that would result in a reduced transdermal flux of $^{14}$C-permethrin activity. This stresses the point that modulating effects are generally very chemical specific, making general extrapolations problematic.

A third finding was that *co-exposure to JP-8 jet fuel significantly increased $^{14}$C-permethrin absorption* (Appendix Manuscript # 4). This is most likely due to the organic nature of the jet fuel functioning both as an efficient delivery vehicle and potentially as a permeability enhancer. *Complete occlusion with cellophane increased absorption of both DEET and to a lessor extent permethrin.* Again, this reduced effect on permethrin is consistent with the above hypothesis that diffusional resistance is not the sole barrier to $^{14}$C-permethrin absorption. Occlusion with fabric did not result in the same level of enhancement for both compounds.

The final, and unexpected, major finding was that *infusion of pyridostigmine bromide into IPPSFs treated with all topical mixtures, blunted the release of inflammatory cytokines*, a finding repeated in human epidermal keratinocyte cell culture studies (Appendix Manuscript # 2). Specifically, infusion of pyridostigmine bromide into IPPSFs resulted in a decrease in release of IL-8, $PGE_2$, and TNF$\alpha$. This finding was the then replicated in human keratinocyte cell cultures where suppression of IL-8 release was observed. These findings suggest that pyridostigmine bromide, at a concentration seen in

soldiers taking the drug, modulates the cutaneous response to topical irritants. This further supports the hypothesis that a systemic agent may alter skin function.

**Miscellaneous Observations and Findings**

Throughout all of these studies in all model systems, significant vehicle effects (e.g ethanol versus aqueous ethanol) were noted. These are fully discussed in the respective manuscripts. It was clearly shown that certain chemical-chemical interactions were only expressed in certain vehicles. The dosing vehicles studied in the present research, represent only a small sample of the infinite combinations of vehicles that could be encountered in the field. Similarly, it was determined that absorption through silastic for these compounds was not representative of in vitro porcine skin. DEET permeability was two orders of magnitude greater in silastic. The stereoisomer selectivity seen in silastic (Second Annual Report) was not seen, and the flux was much greater than, through porcine skin. For both of these compounds, this model system was not representative of porcine skin. In fact, absorption of both compounds though porcine skin even differed from the IPPSF, but was useful as some light was shed on the potential mechanisms of interactions. This lack of direct correlation, similar to that seen between in vitro human diffusion cells and in vivo humans, is also discussed in the manuscripts. The significant finding of the present research is that the IPPSF was predictive of in vivo human absorption for both DEET and permethrin, suggesting that the interaction observed in the IPPSF may be relevant to in vivo human exposure.

**Impact on Future Studies**

It is clear from the data presented that significant chemical-chemical interactions may occur which could modulate both dermal absorption and cutaneous toxicity of

topically applied chemicals encountered in a military environment. However, detection of these interactions requires relatively complex factorial experimental designs with replication of treatment components and vehicle systems. The finding that infused pyridostigmine bromide modulates both chemical absorption and cutaneous cytokine release, raises the possibility that there may be other systemic drugs that could also modulate skin absorption and / or response to irritants. Numerous drugs ranging from over-the-counter anti-inflammatory drugs such as ibuprofen, antimalarial and other anti-infective drugs, as well as other chemicals encountered in the environment, could also have modulating effects on topical irritant absorption. Since all of these could be chemical specific, their experimental detection would require exhaustive studies. Unfortunately, a simplifying structural or functional paradigm to guide such mixture experiments has not yet been defined.

**KEY RESEARCH ACCOMPLISHMENTS**

- Systemic exposure to pyridostigmine bromide and DFP increases the percutaneous absorption of $^{14}C$ permethrin and DEET in isolated perfused porcine skin.
- Topical exposure to low levels of sulfur mustard enhances DEET but decreases $^{14}C$-permethrin absorption.
- Pyridostigmine bromide infusions suppresses cutaneous release of cytokines (IL-8, $PGE_2$) in the IPPSF and in human keratinocyte cell cultures.
- Percutaneous absorption of both DEET and permethrin in the IPPSF was comparable to reported absorption fluxes in humans.
- Co-exposure of permethrin and JP-8 enhances $^{14}C$ permethrin absorption.
- DEET and $^{14}C$ permethrin absorption can be enhanced by complete occlusion.

## REPORTABLE OUTCOMES

### Manuscripts

Riviere JE: Percutaneous absorption of chemical mixtures relevant to the Gulf War. 1999 Annual Report to Congress on Gulf War Illnesses. Appendix. Department of Veterans Affairs, pgs. 169-170, 2001.

Baynes RE, Monteiro-Riviere NA, Riviere JE. Pyridostigmine Bromide Modulates the Dermal Absorption of $^{14}$C-Permethrin. Toxicology and Applied Pharmacology (*In Press*).

Monteiro-Riviere NA, Baynes RE, Riviere JE. Pyridostigmine Bromide Modulates Topical Irritant-Induced Cytokine Release from Human Epidermal Keratinocytes and Isolated Perfused Porcine Skin. (*Submitted*)

Riviere JE, Baynes RE, Brooks JD, Yeatts JL, Monteiro-Riviere NA. Percutaneous Absorption of Topical *N,N*-Diethyl-*m*-toluamide (DEET): Effects of Exposure Variables and Coadministered Toxicants. (*Submitted*).

Riviere JE, Monteiro-Riviere NA, Baynes RE. Gulf War Illness-Related Exposure Factors Influencing Topical Absorption of $^{14}$C-Permethrin. (*Submitted*).

Baynes RE, Yeatts JL, Riviere JE. Analysis of DEET absorption in porcine skin perfusates using solid phase extraction discs and reversed-phase high-performance liquid chromatography. (*Submitted*).

### Abstracts

Baynes RE, Brooks JD, Riviere JE: DEET and pyridostigmine bromide effects on dermal disposition of permethrin. Proc. Conf. Illness Among Gulf War Veterans: A Decade of Scientific Research., pg. 58, 2001. *Poster*

Monteiro-Riviere NA, Inman AO, Riviere JE: Systemic pyridostigmine suppresses inflamatory cytokine release after topical permethrin and DEET exposure. Proc. Conf. Illness Among Gulf War Veterans: A Decade of Scientific Research., pg. 70, 2001. *Selected as an oral platform session.*

Riviere JE, Yeates JL, Brooks JD, Baynes RE: Stereochemistry of dermal absorption of permethrin. Proc. Conf. Illness Among Gulf War Veterans: A Decade of Scientific Research., pg. 72, 2001. *Poster.*

Baynes RE, Brooks JD, Abdullahi AR, Wilkes R, Riviere JE: Influence of DEET and pyridostigmine bromide on dermal disposition of permethrin. Toxicological Sci. 60 (1S): 128-129, 2001. *Poster*

Yeattes JL, Riviere JE, Brooks JD, Baynes RE. Stereoselective absorption of permethrin through silastic membrane and excised porcine skin in vitro flow through diffusion system. Toxicological Sci. 60 (1S): 129, 2001. *Poster*

Riviere JE, Brooks JD, Baynes RE, Monteiro-Riviere NA: Lack of clinically significant effects of sulfur mustard and JP-8 jet fuel on percutaneous absorption of simultaneously administered topical permethrin. Toxicological Sci. 2002 (In Press). *Poster*

Monteiro-Riviere NA, Inman AO, Baynes RE, Riviere JE: Pyridostigmine bromide suppresses IL-8 in human epidermal keratinocytes and in isolated perfused porcine skin exposed to DEET and permethrin. Toxicological Sci. 2002 (In Press) *Poster*

**Other Transfers**

The JP-8 data on permethrin absorption was conveyed to the U.S. Air Force Office of Scientific Research as part of the PI correspondence on JP-8 toxicity research supported by the USAFOSR (F49620-01-1-0080).

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X.R. Xia

## CONCLUSIONS

These research findings support the contention that significant chemical interactions may occur between simultaneously exposed compounds that would modulate the percutaneous absorption of topically applied chemicals such as DEET and permethrin. This includes systemic exposure to agents like pyridostigmine bromide and the nerve agent simulant DFP. Such exposures may also modulate the release of cytokines from epidermal tissue. Low level exposure to chemical warfare agents, at concentrations that would not induce acute toxicosis, modulate dermal absorption of compounds.

The primary question concerning the impact of this research is if the five-fold enhancement seen is sufficient to impact the toxicological potential for either DEET or permethrin relative to playing a significant role in a syndrome such as the Gulf War Illness. Unfortunately, this question cannot be definitively answered. They clearly do not reduce the potential for their involvement in this syndrome. However, these results do question risk assessment practices that ignore the reality of chemical mixtures and concomitant chemical exposures when data from single chemical exposure are used to estimate chemical risk. This work suggests that factors which are capable of increasing permeability through the epidermal barrier may enhance systemic exposure to topical agents. However, the ability of a compound to do this is very dependent upon the mechanism of chemical penetration through the skin. For example, sulfur mustard enhanced DEET absorption, probably though increasing its maximal rate of transdermal flux that is limited by diffusional resistance. However, the same exposure reduced $^{14}C$-

permethrin flux probably due to destruction of the cutaneous enzymes that are required for the dermal metabolism of permethrin.

A major concern that does arise from these studies is that the nature of the interactions observed are dependent upon a number of experimental variables, including vehicle and model system used. Addition of a small percentage of water to a vehicle significantly changes permeability in some systems. However, in perfused skin, the flux of a compound such as DEET appears to have already been saturated making transdermal flux insensitive to further changes in topical availability. In contrast, changes to inherent permeability can increase this base-rate of transdermal flux. Changes in vehicle composition increased $^{14}$C-permethrin partitioning into the stratum corneum that increased availability for absorption or metabolism. The advantage of using a validated system such as the IPPSF or in vivo models is that all potential levels of biological interactions are present in the system. It is the vectorial sum of all potential interactions that determines the final effect of a chemical-chemical or chemical-biological interaction on transdermal flux.

In summary, conditions were identified that enhanced transdermal flux of topically applied permethrin or DEET over normal absorption rates. These included both exposure to topical as well as systemic chemicals. Risk assessments on the role of chemicals such as DEET and permethrin must take into account concomitant exposure to other agents to realistically model the field scenario.

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**APPENDICES** (146 pages)

1. Pyridostigmine Bromide Modulates the Dermal Absorption of $^{14}$C-Permethrin. R.E. Baynes, N.A. Monteiro-Riviere, J.E. Riviere. Toxicology and Applied Pharmacology (*In Press*).

2. Pyridostigmine Bromide Modulates Topical Irritant-Induced Cytokine Release from Human Epidermal Keratinocytes and Isolated Perfused Porcine Skin. N.A. Monteiro-Riviere, R.E. Baynes, J.E. Riviere. (*Submitted*)

3. Percutaneous Absorption of Topical *N,N*-Diethyl-*m*-toluamide (DEET): Effects of Exposure Variables and Coadministered Toxicants. J.E. Riviere, R.E. Baynes, J.D. Brooks, J.L. Yeatts, N.A. Monteiro-Riviere. (*Submitted*).

4. Gulf War Illness-Related Exposure Factors Influencing Topical Absorption of $^{14}$C-Permethrin. J.E. Riviere, N.A. Monteiro-Riviere, R.E. Baynes. (*Submitted*).

5. Analysis of DEET absorption in porcine skin perfusates using solid phase extraction discs and reversed-phase high-performance liquid chromatography. R.E. Baynes, J.L. Yeatts, J.E. Riviere. (*Submitted*).

# PYRIDOSTIGMINE BROMIDE MODULATES THE DERMAL DISPOSITION OF $^{14}$C-PERMETHRIN

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**Abbreviated Title: Pyridostigmine Bromide Influences Permethrin Absorption**

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**Pyridostigmine bromide Modulates the Dermal Disposition of C14-Permethrin.**

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**Toxicology and Applied Pharmacology**

## ABSTRACT

The cause of the Gulf War Syndrome may be related to soldiers being exposed to insecticides (e.g., permethrin, (P)), insect repellents (e.g., *N,N* Diethyl-*m*-toluamide, (DEET)), an organophosphate nerve agent simulant (e.g., diisopropylfluorphosphate, (DFP)), and/or prophylactic treatment (e.g., pyridostigmine bromide, (PB)) against potential nerve gas attacks. The purpose of this study was to assess the dermal disposition of $^{14}$C-permethrin in ethanol or ethanol+water (3:2) in the isolated perfused porcine skin flap (IPPSF) model with simultaneous dermal exposure to DEET or DFP. These IPPSFs were also simultaneously perfused arterially with or without PB, DFP, or DFP+PB. The results indicated that DFP+PB significantly increased $^{14}$C-permethrin absorption compared to controls (1.06% dose vs. 0.14% dose). PB significantly increased $^{14}$C-permethrin disposition in the stratum corneum (SC) in aqueous mixtures only (9.40 vs. 3.35% dose), while topical DEET or topical DFP reduced $^{14}$C-permethrin levels in the SC especially in nonaqueous mixtures. PB also significantly enhanced $^{14}$C-permethrin penetration into all skin tissues and perfusate in aqueous mixtures, while DEET reversed this effect. PB appeared to influence $^{14}$C-permethrin disposition in flow-through diffusion cells suggesting that the mechanism of this interaction may be associated predominantly with epidermal permeability, although muscarinic effects in the vasculature in IPPSFs should not be ruled out and requires further investigation. These experiments suggest that intra-arterial perfusion of PB and/or DFP and topical application of DFP or DEET can alter the disposition of

[$^{14}$C]-permethrin in skin and possibly its bioavailability in soldiers simultaneously exposed to these chemicals

**Key Words: Permethrin; pyridostigmine bromide; skin; absorption.**

# INTRODUCTION

Significant research over the last 10 years suggest that the Persian Gulf War syndrome may be linked to simultaneous exposure to an insect repellent, *N,N*-diethyl-m-toluamide (DEET), a pyrethroid insecticide, permethrin, nerve agents, and an antinerve agent, pyridostigmine bromide (PB) (Abou-Donia *et al.*, 1996; McCain *et al.*, 1997; Hoy *et al.*, 2000;). It should be noted that the primary route of exposure for the insect repellents and insecticides was via the skin, while many soldiers in the Persian Gulf War theater were also taking daily oral doses of PB (Petersdorf *et al.*, 1996). Prophylactic use of PB was initially thought to have little or no health effects in the veterans; however, epidemiological surveys strongly suggest that PB caused more intense side effects than anticipated (Sharabi *et al., 1991*).

Very little is known whether systemic levels of PB can influence the dermal absorption of drugs or chemicals relevant to the Persian Gulf War. One recent study suggested that PB may reduce permethrin distribution to the central nervous system (Buchholz *et al.* 1997) which does not support the hypothesis that PB may enhance permethrin toxicity. Other studies have demonstrated a toxic interaction such as increased lethality and neurotoxicity between PB and DEET (McCain *et al.*, 1997; Chaney *et al.*, 1999). While these studies strongly suggest that these interactions may be related to neurological symptoms in Gulf War veterans, the mechanism of this interaction is not well understood.

The primary objective of this study was to quantitate dermal absorption of $^{14}$C-permethrin after topical application in an ethanol or ethanol-water (3:2) vehicle to isolated perfused porcine skin flaps (IPPSFs) perfused with or without PB, DFP, or DFP+PB. DFP was used as a nerve agent simulant. Our other objective was to determine whether topical exposure to DFP or high

concentrations of DEET influenced permethrin absorption in skin as previously demonstrated with DEET in our *in vitro* model system (Baynes *et. al*, 1997; Baynes and Riviere, 1998). As pig skin is similar anatomically and physiologically to human skin, pig skin is an accepted human surrogate model (Monteiro-Riviere, 1991) to evaluate dermal disposition of $^{14}$C-permethrin. Unlike other *in vitro* skin models, the IPPSF has an intact microvasculature, and therefore physiological, neurohumoral, or immunological changes may be observed in response to drug or chemical exposure as seen with *in vivo* exposures (Riviere and Monteiro-Riviere, 1991). This study also investigated several of the mixtures in a *in vitro* flow-through diffusion system to characterize the effects of surface chemicals and infused PB and/or DFP on the epidermal diffusion of topically applied chemicals. This *in vitro* system unlike the IPPSF does not have an intact microvasuclature, but has a partial full thickness skin with an intact epidermis and minimal dermis through which co-administered pesticides and/or drugs can partition and diffuse before partitioning into the perfusate. This allows for observance of potential chemical interactions on the surface and within epidermis which can influence systemic bioavailability of chemicals that may be relevant to the Persian Gulf War Syndrome.

## MATERIALS AND METHODS

***Chemicals*:**

Radiolabeled $^{14}$C-permethrin (specific activity = 8.1 mCi/mmol) was obtained from Sigma Chemical (St. Louis, MO). 98% pure *N,N* Diethyl-*m*-toluamide (DEET) was purchased from Chem Service Company, West Chester, PA. All other chemicals were HPLC grade. No literature is available regarding topical exposure levels for any of these chemicals in Gulf War veterans. In our laboratory, dosing solutions are usually formulated to deliver doses of 40μg/cm$^2$ for comparison purposes. $^{14}$C-permethrin doses of 40 μg/cm$^2$ in ethanol were applied to porcine skin flaps. DEET (7.5 or 75%) was added to permethrin mixtures because 75% DEET was used by Gulf War soldiers. Dosing at 7.5% was used to evaluate the effects on permethrin disposition with 90% evaporation of DEET in the desert scenario. In order to determine whether skin hydration (e.g., perspiration) influences $^{14}$C-permethrin disposition, water was added to the ethanol based mixtures at a constant ratio of 3:2 ethanol:water. Pyridostigmine bromide (PB) (Mestinon®) was obtained from ICN Biomedicals Inc., Costa Mesa, CA and added to perfusion media at a concentration to mimic the highest PB blood concentrations (50 ng/ml) observed in large population of soldiers taking this prophylactic drug (Marino *et al.*, 1998). Diisopropylfluorphosphate (DFP) was obtained from Sigma Chemical (St. Louis, MO) and was applied topically as (40 μg/cm$^2$) or added to perfusion media (30 ng/ml) to mimic peak DFP concentrations observed in IPPSFs exposed topically to 40 μg/cm$^2$ DFP (Carver *et al.*, 1989).

*Isolated Perfused Porcine Skin Flaps*

IPPSFs were prepared according to procedures previously reported in the literature (Riviere *et al.*, 1986; Monteiro-Riviere, 1990; Bowman *et al.*, 1991). Skin flaps were perfused in a non-recirculating system with oxygenated (95% $O_2$ and 5% $CO_2$) Krebs-Ringer bicarbonate buffer spiked with glucose and bovine serum albumin. The perfusion chambers were maintained at 37°C and a relative humidity of 50-60%. After perfusing the skin flaps for 1 hour, a flexible dosing template measuring 1.0cm x 5.0 cm (Stomahesive, ConvaTec-Squibb, Princeton, NJ) was affixed to the skin surface with Skin Bond (Pfizer Hospital Products, Inc., Largo, FL) to provide a surface area of 5.0 $cm^2$. One hundred microliter (100 µl) doses were applied to each dose site providing a surface concentration of 40 µg/$cm^2$ of $^{14}$C-permethrin. DFP (40 µg/$cm^2$) or DEET (7.5 or 75%) were applied topically to selected skin flaps. Selected flaps were also perfused with intra-arterial pyridostigmine bromide (50 ng/ml media) and/or intra-arterial DFP (30 ng/ml media). Dosing protocols are provided in **Table 1**. Perfusate samples (3 ml) were collected every 10 minutes for the first 30 minutes, then every 15 minutes until 1.5 hours, and then every 30 minutes until termination at 8 hours. Monitoring vascular resistance (VR) (perfusate pressure/flow rate) and glucose utilization (GU) assessed skin flap viability. Arterial perfusate samples were collected hourly and compared with venous samples to determine GU, while VR was defined as the ratio of arterial pressure to perfusate flow rate. At the end of the 8-hour perfusion, the dose area was swabbed with cotton swabs containing an Ivory soapy solution and skin and fat tissues were obtained from the dose site and surrounding areas and digested in Soluene (Packard Chemical Co., Downers Grove, IL) before further analyses as described below.

*Flow through Diffusion Cell Experiments*

The flow-through diffusion cell system, described by Bronaugh and Stewart (1985), was used to perfuse porcine skin sections *in vitro*. Porcine skin was obtained from the dorsal area of weanling female Yorkshire pigs. The skin was dermatomed to a thickness of 200 - 300 μm with a Padgett Dermatome, (Padgett Instruments Inc, Kansas City, MO). Each circular skin was punched to provide a dosing surface area of 0.64 $cm^2$ and then placed into a two-compartment Teflon flow-through diffusion cell. Skin discs were perfused using Krebs-Ringer bicarbonate buffer spiked with dextrose (0.12%) and bovine serum albumin (4.5%). The temperature of the perfusate and flow-through cell was maintained at 37°C using a Brinkmann constant-temperature circulator (Brinkmann Inc., Westbury, NY). The pH was maintained between 7.4 and 7.5. Perfusate flow rate was 4.0 ml/hr and perfusate was spiked with PB, DFP, or PB+DFP at concentrations used in IPPSF experiments described above. Twenty micoliters of the dosing mixtures were topically applied to each diffusion cell (n= 4 – 10 skin sections per treatment) to deliver about 40 $ug/cm^2$ of $^{14}C$-permethrin. Perfusate samples were collected at 0, 10, 20, 30, 45, 60, 75, 90, 105, 120, 150, 180, 240, 300, 360, 420, and 480 minutes. At the end of the perfusion, the dose area was swabbed with a soapy solution to determine surface content, tape-stripped six times with scotch tape to determine stratum corneum content, and then removed from the skin disc with a 0.32 $cm^2$ punch biopsy to determine dose area skin deposition. These tissue and tape strip samples in addition to the remaining peripheral skin were saved for radiochemical analysis described below.

***Stratum Corneum/Vehicle Partition Coefficient Experiments***

The aim of these experiments is to determine the effects that various DEET mixtures have on the partitioning of $^{14}$C-permethrin from the various dosing vehicles into the stratum corneum (SC). The SC was prepared according to method described by Baynes *et al.* (2001). In brief, ten mixtures were formulated with 0%, 1%, 3%, 7% and 10% DEET in either ethanol alone or in equal parts of ethanol and distilled water, 10µl of $^{14}$C-permethrin was added to each mixture. Approximately 500µl of each mixture was added to the SC sample vial (n=4), capped, sealed and allowed to remain undisturbed at room temperature for 24 hours. After the vials were uncapped, 10µl of the vehicle was removed and 15ml of Ecolume (ICN Costa Mesa, CA) was added to each sample. The SC sample was removed, gently blotted on a Kimwipe (Kimberly-Clark Co., Roswell, GA) to remove excess solution and analyzed as described below. After the radioactivity content of the vehicle and SC was determined, these values were normalized to 1000mg SC ($C_{sc}$) and 1000mg vehicle ($C_v$) for comparison. The SC/vehicle partition coefficient was determined by the equation: log $PC=C_{sc}/C_v$.

***Chemical Analysis***

For determination of $^{14}$C-permethrin, tissues, perfusate samples, surface swabs, dosed skin, and stratum corneum samples were combusted in a Packard Model 387 Tissue Oxidizer (Packard Chemical Co., Downers Grove, IL) and then analyzed by Packard Model 1900TR Liquid Scintillation Counter (Packard Chemical Co., Downers Grove, IL).

***Calculations and Statistics***

*Absorption* was defined as the total percentage of initial dose detected in the perfusate for the entire 8-hr perfusion period. *Penetration* was defined as total amount detected in perfusate,

all skin and fat tissues, but not stratum corneum. *Tissue disposition* parameters such as surface, stratum corneum (SC), and dosed skin were described above. All units are expressed in terms of $^{14}$C-permethrin activity. *Absorption ratios* (Mixture/Control) for topical $^{14}$C-permethrin were calculated as {%dose absorption for infused mixture/%dose absorption for control}. Control refers to topical exposure to $^{14}$C-permethrin, but the skin preparation was not infused with PB, DFP, or PB+DFP. Standard errors (SEM) were determined for all data sets. The measurements analyzed were surface, stratum corneum, dose skin, fat, total absorption, and penetration. Analysis of Variance (ANOVA) with multiple comparison tests performed using the LSD method with significance level at 0.05. All analyses were carried out using SAS 8.1 for Windows software (SAS Institute Inc., Cary, NC).

## RESULTS

### *$^{14}$C-Permethrin Absorption*

**Table 1** lists the markers, vehicles, and mixture components used in this study. $^{14}$C-permethrin peak flux in IPPSFs occurred within the first 2 hours for aqueous and nonaqueous mixtures, with peak fluxes ranging from 0.0003 – 0.0055% dose/min for aqueous mixtures **(Figure 1)**. Total absorption over 8 hours ranged from 0.12 – 0.42% dose for nonaqueous mixtures and 0.11 – 1.06% dose for aqueous mixtures. $^{14}$C-Permethrin absorption was greatest (1.06% dose) when applied to IPPSFs as an aqueous mixture (60% ethanol+40% water) and simultaneously perfused with DFP+PB (**Figure 1 and 2**). In addition, DFP arterial perfusion or DFP topical application significantly enhanced $^{14}$C-permethrin absorption when compared to controls ($p < 0.05$), but infusion of PB alone had no effect on absorption. In nonaqueous exposures, $^{14}$C-permethrin absorption was also greatest with PB+DFP arterial perfusion, and DFP topical application or PB+DFP arterial perfusion significantly increased $^{14}$C-permethrin absorption compared to controls. In comparing aqueous and nonaqueous mixtures, only the aqueous mixture in the presence of arterially perfused PB+DFP resulted in significantly greater $^{14}$C-permethrin absorption than the nonaqueous mixture. Simultaneous exposure to 7.5 or 75% DEET did not have a significant effect on $^{14}$C-permethrin absorption, although the data suggest that DEET may be depressing PB-enhanced absorption of $^{14}$C-permethrin.

In the *in vitro* flow-through diffusion cells system, absorption ranged from 0.68 – 1.88% dose for aqueous mixtures (**Figure 3a**) and 0.11 – 0.42% dose for nonaqueous mixtures (**Figure 3b**) and absorption was significantly greater in aqueous than in nonaqueous mixtures for control and PB

arterial perfusion treatments. Arterial perfusion with PB, DFP, or PB+DFP resulted in significantly greater absorption than controls in nonaqueous mixtures, but in aqueous mixtures, only arterial perfused PB significantly increased $^{14}$C-permethrin absorption.

### *$^{14}$C-Permethrin Disposition in Skin*

$^{14}$C-Permethrin penetration was assessed as total amount detected in the perfusate, skin tissues, and fat after termination of the 8-hour perfusion. $^{14}$C-Permethrin penetration was the greatest with skin flaps exposed to aqueous mixtures and simultaneously perfused with DFP+PB (**Figure 2**). This effect was significantly greater than other aqueous mixtures. Furthermore, $^{14}$C-permethrin penetration was significantly greater with arterial perfusion of PB alone than controls, but significantly less than arterial perfusion with DFP+PB. These effects were not observed with DFP exposure or with nonaqueous mixtures. DEET had little or no effect on $^{14}$C-permethrin penetration when compared with controls, but DEET reversed the enhancing effect of perfused PB with aqueous mixtures (**Figure 4**).

In dosed skin, topical DFP significantly reduced $^{14}$C-permethrin deposition in aqueous mixtures and DEET significantly reduced permethrin deposition in aqueous and nonaqueous mixtures. In fat tissue, DFP+PB significantly increased $^{14}$C-permethrin deposition in nonaqueous mixtures when compared to PB or DFP perfusion. DEET significantly enhanced $^{14}$C-permethrin deposition in fat, but when this exposure was combined with perfused PB, $^{14}$C-permethrin deposition in fat was significantly reduced back to levels observed with control or PB perfused skin flaps.

$^{14}$C-Permethrin deposition in the stratum corneum (SC) in aqueous mixtures was significantly increased with PB infusion when compared to controls and other mixtures. In

nonaqueous mixtures, topical or arterial perfusion of DFP significantly reduced $^{14}$C-permethrin deposition in the SC compared to controls. DEET also appears to have the same antagonistic effect as DFP on $^{14}$C-permethrin deposition in SC for aqueous and nonaqueous mixtures. As with $^{14}$C-permethrin penetration in aqueous mixtures, DEET significantly reversed the enhancing effect of perfused PB on [$^{14}$C]-permethrin deposition in SC. $^{14}$C-Permethrin deposition on the surface was, for the most part, increased with infusion of DFP or DFP+PB. Its also worth noting that although infused PB or topical DFP or DEET appeared to have no effect on $^{14}$C-permethrin surface deposition, the combination of topical DEET and infused PB significantly increased surface deposition in aqueous and nonaqueous mixtures.

***SC/Vehilce Partition Coefficient (log PC) for $^{14}$C-permethrin***

**Figure 5** depicts the partitioning behavior of $^{14}$C-permethrin in the presence of DEET in porcine stratum corneum in aqueous and nonaqueous dosing mixtures. The log PC for $^{14}$C-permethrin in SC/vehicle increased from 0.59 – 1.47 as the DEET concentration in the dosing mixtures decreased from 15 to 0% DEET in aqueous mixtures. The log PC for aqueous mixtures containing 3, 7 or 15% DEET were significantly less than the log PC with mixtures containing no DEET ($p < 0.05$). In nonaqueous mixtures, the log PC ranged from 0.41 to 0.53, and there were no significant differences between DEET treatments as observed with aqueous mixtures. The log PC for aqueous mixtures was significantly greater than the log PC for nonaqueous mixtures except for 15% DEET where the presence of water had no effect on the log PC.

## DISCUSSION

### *Effects of PB and DFP Perfusion*

The data from this study utilizing two *in vitro* dermal absorption systems strongly suggest that arterial perfusion with PB and/or DFP can influence $^{14}$C-permethrin absorption as well as skin and stratum corneum deposition. Arterial infusion with PB+DFP had a significant effect on $^{14}$C-permethrin absorption in aqueous mixtures, and a trend in absorption ratios (Mixtures/Controls) of PB+DFP>DFP>PB was observed in skin flaps exposed to $^{14}$C-permethrin in either aqueous or nonaqueous delivery systems (**Table 2**). Intuitively, the almost 6-fold increase in absorption associated with PB+DFP infusion is probably related to effects on the skin microvasculature in these skin flaps, as this is the primary factor that differentiates the two *in vitro* model systems. In flow-through diffusion cells, PB alone had a significant effect in both aqueous and nonaqueous mixtures, and the trend was reversed; that is PB>DFP>PB+DFP. These observations suggest a synergistic interaction between PB and DFP may be influencing $^{14}$C-permethrin absorption in both *in vitro* diffusion systems albeit in opposite directions.

The proposed microvascular mechanism may be linked to a neurohumoral pathway in skin as human keratinocytes are known to synthesize, secrete, and degrade acetylcholine and there are numerous muscarinic receptors in neuronal and non-neuronal cells of the skin (Grando *et al.*, 1993; Ndoye *et al.*, 1998; Haberberger and Bodenbenner, 2000). It is possible that DFP and/or PB can increase acetylcholine levels in the IPPSF by binding to acetylcholinesterases. Significant levels of acetylcholine may then bind to $M_3$ receptors in vascular smooth muscle, and act on the vascular endothelial cells to release endothelium-dependent relaxation factor (EDRF) or nitric oxide which relaxes smooth muscle and result in vasodilation (Furchgott and Zawadzki,

1980). This potential mechanism is supported by data from previous transdermal research in our laboratory with skin flaps (Rogers and Riviere, 1994), which demonstrated that infused acetylcholine and nitroglycerin reduced vascular resistance and thus modulate transdermal delivery. Although similar perfusion work with cerebral vessels in guinea-pig brain demonstrated these effects (Librizzi *et al.*, 2000), these findings contrast with decreased skin blood flow in human subjects given oral doses of PB (Stephenson and Kolka, 1990). Clearly, a PB-induced decrease in skin blood flow in our skin flaps is inconsistent with observed increase of $^{14}$C-permethrin absorption. However, changes in skin "blood flow" is a narrow concept as cutaneous microvasculature is complex, possessing numerous shunts whose dilation may not necessarily result in increased blood flow. In fact, arterio-venous shunt dilation would be expected to decrease blood flow to perfusing micro-capillaries of skin (Riviere and Williams, 1992).

It has also been proposed that oral doses of PB may inhibit protective metabolizing enzymes in the liver thereby increasing permethrin levels in the body and possibly enhancing neurotoxicity (McCain *et al.*, 1997). Previous workers have also demonstrated that DFP can completely inhibit enzyme hydrolysis in skin, as evidenced by lack of drug metabolites in media perfusing skin that was exposed topically to lipophilic drugs and DFP (Bando *et al.*, 1997). By extension, it is possible that PB and/or DFP can inhibit skin carboxylesterases as demonstrated with organophosphates (Heymann *et al.*, 1993), and thereby increasing the dermal bioavailability of permethrin in both of our model systems. Although our study did not fully characterize permethrin metabolism because of limited absorption in the perfusate, it is possible that in the presence of PB and/or DFP, more parent permethrin than its hydrolysis products would have been available for absorption.

Other studies have demonstrated that PB can reduce $^{14}$C-permethrin uptake into the CNS (Buchholz *et al.*, 1997), while others demonstrated that coexposure to PB and/or a nerve agent increased blood brain barrier permeability (Abou-Donia *et al.* 2001; Grauer *et al.* 2001). These findings and our current data suggest that PB may modulate vascular endothelium or keratinocyte membrane permeability in both model systems by pathways not well defined. Our laboratory has also demonstrated that PB influenced keratinocyte IL-8 production in skin flaps and cell culture (Monteiro *et al.*, 2001), which supports a PB/DFP-induced cytotoxic interaction.

***Effects of Topical DFP and DEET Exposure***

Topical DFP exposure significantly increased permethrin absorption irrespective of whether an aqueous or nonaqueous mixture was applied to the skin surface. Once again, the same arguments outlined above can be used to explain these observations, although DFP-induced delayed neuropathy, and not direct cytotoxcity to keratinocytes has been reported in the literature (Abou-Donia, 1983; Damodaran *et al.*, 2001). Previous work demonstrated that DFP does not alter stratum corneum lipids or proteins (Potts *et al.*, 1989) and therefore topical DFP-enhanced absorption may not be related to vehicle effects in skin, but to mechanisms described above.

The enhanced effect of PB perfusion on $^{14}$C-permethrin penetration into skin and perfusate and deposition in the SC was reversed with topical application of DEET in the skin flaps. This reversed effect is not surprising as this was previously demonstrated with permethrin and carbaryl in flow-through diffusion cells (Baynes *et al.*, 1997). DEET also enhanced $^{14}$C-permethrin depot formation in fat and skin surface which supports the argument that it has the capability to simultaneously retain topically applied pesticides (Moody *et al.*, 1987). Our SC/vehicle partition coefficient experiments demonstrated that [$^{14}$C]-permethrin was least likely

to partition into the SC with higher concentrations of DEET and therefore supports our other findings that DEET may enhance permethrin deposition in the vehicle or skin surface but less so in the SC. DEET has a relatively small molecular weight (191.3), it is barely soluble in water (0.99%), and it has a log octanol/water partition coefficient (2.02) (Qiu *et al.* 1998). These physiochemical properties along with our previous experimental findings (Baynes *et al.*, 1997) suggests that although DEET readily penetrates skin, its presence can enhance tissue depot formation of co-diffusing chemicals rather than promote systemic absorption into the skin as previously reported by other investigators (Windheuser *et al.*, 1982).

In conclusion, these studies demonstrated that simultaneous exposure to topical DEET or DFP and/or perfusion with PB, DFP, or DFP+PB can influence $^{14}$C-permethrin disposition in porcine skin. Although the mechanism of enhanced absorption associated with PB and/or DFP arterial perfusion is not well understood, it may be related to known anticholinesterase activity and/or chemically-induced changes in keratinocyte. The data from this study strongly suggest that dermal absorption of permethrin or related insecticides may be increased in combat soldiers simultaneously exposed to a nerve agent simulant or organophosphate insecticide (e.g., DFP) and anti-nerve agent chemicals (e.g., PB). Further work is in progress to understand the specific mechanism of PB and DFP+PB or PB action on chemical absorption and cytotoxicity in skin.

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**TABLE 1.**
**Topical Mixtures and Arterially Infused Chemicals tested in IPPSFs and Porcine Skin Flow-through Diffusion Cells**

| Permethrin Topical Mixtures* | Intra-arterial Chemical | | DEET Topical Mixtures* | Intra-arterial Chemical |
|---|---|---|---|---|
| $^{14}$C-Permethrin+Ethanol[1] | | | 75%DEET+Ethanol+$^{14}$C-permethrin | |
| $^{14}$C-Permethrin+Ethanol[1] | PB | | 75%DEET+Ethanol+$^{14}$C-permethrin | PB |
| $^{14}$C-Permethrin+Ethanol+DFP | | | 7.5%DEET+Ethanol+$^{14}$C-permethrin | |
| $^{14}$C-Permethrin+Ethanol[1] | DFP | | | |
| $^{14}$C-Permethrin+Ethanol[1] | PB+DFP | | | |

*indicates 100% ethanol or 60%ethanol:40%water for all mixtures, except for 7.5%DEET mixture which was only tested with an aqueous mixture
[1]indicates mixtures tested in porcine skin flow-through diffusion cells; PB = pyridostigmine bromide; DFP = Diisopropylfluorphosphate.

**TABLE 2.**
**Absorption ratios (Mixture/Control) for topical $^{14}$C-permethrin following infusion of PB, DFP, and PB+DFP in IPPSFs and Flow-through Diffusion Cells**

| Mixture | IPPSF | Flow-through Diffusion Cells |
|---|---|---|
| **TopAqMix+PB+DFP** | **5.57*** | **1.16** |
| **TopAqMix+PB** | **1.36** | **2.76*** |
| **TopAqMix+DFP** | **2.26** | **2.14** |
| | | |
| **TopNonaqMix+PB+DFP** | **2.47*** | **2.72*** |
| **TopNonaqMix+PB** | **1.35** | **3.82*** |
| **TopNonaqMix+DFP** | **1.64** | **2.91*** |

*= statistically significant differences between controls and mixture
TopAqMix = topical aqueous mixture; TopNonaqMix = topical nonaqueous mixture; PB = pyridostigmine bromide; DFP = Diisopropylfluorphosphate

## LIST OF FIGURES

Figure 5. Log SC/vehicle partitioning coefficient for $^{14}$C-permethrin in aqueous (shaded histograms) and nonaqueous (open histograms) DEET mixtures. Means with different letters represent significant differences between treatments ($p < 0.05$). * indicates significant differences between aqueous and nonaqueous mixtures for each treatment.

Fig 1

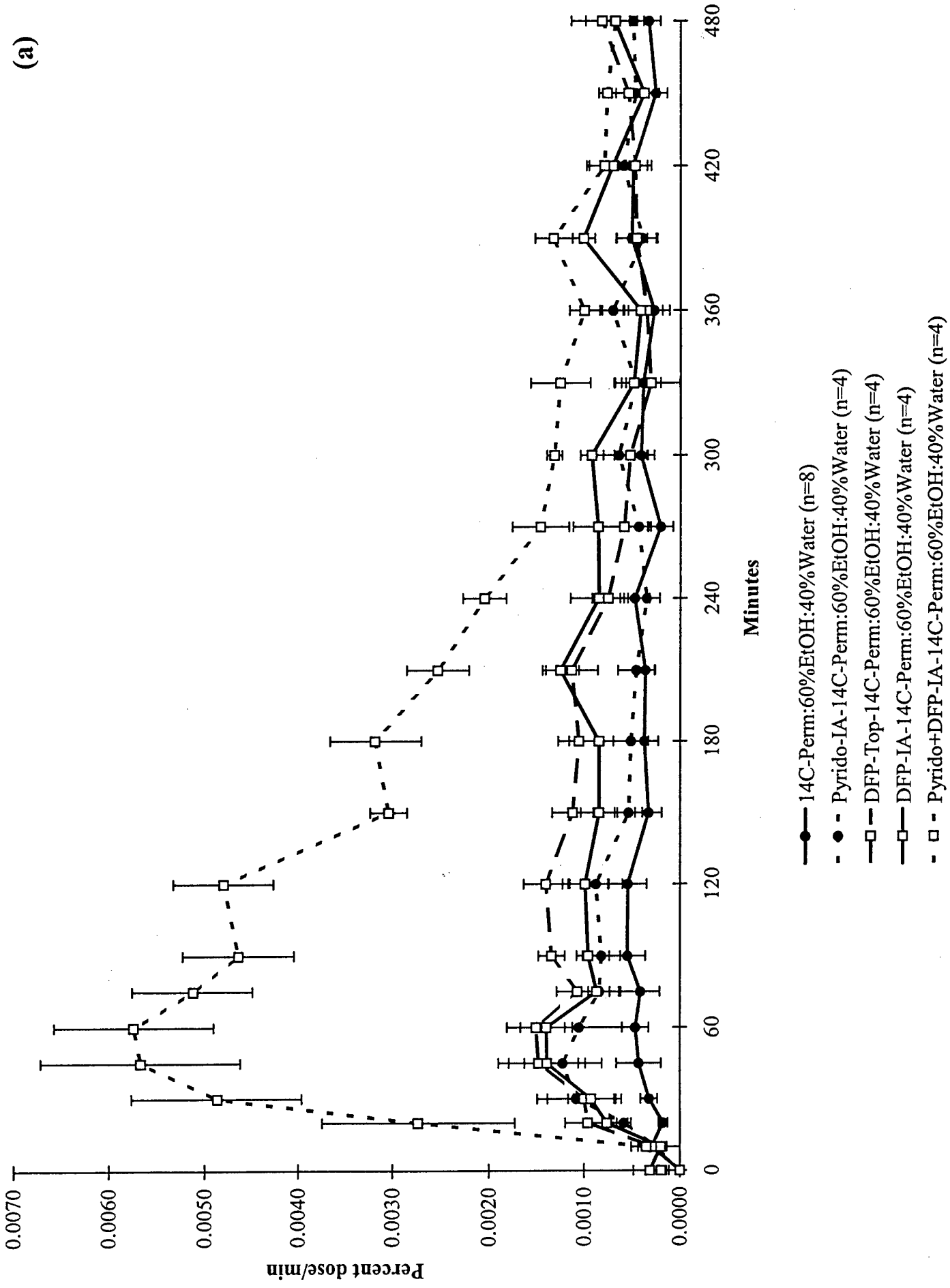
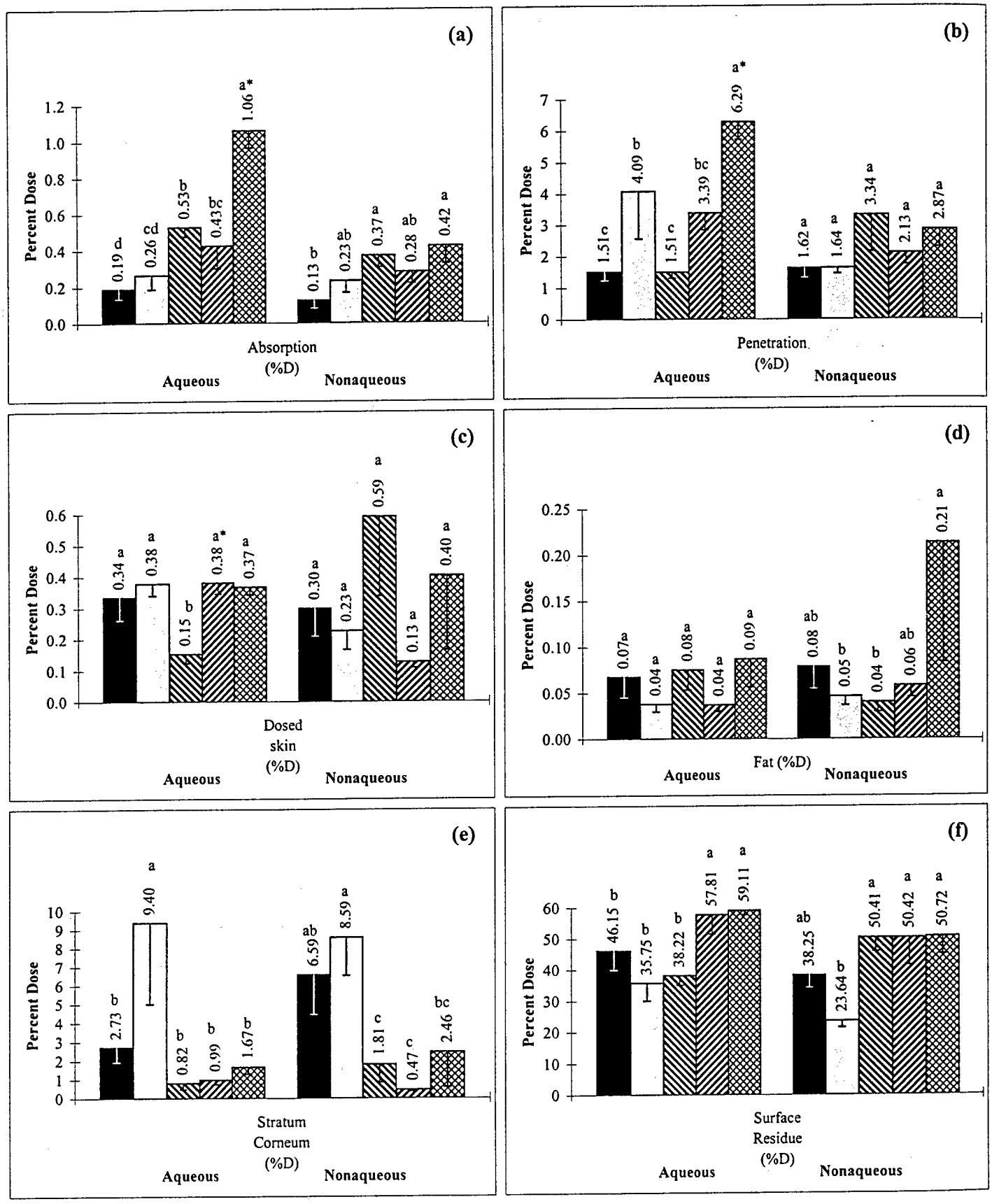

(a)
Percent dose/min
0.0070
0.0060
0.0050
0.0040
0.0030
0.0020
0.0010
0.0000
0
60
120
180
240
300
360
420
480
Minutes
14C-Perm:60%EtOH:40%Water (n=8)
Pyrido-IA-14C-Perm:60%EtOH:40%Water (n=4)
DFP-Top-14C-Perm:60%EtOH:40%Water (n=4)
DFP-IA-14C-Perm:60%EtOH:40%Water (n=4)
Pyrido+DFP-IA-14C-Perm:60%EtOH:40%Water (n=4)

Fig 2

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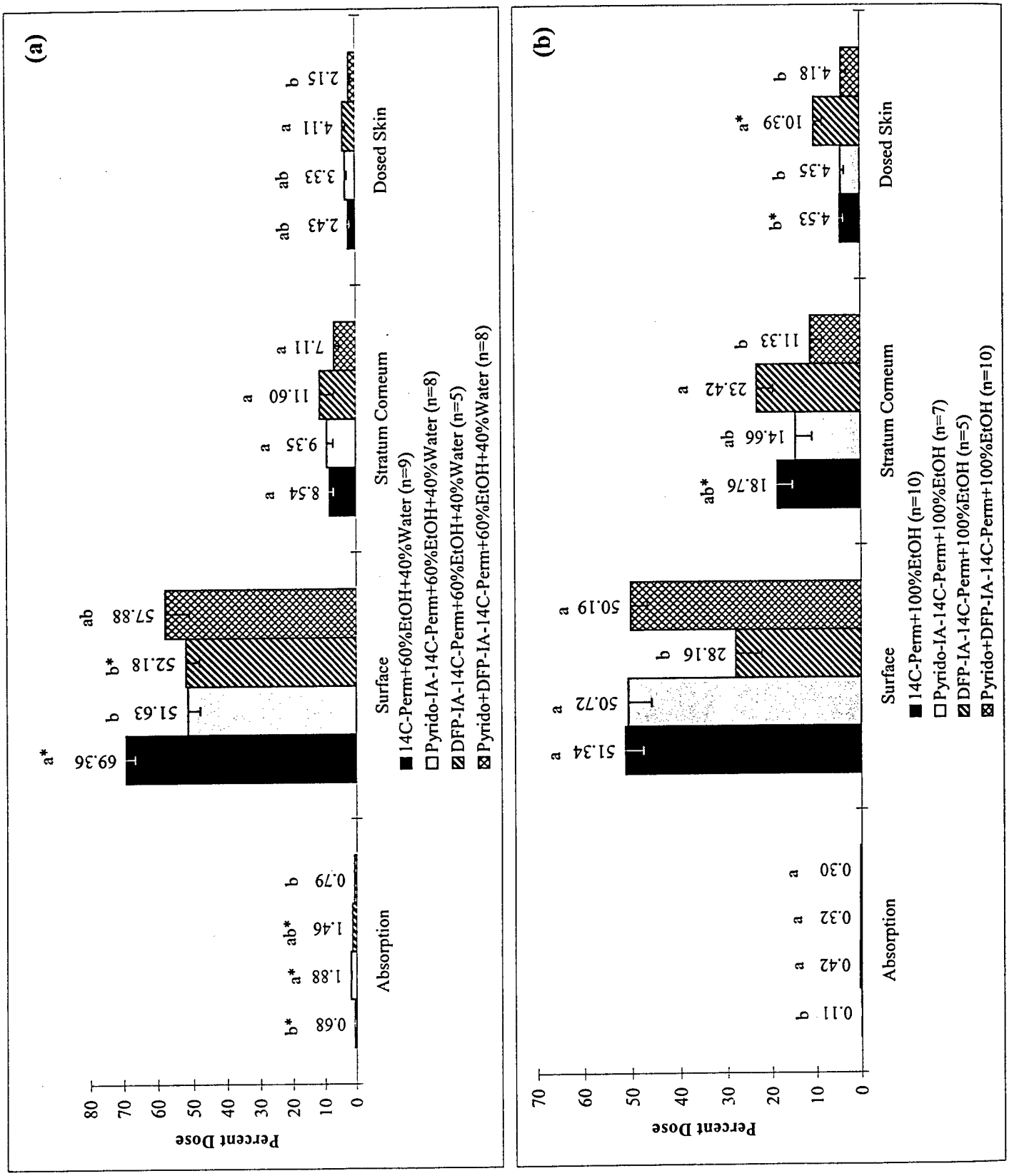
- Pyrido-IA-14C-Perm:60%EtOH:40%Water (n=4)
- DFP-Top-14C-Perm:60%EtOH:40%Water (n=4)
- DFP-IA-14C-Perm:60%EtOH:40%Water (n=4)
- Pyrido+DFP-IA-14C-Perm:60%EtOH:40%Water (n=4)
- 14C-Perm:100%EtOH (n=7)
- Pyrido-IA-14C-Perm:100%EtOH (n=4)
- DFP-Top-14C-Perm:100%EtOH (n=4)
- DFP-IA-14C-Perm:100%EtOH (n=4)
- Pyrido+DFP-IA-14C-Perm:100%EtOH (n=4)

Fig 3


(a)
Percent Dose
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10
20
30
40
50
60
70
80
Absorption
Surface
Stratum Corneum
Dosed Skin
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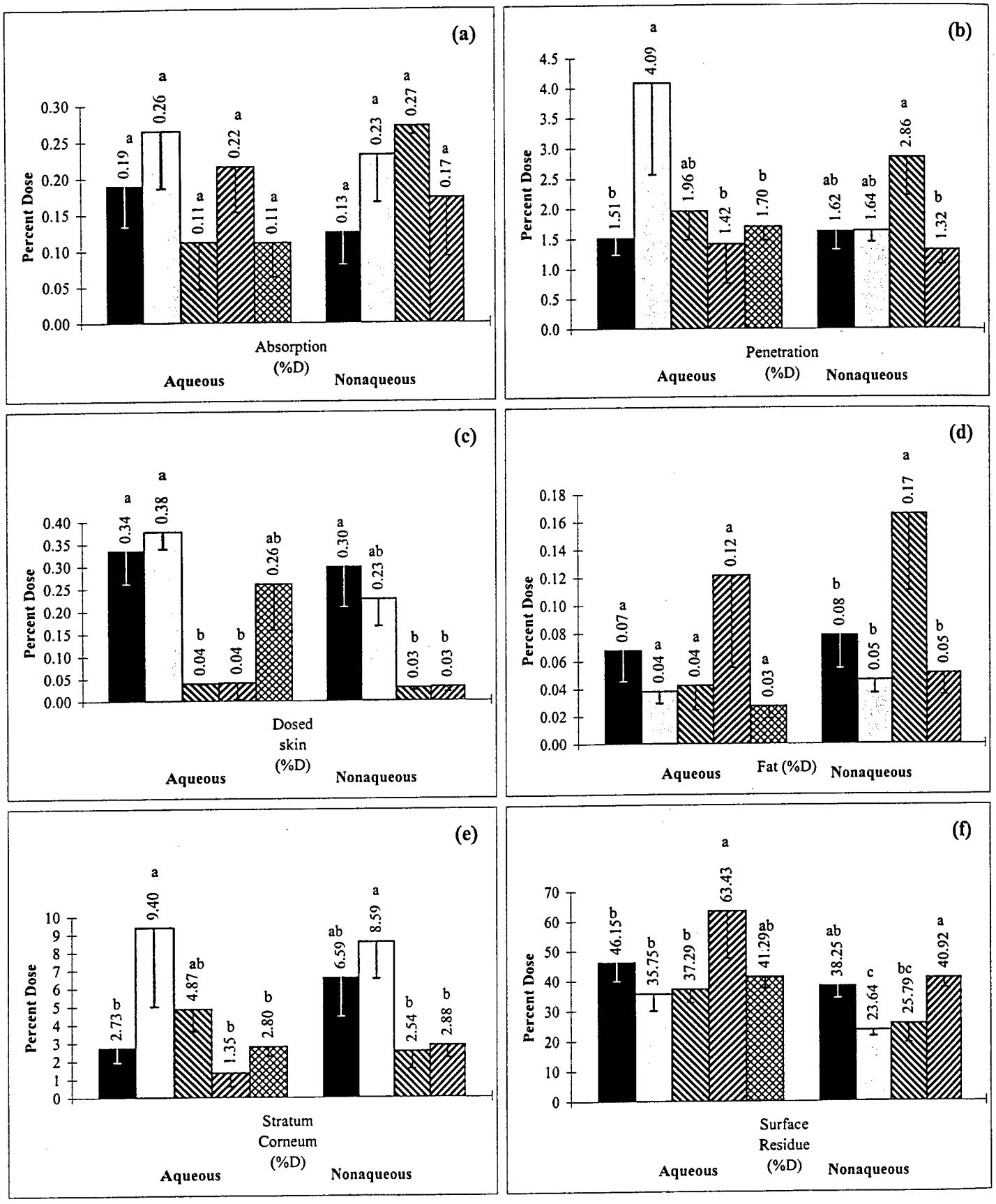
1.88 a*
1.46 ab*
0.79 b
69.36 a*
51.63 b
52.18 b*
57.88 ab
8.54 a
9.35 a
11.60 a
7.11 a
2.43 ab
3.33 ab
4.11 a
2.15 b
14C-Perm+60%EtOH+40%Water (n=9)
Pyrido-IA-14C-Perm+60%EtOH+40%Water (n=8)
DFP-IA-14C-Perm+60%EtOH+40%Water (n=5)
Pyrido+DFP-IA-14C-Perm+60%EtOH+40%Water (n=8)
(b)
Percent Dose
0
10
20
30
40
50
60
70
Absorption
Surface
Stratum Corneum
Dosed Skin
0.11 b
0.42 a
0.32 a
0.30 a
51.34 a
50.72 a
28.16 b
50.19 a
18.76 ab*
14.66 ab
23.42 a
11.33 b
4.53 b*
4.35 b
10.39 a*
4.18 b
14C-Perm+100%EtOH (n=10)
Pyrido-IA-14C-Perm+100%EtOH (n=7)
DFP-IA-14C-Perm+100%EtOH (n=5)
Pyrido+DFP-IA-14C-Perm+100%EtOH (n=10)

Fig 4

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- Pyrido-IA-14C-Perm:60%EtOH:40%Water (n=4)
- 14C-Perm:75%DEET:15%EtOH:10%Water (n=4)
- Pyrido-IA-14C-Perm:75%DEET:15%EtOH:10%Water (n=4)
- 14C-Perm:7.5%DEET:55.5%EtOH:37%Water (n=4)
- 14C-Perm:100%EtOH (n=7)
- Pyrido-IA-14C-Perm:100%EtOH (n=4)
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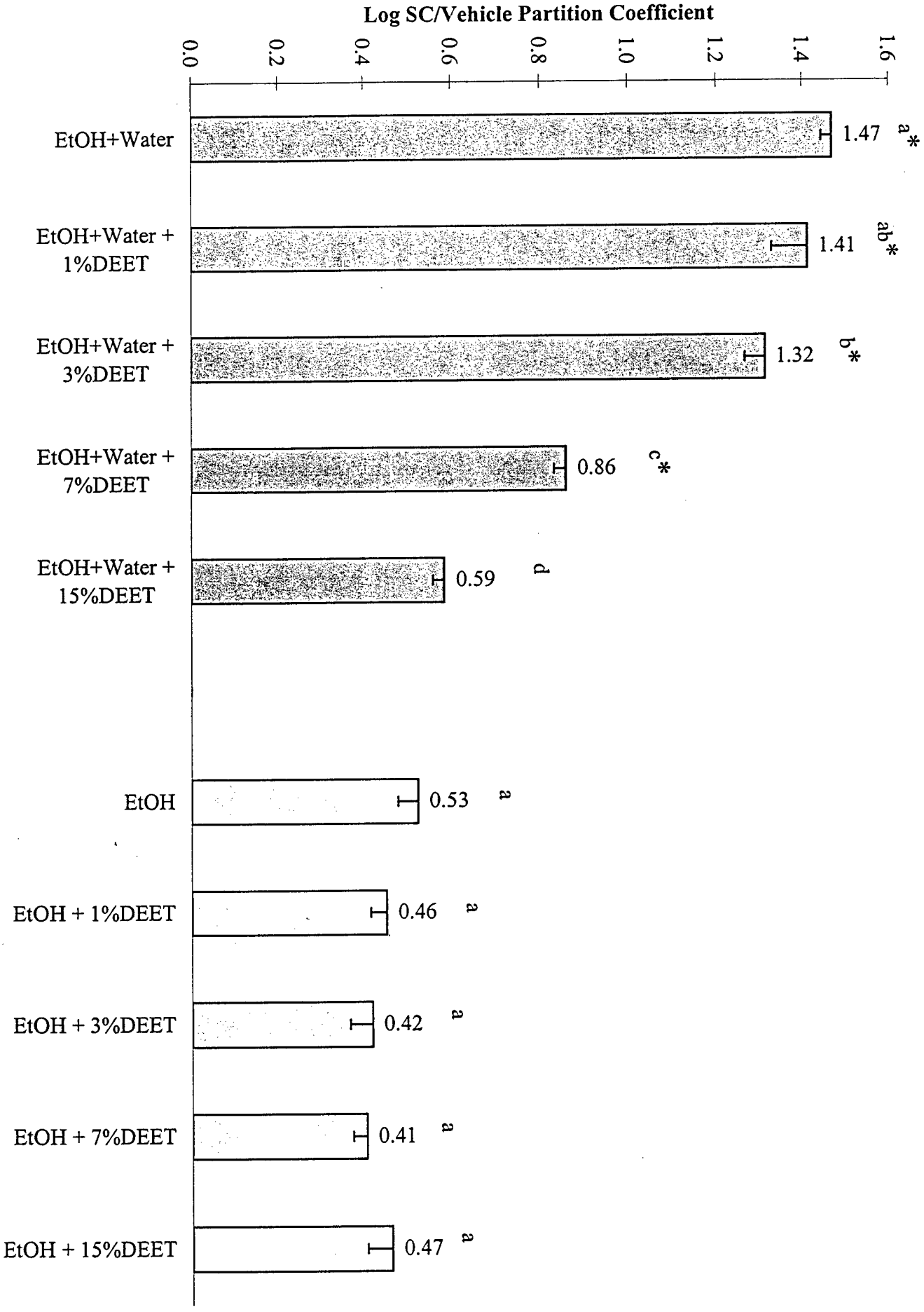
Log SC/Vehicle Partition Coefficient
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0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
EtOH+Water
1.47
a*
EtOH+Water + 1%DEET
1.41
ab*
EtOH+Water + 3%DEET
1.32
b*
EtOH+Water + 7%DEET
0.86
c*
EtOH+Water + 15%DEET
0.59
d
EtOH
0.53
a
EtOH + 1%DEET
0.46
a
EtOH + 3%DEET
0.42
a
EtOH + 7%DEET
0.41
a
EtOH + 15%DEET
0.47
a

Fig 5

# PYRIDOSTIGMINE BROMIDE MODULATES TOPICAL IRRITANT-INDUCED CYTOKINE RELEASE FROM HUMAN EPIDERMAL KERATINOCYTES AND ISOLATED PERFUSED PORCINE SKIN

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Abbreviated Title: Pyridostigmine Bromide Suppresses Cytokines

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# PYRIDOSTIGMINE BROMIDE MODULATES TOPICAL IRRITANT-INDUCED CYTOKINE RELEASE FROM HUMAN EPIDERMAL KERATINOCYTES AND ISOLATED PERFUSED PORCINE SKIN.

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**Toxicology and Applied Pharmacology**

## ABSTRACT

Gulf War personnel were given pyridostigmine bromide (PB) as a prophylactic treatment against organophosphate exposure and then exposed to the insecticide permethrin (P) and the insect repellent DEET. The purpose of this study was to assess the effects of multiple chemical exposure on biomarkers of inflammation. A variety of combinations of the topical mixtures of P and/or DEET in ethanol were applied to isolated perfused porcine skin flaps (IPPSFs). Concentrations of interleukin-8 (IL-8), tumor necrosis factor alpha (TNF-α) and prostaglandin E ($PGE_2$) were assayed to probe for potential inflammatory effects after complex mixture application. IPPSF's (n=4/treatment) were topically dosed with mixtures of P, DEET, P/DEET, in ethanol. Each treatment was repeated with perfusate spiked with 50 ng/ml of PB or 30 ng/ml diisopropylfluorphosphate (DFP). Timed IPPSF venous effluent samples (0.5, 1, 2, 4, and 8 h) were assayed by ELISA for IL-8 and TNF-α and by EIA for $PGE_2$. IPPSFs were also infused with the nerve agent simulant DFP to assess the interaction of simultaneous low level chemical agent exposure.

The overall pattern of PB infusion demonstrated a decrease in IL-8 and $PGE_2$, and an increase in TNF-α with permethrin and DEET in ethanol but a suppression when in combination of one another. To probe the potential mechanism of this PB effect, human

epidermal keratinocyte (HEK) cell cultures were exposed to P, DEET, P/DEET, with and without PB in DMSO. IL-8 was assayed at 1, 2, 4, 8, 12, and 24h. PB suppressed IL-8 in permethrin and ethanol (PE) treatments from 4 to 24 h supporting the IPPSF results. In conclusion, these studies clearly demonstrate that systemic exposure to PB suppressed IL-8 release from keratinocytes. This finding strongly suggests an immunomodulator role for PB in the Gulf War Illness.

**Key Words: pyridostigmine bromide, skin, cytokines, permethrin, DEET, diisopropylfluorphosphate**

## INTRODUCTION

The Gulf War Syndrome is a collection of symptoms experienced by soldiers participating in the Persian Gulf War. These symptoms range from chronic fatigue, muscle and joint pain, gastrointestinal disturbances, respiratory difficulties, sleep disorders, neurocognitive problems and skin manifestations (Jamal 1998). Exposure to numerous environmental adversities including hydrocarbon fumes, oil well fires, diesel exhaust, toxic paints, pesticides, and depleted uranium have been purported to be risk factors for this syndrome. In addition, these veterans were often treated with topical insecticides (e.g., permethrin), insect repellents (e.g., *N, N* diethyl-*m*-toluamide (DEET)) and/or oral pyridostigmine bromide (PB), a prophylactic treatment for nerve agent exposure. Reports have stated that the American Army wore permethrin-impregnated uniforms and also applied DEET topically to the skin to prevent tick-borne encephalitis (Institute of Medicine, 1996).

There has been much speculation and a wealth of research studies as to the possible causes of this syndrome. Several hypotheses suggest that the chemical interactions between PB, DEET and permethrin may contribute to the pathogenesis of Gulf War Illness (Abou-Donia et al., 1996; Baynes et al., 1997; Chaney et al., 1999; 2000; McCain et al., 1997). It is widely accepted that a single chemical may have no toxicological effect, but when used in combination with other agents may exert a detrimental effect (Klaassen and Eaton, 1991; Yang,1994; Mumtaz,1994). Combinations of organophosphate (OP) pesticides and DEET are common among agricultural workers and concern for possible toxic interactions continue to be reported (Chaney et al., 2000). Concurrent DEET and pesticide exposure was present in the Persian Gulf and Balkan

conflicts. However, a consistent confounding factor was the presence of PB. Also, there was potential low level exposure to organophosphate nerve agents. These specific chemical and drug exposures may be significant in light of research studies which demonstrated that neurotoxicity was enhanced in hens following co-exposure to PB, DEET and permethrin (Abou-Donia et al., 1996). Similarly, concurrent oral exposure to PB, DEET and permethrin significantly increased lethality in rats (McCain et al., 1997). These data clearly indicate that at least in laboratory animals, PB exerts a synergistic toxicological interaction with simultaneous exposure to DEET and permethrin. Our laboratory has demonstrated that systemic PB enhances the percutaneous absorption of topically-applied radiolabeled permethrin activity in two *in vitro* porcine dermal absorption models (Baynes et al., 2001). These studies suggest that PB may modulate the toxicological activity and/or systemic exposure to topically applied chemicals such as pesticides or insect repellents.

The anticipation that potential nerve agents would be present during the Persian Gulf conflict warranted the coalition forces to issue PB as a prophylactic agent against nerve gas. PB is a reversible inhibitor of AChE which shields the enzyme from the more potent OP nerve agents which would irreversibly bind to AChE causing severe disability (Sharabi et al., 1991). OP and carbamate pesticides, all cholinesterase inhibitors, are widely used in agriculture for pest control. Also, they are used topically on humans and in our habitats to eliminate a host of insects, many of which may be vectors of human disease. PB itself is an approved human drug (Pyridostigmine Bromide Syrup USP, Mestinon®) used to diagnose and treat myasthenia gravis. Other indications include post-surgical reversal of neuromuscular blockade, senile dementia, as well as for increasing

the neuromuscular tone of the urinary and gastrointestinal tracts (Millard and Broomfield, 1995, USPDI, 2001). When used therapeutically, PB has a wide margin of safety even in pediatric and geriatric populations. Known drug interactions and adverse effects are primarily related to AChE-related neuromuscular mechanisms and or allergic hypersensitivity to the bromide ions (e.g. skin rashes).

These two PB exposure scenarios, its prophylactic use in the Gulf War for protection against nerve agent toxicity and its therapeutic use against myasthenia gravis, suggest that any role for PB in the Gulf War Syndrome would most likely be related to a toxicological interaction with simultaneously exposed chemicals such as DEET, permethrin and/or other environmental stressors or military chemicals. The purpose of this study was to assess the effects of PB systemic exposure on modulating the release of the cytokines interleukin-8 (IL-8), tumor necrosis factor alpha (TNF-$\alpha$), as well as prostaglandin $E_2$ ($PGE_2$), secondary to topical chemical irritation in isolated perfused porcine skin and human epidermal keratinocyte cell cultures. The effect of low level nerve agent exposure was studied by infusion of the nerve agent simulant DFP. These studies are a direct extension of experiments in our laboratory assessing the effects of PB on the percutaneous absorption of permethrin and DEET reported elsewhere (Baynes et al., 2001). Perfusate was collected for cytokine analysis from the perfused skin chemical absorption studies based on the hypothesis that PB-induced alterations in permethrin and/or DEET absorption was secondary to mild PE and/or DEET-induced cutaneous irritation. However, as will be shown in this manuscript, infusion of PB into perfused porcine skin resulted in a marked decrease of IL-8 production, despite slightly enhanced

permethrin percutaneous absorption. This observation was then tested in human keratinocyte cell cultures co-exposed to PB, DEET and permethrin.

## MATERIALS AND METHODS

### Experimental strategy:

Experiments were conducted in two model systems; isolated perfused porcine skin flaps (IPPSF) and human epidermal keratinocyte (HEK) cell cultures. The chemical exposures in perfused skin were based on formulations designed to assess the effects of mixtures of topical chemicals and co-dosed systemic compounds on the percutaneous absorption of topically applied permethrin and DEET. To facilitate the formulation of these topical applications, both aqueous and nonaqueous solvent systems were employed. Systemic exposure to PB and DFP was modeled by including these chemicals in the arterial perfusate of the perfused skin preparation at concentrations which corresponded to that observed in blood after oral administration. Treatment combinations were not selected based on any effect on cytokine release but rather on their ability to result in dermal absorption and or skin deposition of topical chemicals. DFP was infused to simulate concurrent low level nerve agent exposure. Concentrations of IL-8, TNF-α, and $PGE_2$ were determined in samples collected over the duration of an absorption experiment. In contrast, HEK cell culture studies were designed to assess the effects of co-exposure to PB and DEET and/or permethrin on release of IL-8 as a marker of chemical-induced irritation.

### Isolated Perfused Porcine Skin Flaps (IPPSF) Studies:

Female Yorkshire weanling pigs weighing 20-30 kg were purchased commercially and acclimated for one week prior to surgery. The pigs were housed in a temperature (22°C) and light/dark (12 h/12 h) regulated facility on elevated pen floors and provided *ad libitum* with water and 15% protein pig and sow pellets (Wayne Feeds

Division, Chicago, IL). The surgical procedure involved the creation of two single-pedicle, axial pattern, island tubed skin flaps each lateral to the ventral midline on the pig abdomen (Riviere et al., 1986; Monteiro-Riviere et al., 1987; Monteiro-Riviere, 1990; Bowman et al., 1991). Each flap was created during Stage I and harvested 48 h later during Stage II surgery. The flaps were then cannulated, flushed with heparinized saline to clear the vasculature of blood, and transferred to the perfusion chamber maintained in a specially designed fume hood for chemical agents.

Each isolated porcine skin flap was perfused continuously with a modified Krebs-Ringer bicarbonate buffer (pH 7.4, 350 mOsm/kg) containing bovine serum albumin (45 g/l) and glucose (80-120 mg/dl) as the primary energy source. The temperature (37°C) and humidity (60-80%) in the chamber and the media flow rate (1.5 ml/min) were monitored and remained constant throughout the entire perfusion period. In addition, the media was gassed with 95% oxygen and 5% carbon dioxide via a silastic oxygenator. Since the IPPSF is not a sterile organ preparation, antimicrobials (penicillin G and amikacin) were added to the media to prevent bacterial overgrowth from the microflora normally present on the skin surface. Heparin was included to prevent coagulation from residual blood elements in the vasculature of the flap.

Each IPPSF was perfused for 1 h prior to dosing when skin flap viability was then assessed by monitoring vascular resistance (perfusate pressure/ flow rate) and glucose utilization. Viable flaps were then fitted with a flexible dosing template measuring 1.0cm X 5.0cm (Stomahesive, Convatec-Squibb, Princeton, NJ) affixed to the skin surface with Skin Bond (Pfizer Hospital Products, Inc., Largo, FL). A Microman pipette (Gilson Medical Electronics S.A., Villers-le-Bel, France) was used for dosing topical chemicals.

For the inflammatory mediators analyses, 3.5 ml of the venous perfusate were collected at 0, 0.5, 1.0, 2.0, 4.0 and 8.0 h post dose. The effluent was aliquotted into microcentrifuge tubes and then stored at -80°C until use.

**Chemicals and Dosing:** IPPSFs were dosed within the 5.0cm$^2$ dosing area with 100 µl of the topical mixtures listed in Table 1. The dose applied to the dose site was formulated to deliver doses of 40 µg/cm$^2$ of $^{14}$C-permethrin in ethanol (PE). Concentrations of 7.5% or 75% DEET were also added to permethrin mixtures to make up to the 75% DEET used by the soldiers. These solutions contained mixtures of permethrin ($^{14}$C-permethrin, specific activity = 8.1 mCi/mmol, Sigma Chemical, St. Louis, MO); DEET (98% purity, Chem Service Company, West Chester, PA), and ethanol (Sigma Chemical, St. Louis, MO). All of the above chemicals were HPLC grade. In addition, 100% ethanol and 60% ethanol / 40% water controls were studied. The effect of systemic exposure to nerve agents on cutaneous function was assessed by including 30 ng/ml of the simulant DFP (Sigma Chemical, St. Louis, MO) in arterial perfusate. This concentration was previously used in IPPSF experiments to simulate exposure to organophosphate (OP) nerve agents (Carver et al., 1989). To assess the effect of PB on inflammatory mediator release, half the IPPSFs were perfused with intra-arterial PB (Mestinon®, ICN Biomedicals Inc., Costa Mesa, CA) (50ng/ml media). This concentration corresponds to peak blood concentrations of PB achieved following the same prophylactic dose that veterans experienced during the Gulf War (Marino et al., 1998).

**Analysis of IPPSF for *IL-8 and TNF-α ELISA:*** The assays were conducted utilizing Swine IL-8 and Swine TNF-α Enzyme Linked Immunosorbent Assay kits (Cytoscreen™, Biosource International, Inc., Camarillo, CA). The samples were thawed, diluted 1:1 in a

standard diluent buffer, and thoroughly mixed. Samples (triplicate), standards (duplicate), and spiked media for IL-8 and TNF-α were pipetted into the appropriate wells of the antibody-coated ELISA plate. Plates were sealed and incubated for 2 hr at room temperature (RT). After rinsing thoroughly, each well was incubated for 1 hr with 100 µl of biotinylated anti-IL-8 or anti-TNF-α conjugate. Following another rinse, 100 µl of streptavidin-HRP (1:1000) was pipetted into each well and incubated for 30 minutes at RT. Stabilized chromagen (100µl) was placed in each well in the dark for 30 minutes. The absorbance of each well read at 450nm using the Multiskan RC plate reader (Labsystems, Helsinki, Finland). The absorbance of each sample was converted to concentration (pg/ml) by fitting to the standard curve using Genesis-Lite Windows based microplate software version 3.0 (Life Sciences International UK Ltd). The values were adjusted for dilution and sample recovery (determined from spiked media) for the final timed concentrations.

**$PGE_2$ Enzyme Immunoassay (EIA):** This assay was conducted using a prostaglandin $E_2$ EIA kit (Caymay Chemical, Ann Arbor, MI). The 15 ml C18 columns (Spec Plus™, MetaChem, Torrance, CA) used to purify the media were preconditioned with 500 µl of methanol followed by 500 µl of fresh ultrapure water. The samples were thawed and 250 µl pipetted into 8ml of 0.1M potassium phosphate monobasic, pH 5.0 and primed with a slight vacuum to a flow rate of approximately 1.0 ml/min. Each column was eluted with 8.0 ml of ethyl acetate to remove the bound $PGE_2$ from the C18 column. The ethyl acetate was evaporated on a Zymark TurboVap LV Evaporator under nitrogen until dry. The samples were reconstituted with 1000 µl of EIA buffer. Then 50 µl samples (triplicate), standards (duplicate), and spiked media (1000pg/ml) were placed in the wells

followed by 50µl of $PGE_2$ tracer and 50µl of $PGE_2$ antibody. The plate was covered and incubated for 18 hours at 4°C. After thoroughly rinsing the plates, 200 µl of Ellman's Reagent was placed into each well and incubated for 90 min in the dark on the orbital shaker (100 revolutions/min). The absorbance of each well was read at 405 nm using the Multiskan RC plate reader (Labsystems, Helsinki, Finland). The $\%B/B_o$ (percent bound /maximum bound) of the standards were calculated and plotted against the standards concentration. A logarithmic trend line was applied to the points and the concentration of the samples calculated. The values were adjusted for dilution and sample recovery (determined from spiked media) for the final timed concentrations.

**Human Epidermal Keratinocytes (HEK) Studies:**

Cryopreserved HEK were purchased from Clonetics Corp. (San Diego, CA) and stored under liquid nitrogen until passage. Cells (approximately 263,000) were diluted in 15ml of KGM-2 (serum-free keratinocyte basal media supplemented with human epidermal growth factor, insulin, bovine pituitary extract, gentamycin, and amphotericin-B) in each $75cm^2$ culture flask and grown in a humidified 5% $CO_2$ environment at 37°C. The keratinocytes were harvested once they reached 80% confluency, stored under liquid nitrogen until plated, or plated immediately in 24-well culture plates ($2.0cm^2$ /well area) at a density of approximately 20,000 cells per well. Once HEK were grown to 80% confluency they were dosed either with or without PB at 50ng/ml and treated with PE ($40\mu g/cm^2$), DEET ($7.5\mu l/cm^2$), or a PE/DEET mixture in the DMSO vehicle (final concentration 1.0%) diluted in KGM-2 media. The dosing treatments were the same concentration per unit area as those applied to the IPPSF making it the maximum dose

that *in situ* keratinocytes could be exposed to. This would result in a greater exposure of HEKs to both DEET and permethrin compared to the IPPSF. However, since the focus of these studies was to assess the ability of PB to modulate subsequent IL-8 release, an increase exposure to irritants is consistent with these experimental goals. Media samples were taken at 1, 2, 4, 8, 12, and 24 h post-treatment and stored at -80°C until assayed. Each compound was conducted in a minimum of four plates, four wells per time point.

**IL-8 Immunoassay:** Cell media was thawed and assayed in triplicate for IL-8 using human IL-8 Cytoset (Biosource International, Camarillo CA). Briefly, 96-well plates were coated with an IL-8 capture antibody for 18 h at 4°C and nonspecific binding sites blocked with 0.5% bovine serum albumin. Samples and standards were placed in the wells along with a biotinylated monoclonal mouse anti-human IL-8 antibody. Following a 2 h incubation at room temperature, the biotin was tagged with streptavidin/horseradish peroxidase. Washing was performed after each step. The chromagen (Sigmafast, Sigma Chemical Company) was placed in each well and incubated in the dark for 30min. The plate was read at 450nm on a Multiskan RC plate reader (Labsystems, Helsinki, Finland). The mean IL-8 concentration (pg/ml) for each treatment and time point was calculated and data normalized by dividing by the 1 h values.

**Statistics:**

In the IPPSF experiments, IL-8, TNFα and $PGE_2$ sample concentration means were generated for each time point collected and adjusted for pre-treatment background levels by subtracting time zero concentrations from the observed time point. In the HEK cultures, the 2, 4, 8, 12 and 24 h sample concentrations were divided by the 1 h value (fraction of initial value) to obtain results. Concentrations were expressed as pg

cytokine/mL media as determined using the Genesis Life Version 3 for Windows software (Labsytems). Inflammatory mediator release occurs from IPPSF controls secondary to the surgical procedure that creates this preparation (Monteiro-Riviere, 1992; Monteiro-Riviere et al., 1994; Zhang et al., 1995a,b). This confounding factor, as well as vehicle effects, are factored in these analyses by subtracting initial zero-time concentrations from subsequent values, to remove this background and reveal true PB-related treatment effects. Significant difference ($p<0.05$) between post-treatment times were determined using LSD Duncan test in the ANOVA (SAS 6.12 for Windows; SAS Institute, Cary, N.C.).

## RESULTS

Figures 1, 3, and 5 depict the effect of co-exposure of PB on the concentration versus time profiles of IL-8, TNF-α, and $PGE_2$, respectively, in IPPSFs exposed to the seven treatment combinations outlined in Table 1. Figures 2, 4, and 6 directly compare the effect of PB treatment on mediator release by pooling all treatments within time points for each mediator. Inflammatory mediator release generally increased with time from 0.5 to 8 h. IL-8 concentrations were continuing to increase by 8 h while $PGE_2$ concentrations appeared to plateau at 4 h. In contrast, TNF-α concentrations peaked at 1-2 h. A similar pattern of mediator release was also seen in ethanol and ethanol/water vehicle controls.

Table 2 summarizes the significant effects seen after PB administration. PB suppressed IL-8 release in all treatment combinations except for DE and PDE. IL-8 release was reduced in DEW and PDEW treatments, supporting the contention that vehicle selection, in these cases, three containing DEET, plays an important role in interpreting cutaneous toxicological effects. When all treatment combinations are pooled (Fig. 2), IL-8 suppression is still clearly seen. The effects of PB on TNF-α release is fundamentally different, in that PB increased release in PE and DE treatments, but suppressed the release when DEET / PE combinations were present in the PDEW and PDE treatments (Fig. 3). When all treatment combinations were pooled (Fig. 4), PB suppression of TNF-α release is seen at the 1 and 2 h time points. The effects of PB exposure on $PGE_2$ expression is time-dependent, with a tendency for suppression at the 0.5, 1.0 and 2.0 h time points in the DE, PEW, DEW, and PDEW treatments (Fig. 5). This is clearly evident when all preparations are pooled across treatments (Fig. 6).

In order to confirm the observed effects of PB on IL-8 release, mixtures of permethrin and DEET were dosed in HEK. Fig. 7 distinctly shows that IL-8 release was also suppressed at all time points in the permethrin dose group. As in the IPPSFs, IL-8 release was not as pronounced when DEET was present in the media, due to the fact that DEET was toxic to the cells and therefore not shown. DMSO was used to solubilize permethrin in these cell culture studies instead of ethanol because ethanol is toxic to HEK. PB also reduced IL-8 release from DMSO treated HEK cultures.

## DISCUSSION

These results clearly demonstrate that PB exposure modulates cytokine release in both IPPSF and HEK experimental model systems. The most consistent effect observed was with IL-8 suppression in both models. It is important to note that PB significantly reduced IL-8 in ethanol/water mixtures. This finding is in agreement with our observations that PB also enhanced PE percutaneous absorption and skin deposition in ethanol/water mixtures (Baynes et al., 2001). This observation suggest that the ethanol / water mixture is required for PE (or metabolite of) to penetrate into skin to reach the keratinocytes to cause IL-8 release. It is interesting to note that treatments that tended to suppress IL-8 were similar to those that increased permethrin absorption ruling out that simple inflammation is responsible for enhanced absorption. These IPPSF findings are consistent with the HEK data where PE alone was the primary inducer of IL-8 release. In both scenarios, PB co-exposure clearly dampened (depressed) this effect.

The effect of concurrent DEET exposure is not as evident in either model system. In the IPPSF, DEET tends to blunt the effect while in HEK, it drastically reduced IL-8 release due to direct cellular toxicity. Previous workers, including our laboratory, have shown an antagonistic effect of combined DEET and PE exposure. This includes decreased lethality in rats after oral administration of the combination versus either compound alone (McCain et al., 1997). However, in this rodent study, addition of PB produced a synergistic response. In our absorption studies (Baynes et al., 2001), DEET similarly reduced the ability of PB infusions to increase permethrin absorption, further supporting this antagonist when present as a mixture.

The effects of PB on other mediators is less clear, although consistent within treatment effects are observed. In general, $PGE_2$ release seems to be depressed by PB exposure at early time points. No combination treatments clearly increased the $PGE_2$ response. The effects of PB on IL-8 and $PGE_2$ are consistent with a general decrease in cutaneous irritation. In contrast, either permethrin or DEET alone in an ethanol vehicle increased TNF-$\alpha$ release. However, when these chemicals are combined, TNF-$\alpha$ release was suppressed.

It has been shown that keratinocytes can trigger the release of pro-inflammatory cytokines, chemokines, and growth factors (Kondo, 1999) and this "network" of soluble molecules which is responsible for the initiation of the inflammatory response in the skin (Luger et al.,1990; Nickoloff, 1991). Many of these molecules are produced constitutively in the keratinocyte, but are up-regulated in response to an inflammatory agent (Corsini and Galli, 1998). It has also been suggested that each inflammatory agent or compound may have a certain cytokine profile individually but may be different when comparing multiple agents concurrently (Wilmer et al., 1994).

A major effector in the development of inflammation in the skin is keratinocyte-derived IL-8. IL-8 is known for its role in neutrophil chemotaxis, and as a chemoattractant for T cells and basophils (Larsen et al., 1989; Leonard et al., 1991a), and has been implicated in keratinocyte proliferation by modulating cytosolic free $Ca^{2+}$ transients (Tuschil et al., 1992). A few studies have shown that injecting IL-8 stimulates an inflammatory response, characterized by the accumulation of neutrophils and lymphocytes at the injection site (Leonard et al., 1991b; Swensson et al., 1991). IL-8 release from keratinocytes is normally precluded by the release of tumor necrosis factor

alpha (TNF-α). Such interactions could be responsible for the confounding responses seen when full chemical mixtures are dosed. TNF-α may function in autocrine fashion to stimulate the production and secretion of multiple cytokines and chemokines, as well as adhesion molecules such as ICAM-1. TNF-α has been shown to modulate other cell types in the skin and activate infiltrating cells (e.g. macrophages).

Inflammatory mediator release is thus difficult to interpret since they are time dependent and their release is regulated by a complex web of feedback by other mediators. Factors which cause release of IL-8, TNF-α and $PGE_2$ do not work through the same signal transduction mechanisms. As proinflammatory mediators, these cytokines were selected as potential early biomarkers of cutaneous irritation of an impending inflammatory response and have been used previously in our laboratory to characterize chemical induced skin toxicity in the IPPSF exposed to other irritants.

The IPPSF has been utilized to characterize sulfur mustard [bis(2-chloroethyl) sulfide, HD ] toxicity, an alkylating agent that causes severe cutaneous injury. $PGE_2$ and $PGF_{2\alpha}$ significantly increased by 6 h upon exposure to 5.0mg/ml of HD. IL-$1_\alpha$ levels were significantly increased by 6 h in the 10 mg/ml of HD (Monteiro-Riviere, 1992; Zhang et al., 1995a). The IPPSF has been used to assess early phototoxicity and has demonstrated the release of $PGE_2$ after exposure to mid-wave UVB (290-320nm) radiation at 630mJ/cm$^2$ (Monteiro-Riviere et al., 1994). Mechanisms of HD-induced cytotoxicity examined the protective effects by using pharmacological agents with different mechanisms of action and the antiinflammatory effect of indomethacin inhibited the $PGE_2$ release normally seen with HD exposure (Zhang et al., 1995b). These previous studies support the use of the IPPSF as a model for characterizing

inflammatory mediators involved in cutaneous irritation secondary to both physical and chemical agents.

Similarly, it is widely accepted that keratinocytes are capable of releasing the cascade of cytokines after chemical irritant exposure. We have previously demonstrated this with the release of IL-8 and TNF-α in HEK after exposure to jet fuels (Allen et al., 2000) and in porcine keratinocytes (Allen et al., 2001). In the present study, the pattern of cytokine inhibition by PB was similar in both HEK and IPPSF systems, suggesting that the primary effect seen may be related to PB modulation of keratinocyte function, the common denominator in both models.

Topical PE appears to be capable of causing release of these mediators when dosed in the appropriate vehicle. Our previous studies discussed earlier on PE absorption in multiple model system shows minimal PE percutaneous absorption (Baynes et al., 1997; Baynes et al., 2001). Pyrethroids have been associated with contact sensitization (Flannigan et al, 1985). However, the primary observations in the present studies are an immunosuppressive effect of PB on PE induced cytokine release.

One hypothesis of Gulf War illness is related to immunomodulation, although the precise mechanism is not known. Putative factors include stress, multiple vaccinations, as well as other unique Persian Gulf environmental factors. The significant finding of the present study is that systemic exposure to PB, modeled in these studies as arterial infusion into the IPPSF, significantly decreased the release of IL-8 and $PGE_2$ secondary to topical dermal application of PE in various vehicles. The effects on TNF-α is not as clear. This would support a role for PB as an immunomodulating factor in Gulf War Illness.

Mechanistically, the HEK studies suggest that basal keratinocytes are the target cell because this PB-effect was seen in this monolayer culture. The precise mode of action would be pure speculation. A PB involved neuromuscular receptor interaction would be most consistent with both the pharmacological activity of PB. However, this mechanism is unlikely as the suppression was also seen in HEK, which consists of a monolayer of cells without neural elements. It is most likely that PB is modulating other receptor classes in keratinocytes that down regulate cytokine release, or alternatively cause the release of another mediator (e.g. neuropeptides), not assayed in the present study, which then decreases IL-8 and $PGE_2$ release. Numerous studies have shown that human skin may express many types of neuropeptides that are directly derived from sensory neurons or from keratinocytes (Scholzen et al., 1998). The ideal experimental endpoint would be a full proteomic analysis whereby multiple proteins could be more easily followed. However, this technique was not available when these studies were conducted. Such a mechanism could also be teased out with future inhibitor studies. Whatever the mechanism, these data strongly support an immunomodulator role for PB in the Gulf War Illness.

## ACKNOWLEDGMENTS

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assay analysis. Portions of this work was presented as an invited presentation at the Conference on Illnesses Among Gulf War Veterans: A Decade of Scientific Research, Alexandria, Va.

## TABLE 1

### Chemical Treatments Used in IPPSF Exposures

| | |
|---|---|
| PE | 40 μg/cm$^2$ Permethrin in ethanol |
| DE | 75% DEET in 25% ethanol |
| PEW | 40 μg/cm$^2$ Permethrin in 60% ethanol / 40% water |
| DEW | 75% DEET in 15% ethanol / 10% water |
| PDE | 40 μg/cm$^2$ Permethrin in 75% DEET / 25% ethanol |
| PDEW | 40 μg/cm$^2$ Permethrin and 75% DEET / 15% ethanol / 10% water |
| DFP PE | 40 μg/cm$^2$ Permethrin in ethanol co-infused with 30 ng/ml DFP |

All of the above solutions were dosed in a total volume of 100 μl in a 5 cm$^2$ IPPSF dosing area. All treatments were repeated (n=4 / treatment combination) in IPPSFs containing 50 ng/ml PB in arterial perfusate.

## TABLE 2

**Overall Pattern of Pyridostigmine Treatment Effects on Inflammatory Mediator Release in IPPSF**

| Treatment | IL-8 | TNF-α | $PGE_2$ * |
|---|---|---|---|
| PE | ↓ | ↑ | ↔ |
| DE | ↔ | ↑ | ↓ |
| PEW | ↓ | ↔ | ↓ |
| DEW | ↓ | ↔ | ↓ |
| PDE | ↔ | ↓ | ↔ |
| PDEW | ↓ | ↓ | ↓ |
| DFP PE | ↓ | ↔ | ↓ |

* At 0.5, 1.0 and 2.0 h time points.

## FIGURE LEGENDS

**Figure One:** Concentrations of IL-8 versus time in IPPSFs exposed to seven chemical mixtures with and without 50 ng/ml PB in the perfusate. Mean +/- SD. P = permethrin, E = ethanol, D = DEET, W = water. (See text for full discussion of treatments). Different letters in pairwise comparisons are significantly different ($p < 0.05$) from non-PB control.

**Figure Two:** Concentrations of IL-8 versus time in IPPSFs when treatments are pooled and compared with and without 50 ng/ml PB in the perfusate. Mean +/- SD. Different letters in pairwise comparisons are significantly different ($p < 0.05$) from non-PB control.

**Figure Three:** Concentrations of TNF-$\alpha$ versus time in IPPSFs exposed to seven chemical mixtures with and without 50 ng/ml PB in the perfusate. Mean +/- SD. P = permethrin, E = ethanol, D = DEET, W = water. (See text for full discussion of treatments). Different letters in pairwise comparisons are significantly different ($p < 0.05$) from non-PB control.

**Figure Four:** Concentrations of TNF-$\alpha$ versus time in IPPSFs when treatments are pooled and compared with and without 50 ng/ml PB in the perfusate. Mean +/- SD. Different letters in pairwise comparisons are significantly different ($p < 0.05$) from non-PB control.

**Figure Five:** Concentrations of $PGE_2$ versus time in IPPSFs exposed to seven chemical mixtures with and without 50 ng/ml PB in the perfusate. Mean +/- SD. P = permethrin, E = ethanol, D = DEET, W = water. (See text for full discussion of treatments). Different letters in pairwise comparisons are significantly different ($p < 0.05$) from non-PB control.

**Figure Six:** Concentrations of $PGE_2$ versus time in IPPSFs when treatments are pooled and compared with and without 50 ng/ml PB in the perfusate. Mean +/- SD. Different letters in pairwise comparisons are significantly different ($p < 0.05$) from non-PB control.

**Figure Seven:** Concentrations of IL-8 in culture media after exposure to mixtures of permethrin, DEET and PB in NHEK. Mean +/- SD. Different letters in pairwise comparisons are significantly different ($p < 0.05$) from non-PB control.

**Figure Eight:** Concentrations of IL-8 versus time in culture media when treatments are pooled and compared with and without 50 ng/ml PB in the media. Mean +/- SD. Different letters in pairwise comparisons are significantly different ($p < 0.05$) from non-PB control.

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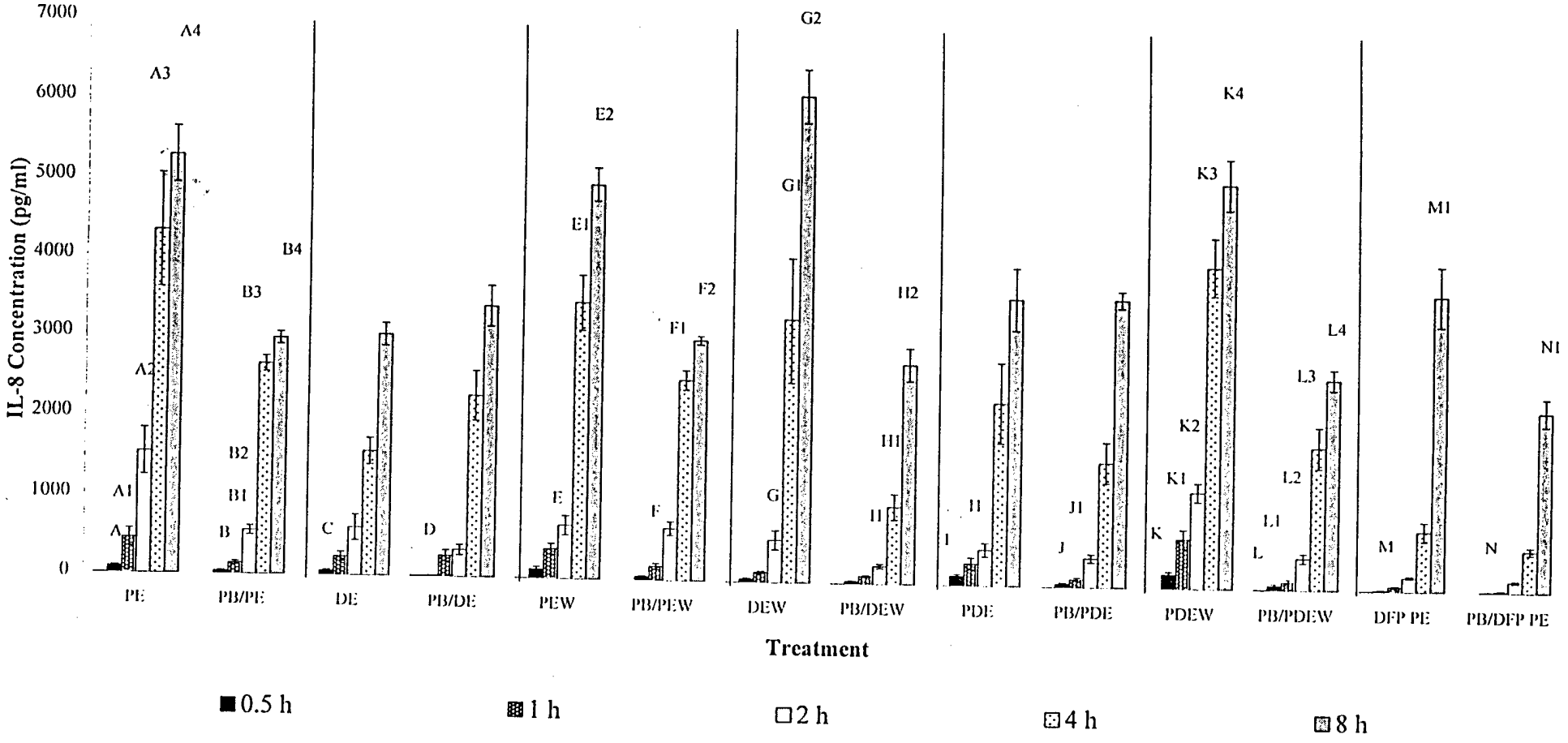
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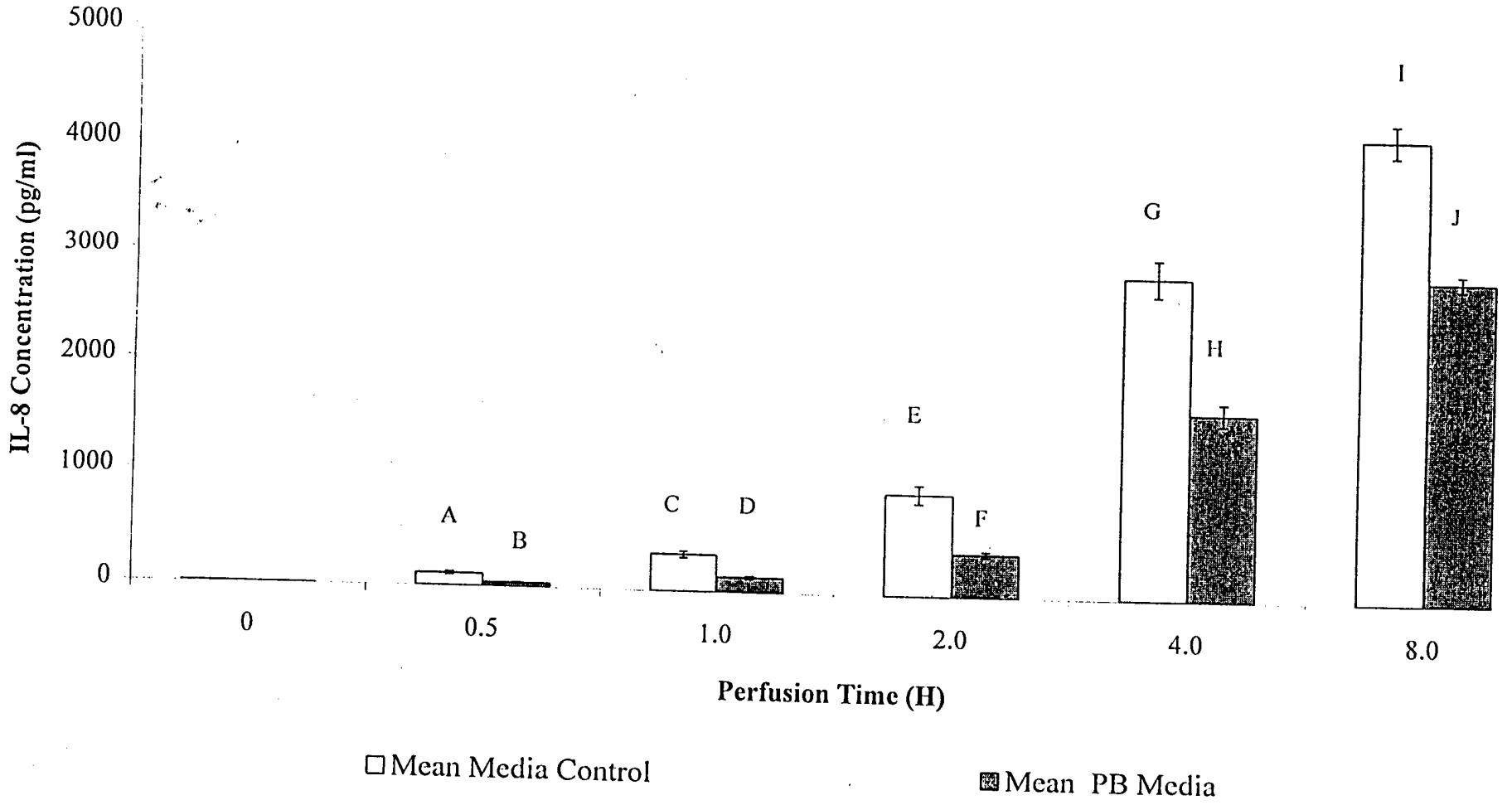
6000
5000
4000
3000
2000
1000
0
A
A1
A2
A3
A4
B
B1
B2
B3
B4
C
D
E
E1
E2
F
F1
F2
G
G1
G2
H
H1
H2
I
J
J1
K
K1
K2
K3
K4
L
L1
L2
L3
L4
M
M1
N
N1
PE
PB/PE
DE
PB/DE
PEW
PB/PEW
DEW
PB/DEW
PDE
PB/PDE
PDEW
PB/PDEW
DFP PE
PB/DFP PE
Treatment
0.5 h
1 h
2 h
4 h
8 h

Figure 1


5000
4000
3000
2000
1000
0
IL-8 Concentration (pg/ml)
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B
C
D
E
F
G
H
I
J
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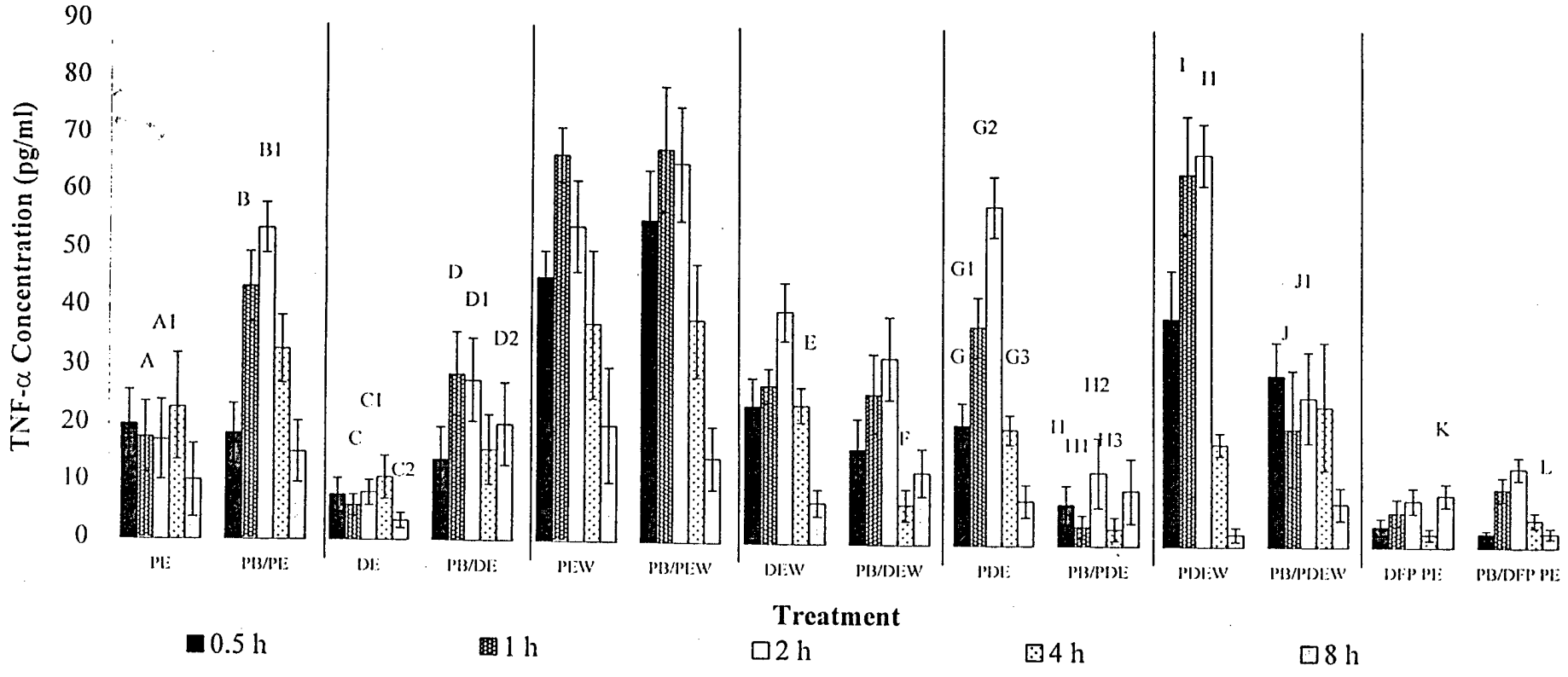
2.0
4.0
8.0
Perfusion Time (H)
Mean Media Control
Mean PB Media

Figure 2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40
30
20
10
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B
B1
C
C1
C2
D
D1
D2
E
F
G
G1
G2
G3
H
H1
H2
H3
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L
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DE
PB/DE
PEW
PB/PEW
DEW
PB/DEW
PDE
PB/PDE
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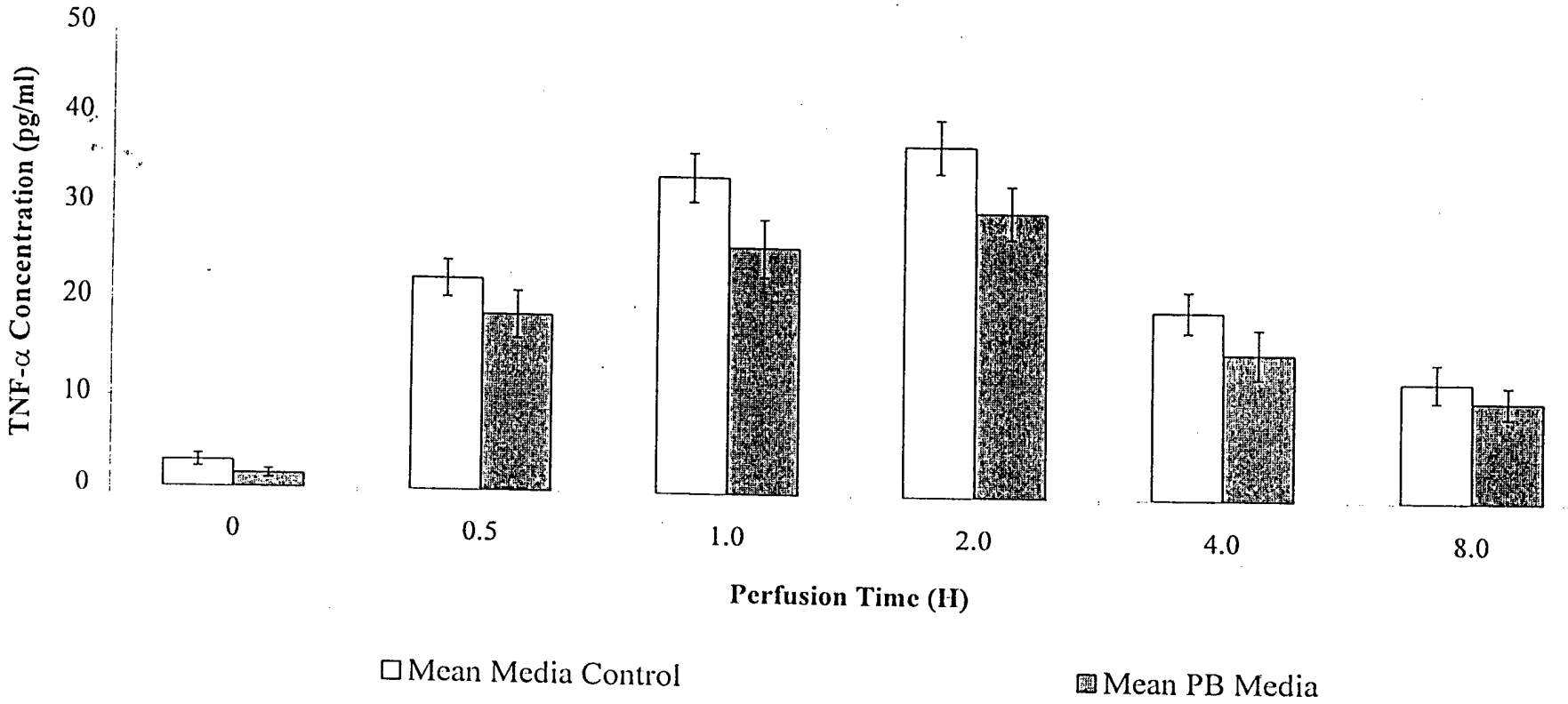
DFP PE
PB/DFP PE
Treatment
0.5 h
1 h
2 h
4 h
8 h

Figure 3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30
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10
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TNF-α Concentration (pg/ml)
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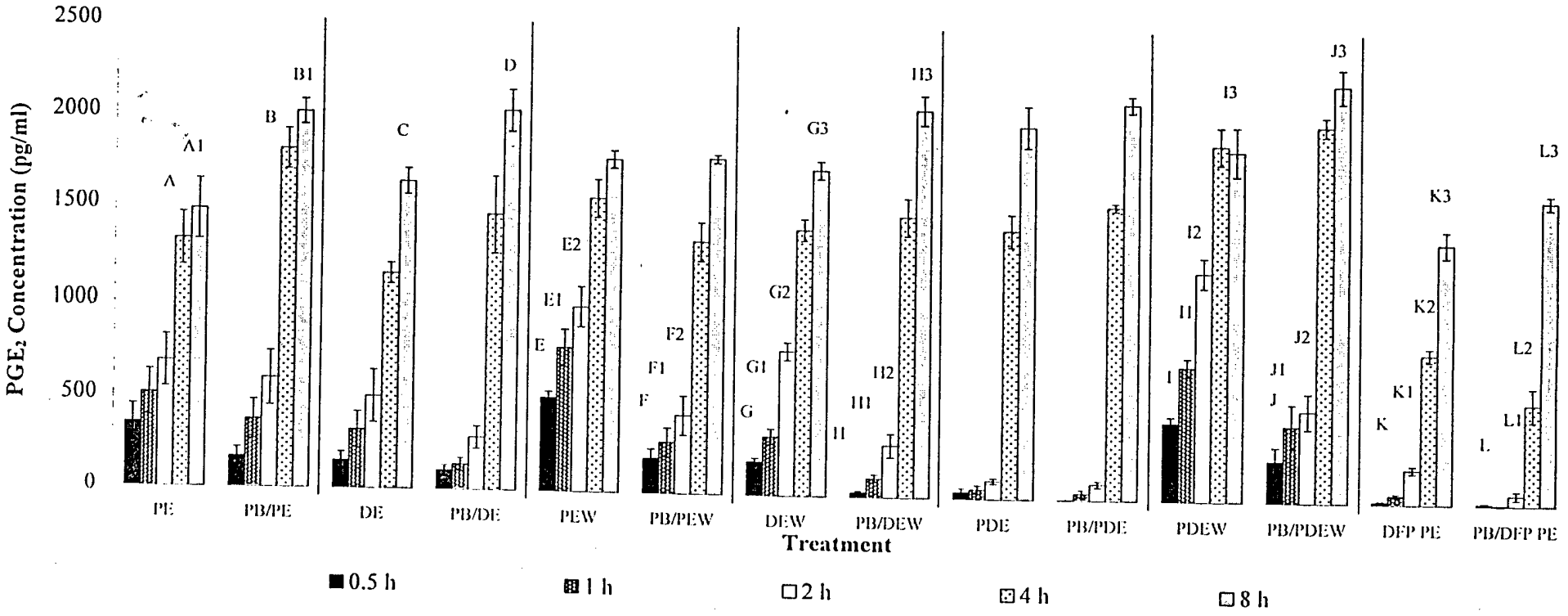
Perfusion Time (H)
Mean Media Control
Mean PB Media

Figure 4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B
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C
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F
F1
F2
G
G1
G2
G3
H
H1
H2
H3
I
I1
I2
I3
J
J1
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L3
PE
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DE
PB/DE
PEW
PB/PEW
DEW
PB/DEW
PDE
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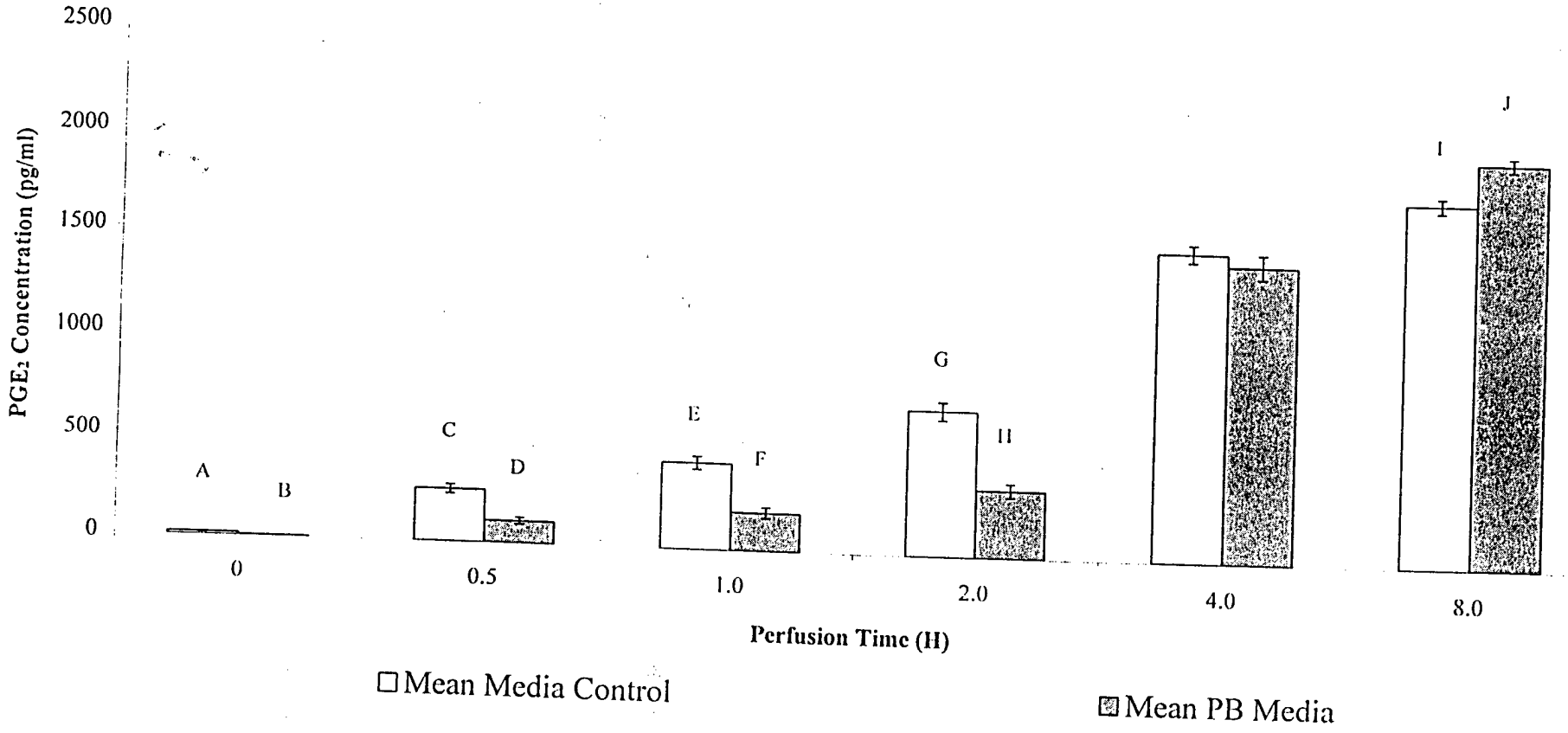
PDEW
PB/PDEW
DFP PE
PB/DFP PE
Treatment
0.5 h
1 h
2 h
4 h
8 h

Figure 5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PGE2 Concentration (pg/ml)
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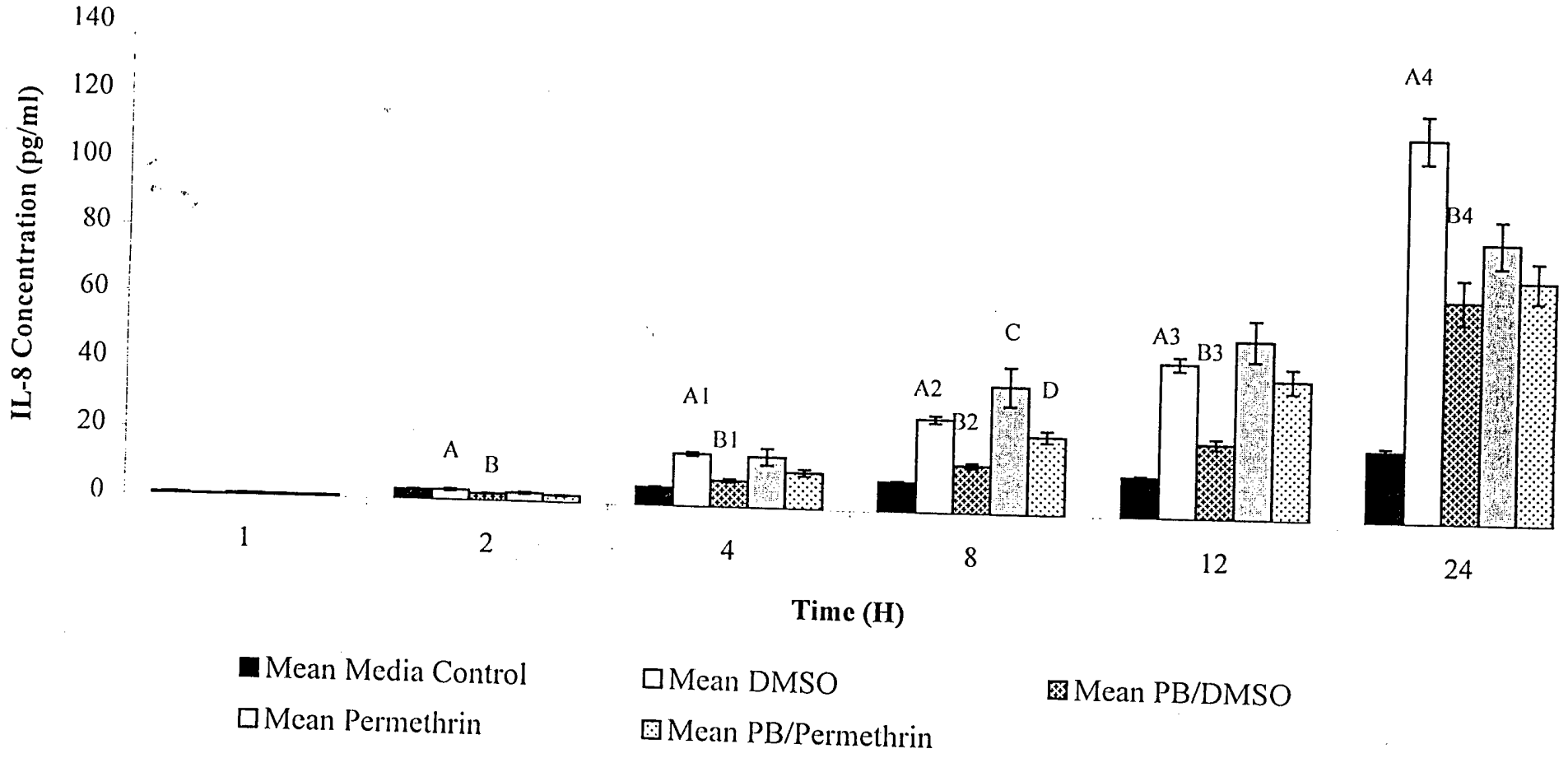
4.0
8.0
Perfusion Time (H)
Mean Media Control
Mean PB Media

Figure 6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B1
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A3
B3
A4
B4
1
2
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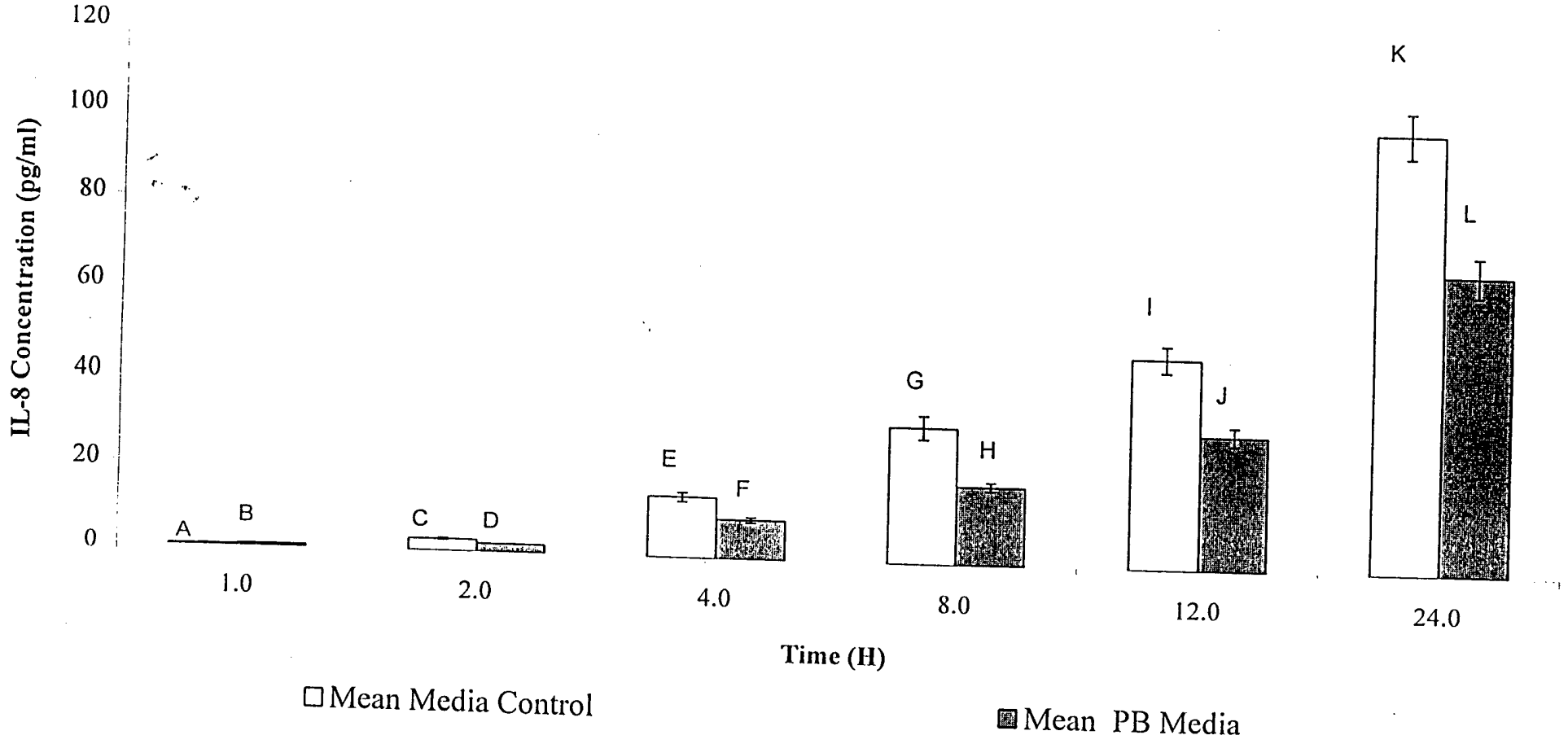
8
12
24
Time (H)
Mean Media Control
Mean Permethrin
Mean DMSO
Mean PB/Permethrin
Mean PB/DMSO

Figure 7


120
100
80
60
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IL-8 Concentration (pg/ml)
A
B
C
D
E
F
G
H
I
J
K
L
1.0
2.0
4.0
8.0
12.0
24.0
Time (H)
Mean Media Control
Mean PB Media

Figure 8

# PERCUTANEOUS ABSORPTION OF TOPICAL *N,N*-DIETHYL-*m*-TOLUAMIDE (DEET): EFFECTS OF EXPOSURE VARIABLES AND CO-ADMINISTERED TOXICANTS

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Abbreviated Title: Percutaneous Absorption of DEET

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## ABSTRACT

Exposure to *N,N*-diethyl-*m*-toluamide (DEET) commonly occurs in the general population and has been implicated as a contributory factor to the Gulf War Illness. The focus of the present studies was to determine the effect of co-exposure factors, potentially encountered in a military environment, which could modulate transdermal flux of topically applied DEET. Factors investigated were vehicle, dose, co-exposure to permethrin, low-level sulfur mustard, occlusion, and simultaneous systemic exposure to pyridostigmine bromide and the nerve agent simulant diisopropylfluorophosphate (DFP). Studies were conducted using the isolated perfused porcine skin flap (IPPSF), with a few mechanistically oriented studies conducted using in vitro porcine skin and silastic membrane diffusion cells. DEET was quantitated using high performance liquid chromatography. The vehicle-control transdermal DEET flux in the IPPSF was approximately 2 $\mu g/cm^2/hr$ for both 7.5 and 75% DEET concentrations, a value similar to that reported in humans. DEET absorption was enhanced by co-infusion of pyridostigmine bromide and DFP, by the presence of sulfur mustard, or by dosing under complete occlusion. The greatest increase in baseline flux was five-fold. In vitro diffusion cell studies indicated that silastic membranes had two orders of magnitude greater permeability than porcine skin, and showed vehicle effects on flux which were not detected in the IPPSF. These results suggest that co-exposure to a number of chemicals which potentially could be encountered in a military environment may modulate the percutaneous absorption of topically applied DEET beyond that seen for normal vehicles at typically applied concentrations.

**Key Words:** DEET, Gulf War, pyridostigmine bromide, skin, absorption

## INTRODUCTION

The focus of research into the pathogenesis of the Gulf War Illness has focused on chemical and non-chemical factors that could result in the observed syndrome. Potential factors include repeated vaccinations, oil-well fires, sand, depleted uranium, stress and exposure to chemicals; the primary candidates being the nerve-agent sarin, the nerve agent prophylactic drug pyridostigmine bromide, the insecticide permethrin, and the insect repellent *N,N*-diethyl-*m*-toluamide (DEET) (Fulco et al., 2000; Jagannathan et al., 2000; Wessely, 2001). A great deal of research has focused on the potential interactions of pyridostigmine bromide, permethrin and DEET which could result in symptoms consistent with the Gulf War Illness (Abou-Donia et al., 1996; McCain et al., 1997; Hoy et al., 2000).

In order for these chemicals to exert a toxicologic effect, they must be present in the systemic circulation. Pyridostigmine bromide is formulated as an oral drug (Mestinol®) and achieves effective concentrations in blood (Marino et al., 1998). However, permethrin and DEET must be absorbed across the skin after topical exposure to exert a toxicologic effect. Research efforts in this context have focused on the dermal absorption of permethrin, since this compound is minimally absorbed after topical exposure and would have to show significantly enhanced absorption to play a role in the pathogenesis of the Gulf War Illness (Baynes et al., 1997; NRC, 1994). Our previous studies have demonstrated that systemic pyridostigmine may enhance the absorption of $^{14}$C-permethrin activity after topical exposure (Baynes et al., 2002). However, very little effort has been spent on addressing the dermal absorption of DEET and how co-administered agents could modulate its absorption.

DEET is rapidly and efficiently ( $\leq$ 25% of applied dose) absorbed across the skin after topical application (Baynes et al., 1997; Ross and Shah, 2000; Selim at al., 1995, Moody et al., 1995; Reifenrath and Robinson, 1982), thus allowing it to potentially be a toxicologic factor in the Gulf War Illness. It is widely used in over-the-counter civilian preparations and is generally considered to possess a relatively moderate toxicity index if used appropriately, although acute massive overdoses can occur (Osimitz and Grothaus, 1995; Qiu et al., 1998; Young and Evans, 1998). What remains unclear is how other exposure variables and simultaneously administered drugs and chemicals affect percutaneous absorption and dermal penetration of DEET. These include co-exposure to permethrin, pyridostigmine bromide, low-level chemical warfare agents, as well as a number of other formulation variables that could modulate DEET absorption. These factors are important to investigate since they could significantly modulate exposure and facilitate systemic toxicosis.

The focus of the present manuscript is too assess the effect of these exposure factors on the percutaneous absorption of topically applied DEET in two accepted experimental models of skin absorption; in vitro flow-through diffusion cells (Bronaugh at el., 1999) and the isolated perfused porcine skin flap (IPPSF) (Wester et al., 1998; Riviere et al., 1992). Porcine skin is widely accepted to be an appropriate animal model for assessing chemical absorption in humans (Monteiro-Riviere, 1991; Wester and Maibach, 1993). Use of both avascular (diffusion cells) and vascular (IPPSF) models allows for obtaining mechanistic insight into the nature of the observed interactions. Secondly, the IPPSF allows simultaneous systemic exposure to a drug or toxicant to be simulated by infusing the compound into the arterial cannula. Finally, diffusion cell

studies were also conducted using an inert silastic membrane to directly assess the vehicle effects on DEET absorption. The exposure factors investigated in IPPSFs in the present study include topical permethrin, infused pyridostigmine bromide, infused nerve agent simulant diisopropylfluorophosphate (DFP), topical vesicant agent sulfur mustard (HD), fabric occlusion, as well as altering concentrations of vehicle components (ethanol / water) and concentration of DEET (7.5 / 75%). These studies will clearly illustrate the complexity of these interactions that can occur when topical exposure to an individual chemical such as DEET is confounded with simultaneous exposure to topical and systemic agents.

## MATERIAL AND METHODS

**Chemicals and Doses**: *N,N*-Diethyl-*m*-toluamide (DEET) (98% pure) was purchased from Chem Service Company, West Chester, PA. DEET was generally applied at a surface concentration of 75% to mimic that used by Gulf War veterans. One exposure was at 7.5% to assess low dose effects. Permethrin was obtained from Sigma Chemical (St. Louis. MO) and used at a surface concentration of 40 $\mu g/cm^2$ in all studies. Pyridostigmine bromide (Mestinol®) was obtained from ICN Biomedicals Inc., Costa Mesa, CA and was added to the IPPSF perfusion media at a concentration (50 ng/ml) to simulate the highest pyridostigmine concentrations seen in soldiers taking this drug as a prophylactic treatment for nerve agent exposure (Marion et al., 1998). Diisopropylfluorophosphate (DFP), employed as a chemical warfare nerve agent simulant in these studies, was obtained from Sigma Chemical (St. Louis, MO) and was added to the perfusion media (30 ng/ml) to mimic previously modeled DFP concentrations in the IPPSF (Carver et al., 1989). Stock surety sulfur mustard in ethanol was obtained from the U.S. Army Medical Research and Development Command, and was dosed at a surface concentration of 40 $\mu g/cm^2$, an amount previously determined to be a subvesicating dose in the IPPSF (Monteiro-Riviere and Inman, 1995, 1997). A sub-vesicating dose was selected since such exposure would go undetected and thus could potentially modify DEET absorption. In contrast, a blister producing dose of sulfur mustard would be detected and appropriate medical attention secured, removing this factor as a potential modulator of DEET absorption. Vehicles consisted of ethanol or ethanol/water (3:2 fixed v/v ratio), the latter to assess effects of hydration conditions as would be seen with perspiration. All water was purified with an ultra high purity water filtration system

(Dracor Water Systems, Durham, NC). HPLC grade acetonitrile, ammonium acetate, and reagent grade glacial acetic acid was purchased from Fisher Scientific (Fair Lawn, NJ). All other chemicals used in these experiments were HPLC grade and obtained from Sigma Chemicals or Fisher Scientific.

**Isolated Perfused Porcine Skin Flaps**: IPPSFs were prepared and perfused as described in detail elsewhere (Riviere et al., 1986; Monteiro-Riviere, 1990; Bowman et al., 1991; Riviere and Monteiro-Riviere, 1991). Skin flaps were perfused in a non-recirculating perfusion chamber maintained at 37°C and 50-60% relative humidity, and perfused at 1 ml/min with an oxygenated (95% $O_2$ / 5% $CO_2$) Krebs-Ringer bicarbonate buffer solution, maintained between pH 7.4-7.5, and spiked with dextrose (0.12%) and bovine serum albumin (4.5%). In some treatments, either pyridostigmine bromide and/or DFP were included in the perfusate to simulate systemic exposure conditions. After one hour of equillibration, a flexible 1 cm x 5 cm dosing template (Stomahesive®, ConvaTec-Squibb, Princeton, NJ) was affixed to the skin flap surface with Skin Bond® (Pfizer Hospital Products, Largo, FL). Test solutions (100 µl), containing chemicals at the doses stated above, were applied to the 5 $cm^2$ dosing sites according to the experimental design protocols (n=4 replicates) in Table 1. In two groups (n=4 each), 100% cotton fabric or cellophane tape was applied over the dose site using a double Stomahesive® patch to simulate conditions of a uniform versus complete occlusion. Glucose utilization and vascular resistance were monitored hourly to insure viable skin flap preparations. Perfusate samples (3 ml) were collected at 0, 10, 20, 30, 45, 60, 75, and 90 minutes, and every ½ hour until termination at 8 hours, and then analyzed for absorbed DEET

concentrations as described below. All non-sampled venous perfusate effluent was collected and sampled for DEET analysis.

**Flow-Through Diffusion Cell Experiments:** Flow-through diffusion cells, as originally described by Bronaugh and Stewart (1985), were used in these studies. Perfusate (4 ml/hr) and perfusion conditions (temperature, pH) were similar to that described above for IPPSFs. Inert 250 μm silastic (polydimethylsiloxane) membranes (Dow Corning, Midland, MI) or dermatomed (500 μm) porcine skin, obtained from the dorsal area of female weanling Yorkshire-cross pigs and punched biopsied to obtain a dosing area of 0.64 $cm^2$, were used in these experiments. All animal protocols were approved by the North Carolina State University Institutional Animal Care and Use Committee. Dosing solutions of 20 μl containing DEET, ethanol, water and/or permethrin were dosed according to the treatments (n = 5 replicates) listed in Table 1. Perfusate samples were collected using the same times as for the IPPSFs above, and assayed for DEET as described below.

**Chemical Analysis:** A modified solid phase extraction method (Qiu and Jun, 1996) was used to extract DEET from the sample media. Solid phase extraction discs (Ansys SPEC PLUS 3ml C18 15mg, Lake Forest, CA., USA) were preconditioned with 500 μl of acetonitrile followed by 500 μl of water. The discs were not allowed to dry out during the preconditioning or sample loading steps. The 1 ml samples were loaded onto the discs and drained slowly (1 to 2 drops per second) using low pressure or gravity. This was followed by a solvent (10:90 Acetonitrile: pH 4.5 ammonium acetate buffer 0.03M) wash and strong vacuum (15 in. Hg) to dry out the discs before the final elution step. Samples were then eluted with 1 ml of elution solvent (40:60 acetonitrile: pH 4.5 ammonium

acetate buffer 0.03M) and placed directly into HPLC vials for analysis. The Waters HPLC system (Waters, Inc., Milford, MA) was equipped with a 996 PDA detector, 717 plus autosampler, 600 controller, temperature control module, and model 60F solvent pumping system. All data were collected on a Gateway E3110 computer utilizing Waters Millennium 32 version 3.05.01 software. A Waters Symmetry Shield RP18 (3.5-micron, 4.6x150mm) column was used for the separations. The mobile phase consisted of 36% acetonitrile and 64% pH 4.5 ammonium acetate buffer, 0.03M. The column temperature was 35° C, detector wavelength 220 nm, flow rate 1.0 ml/min and injection volume 10µL. Recoveries and blanks were run with every batch of samples. The standard curves, prepared by spiking the same volumes of elution solvent with the same amounts of DEET used for spiking the media, revealed a minimum $R^2$ of 0.999. The average recoveries for sample media spiked with 0.5 to 5.0 ppm DEET ranged from 93 to 117%, with coefficient of variations generally averaging 2 – 13%. The limit of detection of the assay was 0.02 µg/ml and the limit of quantitation was 0.08 µg/ml.

**Calculation and Statistics:** DEET absorption (µg) into perfusate was quantitated by determining the area under the curve (AUC), peak flux, and total mass of DEET absorbed. Total mass absorbed is the summation of the DEET in the perfusate samples and final venous effluent. All parameters are expressed as mean ± standard error. Statistical significance ($p \leq 0.05$) between treatments were determined by analysis of variance (ANOVA) with multiple comparison tests performed using the LSD method (SAS 8.1 for Windows, SAS Institute Inc., Cary, NC).

The in vitro diffusion cell data was further analyzed to determine diffusion parameters. *Extrapolated steady state flux*, $J_{ss}$ (µg/cm$^2$/hr), was determined at steady state

from the slope of the cumulative mass per unit area vs time (hr) curve. Steady state was determined as that time interval in the DEET concentration vs. time plots when there is no significant change in DEET concentration with time. *Permeability, P* (cm/hr) was determined from the ratio of individual fluxes to the concentration ($\mu g/cm^3$) of applied dose. *Diffusivity, D* ($cm^2/hr$), was determined by obtaining the lag time before steady-state flux is reached. This lag time ($\tau$) was obtained by extrapolating the steady state portion of the curve back to the time- or x-axis. Lag time is related to diffusivity (D) and membrane thickness (L) by the following equation: $D = L^2/6\tau$, where L = 250 μm for silastic and 500 μm for porcine skin. The partition coefficient between the skin and dosing vehicle ($K_{sv}$), was then determined as: $K_{sv} = P \times [L/D]$

## RESULTS

The results of these exposures are presented in graphical and tabulated forms as Figures One through Seven. The effects are best assessed by examining the tabulated data where the statistical comparisons are presented. The graphics in turn illustrate the effect that additives have on the shape of the DEET flux profiles in both model systems. Statistical comparisons are not shown on the flux profiles. In examining these data, one must take into consideration that the vehicle in which DEET is dosed, ethanol and ethanol/water (aqueous ethanol), also affects disposition making comparisons of additive effects within vehicles necessary.

**Isolated Perfused Porcine Skin Flaps:** The percutaneous absorption parameters of DEET are tabulated and depicted in Figures One through Five. Note that some of the specific treatment combinations are repeated in these tabulations to facilitate making statistical comparisons between treatments. DEET absorption was not significantly different (this cross-figure comparison is not shown in the tabulations) between ethanol (Figure One) and ethanol / water (Figure Two) solutions (absorption: 69 vs. 67 μg, AUC: 77 vs. 83 μg-min/ml; Peak Flux: 0.22 vs. 0.27 μg/min; all $p>0.05$). Addition of permethrin to DEET in ethanol (Figure One) slightly enhanced absorption (not significant) by 180 minutes. Permethrin added to aqueous ethanol solutions (Figure Two) had no effect on DEET absorption.

As seen in Figures One and Two, there were no statistically significant differences in DEET absorption parameters when pyridostigmine, or permethrin and pyridostigmine were present in a 75% DEET / 25% ethanol or 75%DEET/15% ethanol vehicles. However, co-infusion of DFP with the complete chemical exposure scenario

(Figure Two) resulted in statistically significant, almost two-fold, increased DEET absorption compared to all other treatment conditions. Finally, application of a low concentration (7.5%) of DEET resulted in similar levels of DEET absorption.

The final comparisons involve the effect of sulfur mustard (HD) co-exposure. In these sets of experiments, aqueous vehicles could not be employed due to the hydrolysis of HD when water is present. Figures Three compares the effect of HD in permethrin-ethanol-DEET vehicles, with and without simultaneous pyridostigmine infusions. Figure Four adds DFP infusions to this matrix. As can be seen in Figure Three, HD significantly increased DEET absorption when pyridostigmine was not present. Similarly in Figure Four, DFP blocked this HD effect. However, unlike all other DEET perfusate flux profiles previously compared, the full mixture profile (Figure Four with DFP + Pyridostigmine) had a very different shape with a clear peak around 180 mins. A similar but delayed peak was seen in the DFP profile. In summary, HD alone clearly enhances DEET absorption. The presence of DFP creates a flux pattern different than that seen for all other treatment combinations in Figures One through Three.

The effects of occlusion on DEET absorption parameters and flux profiles is presented in Figure Five. As expected, complete occlusion with cellophane statistically increased all parameters of DEET absorption. Fabric occlusion, as would be seen with wearing a uniform, resulted in an intermediate state of enhanced absorption which was not statistically different from the non-occluded treatment. The flux profile clearly shows this intermediate absorption rate.

**In Vitro Diffusion Cell Studies**: Figure Six depicts the porcine skin flow-through diffusion cell data. The primary effect observed in porcine skin diffusion cell studies was

a statistically significant increase in DEET absorption, perfusate AUC and peak flux when DEET was dosed in ethanol vehicles containing water. Figure Six depicts an almost two-fold increase in DEET AUC when dosed in an aqueous ethanol vehicle. In the aqueous vehicle only, permethrin also tended to decrease the absorptive flux up through 150 mins, after which time there was no effect.

Figure Seven depicts DEET absorption parameters from silastic membrane diffusion cell experiments. DEET absorption through silastic was orders of magnitude greater than through skin. Consistent with the porcine skin, DEET absorption was significantly increased in aqueous ethanol vehicles as assessed by absorption, perfusate AUC and peak flux parameters. In contrast, the effect of co-dosed permethrin was variable, showing an increase in ethanol and a decrease in the aqueous vehicle. This is similar to the blunting of the DEET aqueous flux profile in porcine skin diffusion cells seen from 45 to 150 mins (Figure Six). This blunting in ethanol vehicle was not seen in the aqueous IPPSF data in Figure Two. In contrast, the increase in DEET flux with permethrin in ethanol was not seen in porcine skin diffusion cells, but was marginally seen in the IPPSF data in Figure One.

Diffusion parameters of DEET in both porcine skin and silastic diffusion cells are tabulated in Table 2. The noteworthy observations in this analysis are that permeability in skin changed modestly under different solvent conditions, which was a function of altered diffusivity and stratum corneum to vehicle partition coefficients. Secondly, as observed with the raw flux data, permeability and flux through silastic was two orders of magnitude greater than through porcine skin.

**Summary of Results:** In summary, these results indicate that DEET is well absorbed through porcine skin. There are significant differences between model systems, both relative to absolute absorption, as well as relative to the effects of concomitant chemical administration. When one compares total DEET absorption over 8 hrs normalized to surface area, between the IPPSF and the porcine skin diffusion cell model, using a comparable aqueous ethanol vehicle control group, absorption in the porcine skin diffusion cells was approximately 90 μg / $cm^2$, compared to 13 μg / $cm^2$ in the IPPSF. Across all treatment combinations in the IPPSF, DEET absorption into the perfusate was enhanced by co-infusion of pyridostigmine bromide and the organophosphate nerve agent simulant DFP, in the presence of HD, and by dosing under occlusive conditions. Maximum extents of enhanced absorption (treatment/control) under non-occluded conditions, ranged from approximately 1.8 to 2.2 fold. When dose sites were occluded, absorption increased another two-fold. The "worst-case" maximal enhancement scenario seen in this study was a five fold increase when DEET absorption in the {occluded / pyridostigmine / DFP / permethrin / 15% ethanol / 10% water } group (approximately 340 μg / 5 $cm^2$; Figure Five) was compared to its vehicle control (approximately 67 μg / 5 $cm^2$; Figure Two). This would be equivalent, in dosed surface area normalized units, to 68 versus 13 μg / $cm^2$, a five-fold increase.

## DISCUSSION

The focus of these studies was to assess the effects of co-exposure factors on the percutaneous absorption of topically applied DEET. These studies clearly indicate that DEET is well absorbed when topically applied to porcine skin. Depending on the exposure conditions in the IPPSF, 13 to 68 μg / $cm^2$ were absorbed into perfusate over an 8 hr period. This is the amount of DEET that would be considered bioavailable to cause a systemic effect. Selim et al. (1995) has reported on DEET absorption in vivo in humans applying neat DEET, or 15% DEET in ethanol, to a 24 $cm^2$ dosing site. Absorption was determined by quantitative collection of urine and feces over various time periods for up to 128 hrs. Using the Selim urine data through 8 hrs to take into account the IPPSF study duration, and normalizing to unit surface area to correct for the IPPSF 5 $cm^2$ dosing site compared to the 24 $cm^2$ human site, DEET absorption in humans ranged from 14 to 20 μg / $cm^2$. These values are essentially identical to the approximately 13 – 16 μg / $cm^2$ absorption, from 7.5% and 75% DEET vehicle controls, seen in the IPPSF experiment. This concurrence supports previous work with other drugs and chemicals (Riviere and Monteiro-Riviere, 1991; Riviere et al., 1992, Wester et al., 1998) that the IPPSF data reported here should be predictive of DEET absorption in vivo in humans.

These studies suggest that for the exposure conditions examined, DEET absorptive flux is relatively constant at a rate of approximately 2 μg/$cm^2$/hr, even when 7.5% of 75% concentrations are compared. Also, this is supported by this similar rate seen for DEET absorption in humans when neat or 15% DEET was applied (Selim et al., 1995). These data suggest that transdermal DEET flux is operationally saturated through in vivo skin. Viewed in a pharmacokinetic context, DEET transdermal flux across human

skin and in the IPPSF can be viewed as a zero-order kinetic process with a $k_0$ of 2 $\mu g/cm^2/hr$. Factors which could further increase this absorption would have to do so by altering the inherent permeability of the skin barrier, and increasing $k_0$. In the present experiments, flux was increased significantly when low levels of topical sulfur mustard were present, when systemic exposure to both pyridostigmine bromide and DFP occurred, or when occlusive dosing conditions were present.

The enhanced DEET flux seen with co-exposure to low-level sulfur mustard is not surprising as this highly reactive vesicant would be expected to alter epidermal barrier function based on it mechanism of action in skin (Monteiro-Riviere and Inman, 1995, 1997). Low level sulfur mustard in the IPPSF has previously been shown to alter enzyme activity, detected using histochemistry techniques, in all layers of the epidermis supporting the non-specific biochemical toxicologic effects of this compound (Monteiro-Riviere and Inman, 2000). Sulfur mustard exposure can also result in release of inflammatory mediators from skin. This was detected in IPPSFs after relatively low-level sulfur mustard exposure, which could modulate vascular permeability and enhance percutaneous absorption (Zhang et al., 1995). In a toxicokinetic study of sulfur mustard percutaneous absorption in IPPSFs, absorbed sulfur mustard induced changes in vascular permeability inferred by time-varying mustard fluxes and directly assessed by altered inulin (extracellular space marker) distribution (Riviere et al., 1995). Therefore, changes in either epidermal permeability due to mustard-induced structural or biochemical damage to keratinocytes, or increased vascular uptake due to increases in capillary permeability (direct toxicity or secondary to cytokine release), would be consistent with the increased DEET absorption seen in this study.

In contrast to this enhancing effect of sulfur mustard exposure on DEET, $^{14}$C-permethrin absorption was marginally decreased (Unpublished data) when co-exposed to this toxicant. In the case of $^{14}$C-permethrin, it is probably a metabolite of permethrin that is actually being absorbed across the skin (Baynes et al., 2002). Permethrin absorption is thus limited by both a diffusion and metabolic barrier. Parent compound does not readily penetrate skin. Consistent with the effects of sulfur mustard on enzyme histochemistry discussed above, damage to epidermal enzymes would be expected to result in a decreased transdermal flux of $^{14}$C-permethrin if a metabolite were the primary absorbed moiety. In the case of DEET, HD-induced enhanced permeability through a diffusion barrier would increase transdermal flux.

The enhanced DEET absorption seen with co-infusion of pyridostigmine bromide and DFP is in this case similar to that seen with $^{14}$C-permethrin absorption (Baynes et al., 2002). $^{14}$C-permethrin absorption increased 5.6 fold from control in a scenario where both compounds were co-infused. Unlike DEET, infusion of DFP or pyridostigmine alone slightly increased $^{14}$C-permethrin absorption compared to controls. The mechanism behind this effect is not clear. Pyridostigmine and DFP are both drugs targeted to the cholinergic nervous system, making modulation of the skin's microcirculation a potential mechanism. A second potential target of interaction would be on enzyme metabolism. The significant finding of these studies is that co-infusion of these two compounds also enhances DEET absorption, as was seen for $^{14}$C-permethrin. The interactions of pyridostigmine bromide and DFP on the sulfur mustard-induced DEET effect, seen in Figure Four, would also point to a more complex pharmacologic mechanism whose explanation at this time would be speculative.

The final significant effect seen with DEET was the increase absorption observed after complete occlusion. This may have been secondary to a combined effect of occlusion that increases skin permeability due to hydration, as well as some retention of evaporated DEET. This effect was not as pronounced when fabric occlusion was employed. Occlusion-induced hydration of the epidermal permeability barrier may also be similar to the hydration seen in conditions of high humidity, perspiration or water exposure.

The exposure variables that proved significant in these studies may shed light on the vehicle effects seen in the in vitro diffusion cells that were not present in the IPPSFs. In vitro absorption studies were conducted to probe mechanisms of interactions that might shed light on the absorption changes seen in the IPPSF experiments. Absorption, as well as permeability, through silastic membranes were orders of magnitude above that seen in the skin systems suggesting that this membrane is not an appropriate model for studying DEET in skin. In contrast, percutaneous absorption in the in vitro porcine skin diffusion cells is in the low end of the range seen for DEET absorption reported using in vitro human skin diffusion cells (Ross and Shah, 2000; Stinecipher and Shah, 1997; Moody et al., 1995). Our marginally lower fluxes are consistent with differences in techniques, where the human data was often conducted using fully hydrated static Franz cells with prolonged experimental durations. However, all in vitro results were much higher than those seen in the IPPSF, and by extension, also in humans.

In the in vitro studies, changes in vehicle composition generally altered permeability by changing $K_{sv}$, and diffusivity. This change in $K_{sv}$, secondary to formulation, resulting in a change in permeability is the same effect reported in other in

vitro formulation studies (Ross and Shah, 2000). Aqueous vehicles significantly increase DEET absorption in both porcine skin and silastic diffusion cell experiments. The permeability of DEET in silastic membranes is very large and probably precludes these model membranes from being used to study factors that modulate DEET absorption in vitro.

These vehicle effects did not alter DEET absorption in the IPPSF. It is possible that changes in vehicle to membrane partitioning were decreasing DEET diffusion in the in vitro experiments. These effects are not detectable with the lower absorption fluxes seen in the more biologically-complex IPPSF model. That is, in the IPPSF and most probably in humans based on the similar fluxes seen with in vivo studies with DEET vehicles, the rate-limiting step for absorption is the intact skin. Permeability through this intact barrier modulates overall absorptive flux. Factors which further increase DEET availability on the surface of skin may not translate into increased transdermal flux, much as increasing the applied concentration from 7.5% to 75% does not result in increased flux.

From the perspective of pharmacokinetics, DEET diffusion in the in vitro models is usually governed by Fick's law of diffusion which is a first-order kinetic process (Riviere, 1999). Factors which alter partitioning may modulate permeability, a first order rate constant, which when multiplied by available dose yields the observed transdermal flux ($\mu g/cm^2/hr$). In contrast, it appears that in the IPPSF and in vivo, transdermal flux is now fixed ($k_0 = 2\ \mu g/cm^2/hr$) and insensitive to vehicle modulation. However, conditions where $k_0$ are increased, such as with sulfur mustard or occlusion, result in an increase in transdermal flux to levels approaching 10 $\mu g/cm^2/hr$.

This phenomenon is often encountered in formulation of dermatologic drugs where the inherent skin permeability is too low for such manipulations to actually increase transdermal flux. Instead, topical activity is increased in the absence of an increase in systemic absorption. The only effects that resulted in enhanced absorption in the IPPSF were those that changed permeability, effectively increasing absorption above the plateau seen with all other DEET exposure conditions. In the case of in vitro studies, this "base flux" is large enough to allow first order kinetics to be operative, and thus altered transdermal flux due to changing formulations.

What are the practical applications of these findings? First one must assume that the "base level" of DEET flux seen under normal application conditions in the IPPSF and in vivo human studies, of 14-20 μg / $cm^2$ / 8hrs, or approximately 1-3 μg / $cm^2$ /hr, does not result in significant systemic toxicity. Adverse reactions to DEET are rare considering the extremely large numbers of people in the general population whom routinely apply this as a insect repellent in the summer. Signs of acute toxicity, when it does occur, include tremor, restlessness, slurred speech, seizures, impaired cognitive functions, and coma (Young and Evans, 1998). Cases of toxicosis are related to extensive and repeated topical applications, or ingestion with suicidal intent. The focus of the present study was not to relate DEET absorption to acute toxicosis, but rather to assess factors that might modulate percutaneous absorption, and thus affect DEET's role in the Gulf War Illness. Are there exposure scenarios that would result in higher levels of DEET systemic exposure than anticipated under normal conditions?

Our data suggests that co-exposure to low level mustard, systemic pyridostigmine or the organophosphate DFP, and occlusion may increase base-level DEET absorption up

to five-fold over normal levels. Systemic exposure is a function of applied surface area, as well as integrity of the skin barrier, and not the concentration of DEET applied. In contrast, efficacy against mosquitoes is related to applied surface concentration as evaporative loss is directly related to surface concentration. Taking our worse case scenario, these modulating factors would be equivalent, in terms of systemic DEET exposure, to increasing the applied surface area by a factor of five. It must be stressed that all of the conditions selected for study in the present report were not those that were actually encountered in the Gulf War, except for pyridostigmine bromide, permethrin and fabric (uniform) occlusion. Instead, they included examples of toxic chemicals that might be encountered in a military environment.

The important finding is that vehicle and concentration seemed to have minimal effects on absorption, while exposure to systemic drugs or a topical toxicant such as sub-vesicating doses of sulfur mustard, did increase absorption, thereby accentuating any role that DEET might play in a syndrome such as the Gulf War Illness. The most obvious finding, and one that is easily addressable in an occupational environment, was that covering DEET dose areas with an occlusive dressing or even fabric, enhances absorption. Care should be taken to avoid this scenario after DEET has been applied.

The relevance of these findings to Gulf War Illness is not known except for confirming that significant amounts of DEET may be absorbed across the skin. From a scientific perspective, co-administration of a cutaneous toxicant may modulate DEET absorption. Significantly, systemic exposure to certain chemicals, such as pyridostigmine and DFP, can modulate the percutaneous absorption of an unrelated chemical. This scenario is not usually assumed to be relevant to dermal risk assessment, and further

supports the contention that risk assessment of chemical mixtures cannot be based solely on individual chemical studies.

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## FIGURE CAPTIONS

**Figure One.** Mean (SEM) DEET IPPSF perfusate absorption flux (μg/min) profiles and absorption parameters for nonaqueous mixtures.

**Figure Two.** Mean (SEM) DEET IPPSF perfusate absorption flux (μg/min) profiles and absorption parameters for aqueous mixtures.

**Figure Three.** Mean (SEM) DEET IPPSF perfusate absorption flux (μg/min) profiles and absorption parameters for sulfur mustard (HD) versus no HD mixtures.

**Figure Four.** Mean (SEM) DEET IPPSF perfusate absorption flux (μg/min) profiles and absorption parameters for nonaqueous sulfur mustard (HD) mixtures.

**Figure Five.** Mean (SEM) DEET IPPSF perfusate absorption flux (μg/min) profiles and absorption parameters for aqueous mixtures with complete cellophane tapeor fabric occlusion versus control.

**Figure Six.** Mean (SEM) DEET porcine skin flow-through diffusion cell perfusate absorption flux (μg/min) profiles and absorption parameters in aqueous and nonaqueous mixtures.

**Figure Seven.** Mean (SEM) DEET silastic flow-through diffusion cell perfusate absorption flux (μg/min) profiles and absorption parameters in aqueous and nonaqueous mixtures.

**Table 1. Topical DEET Dosing Protocols**

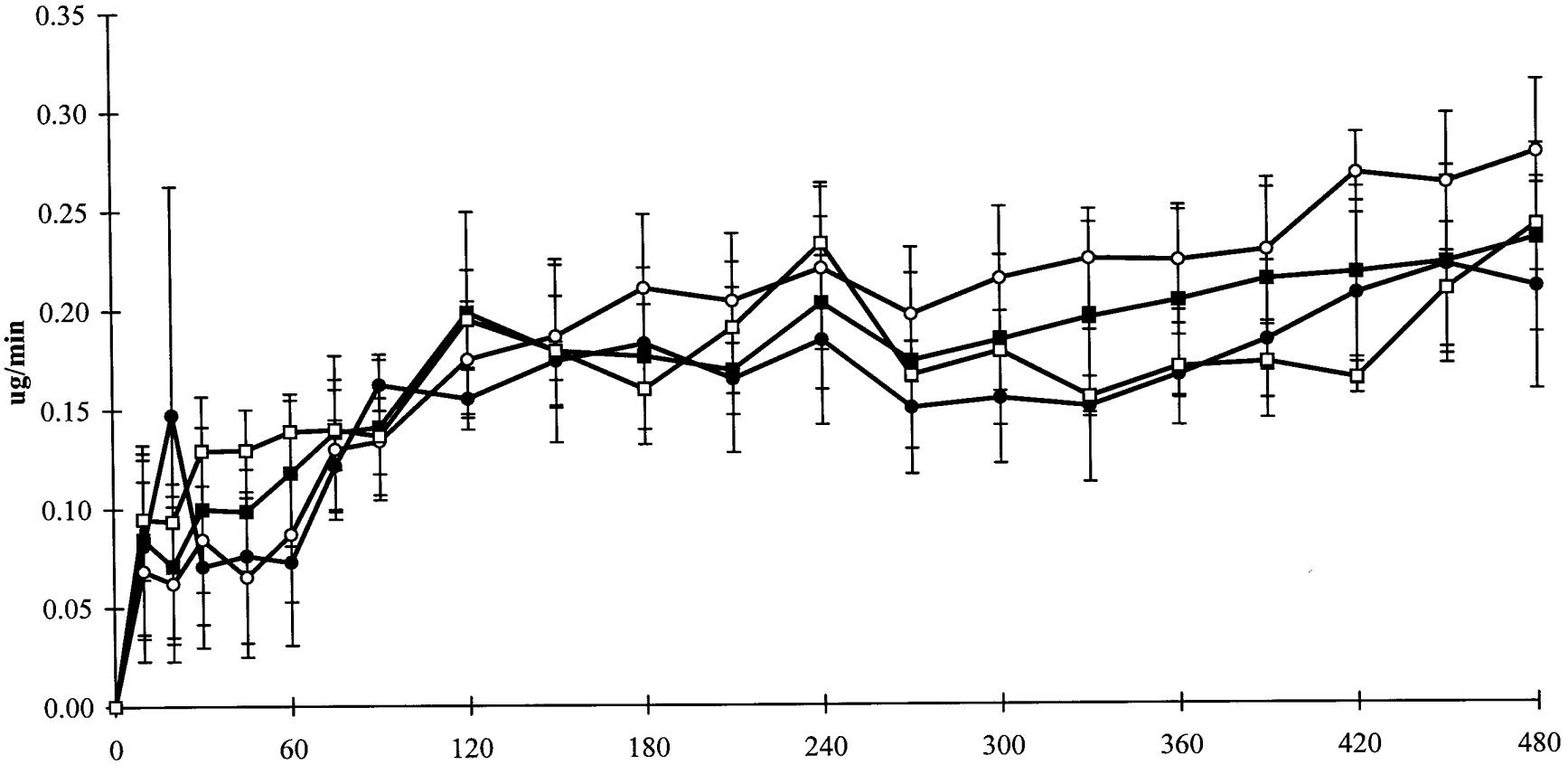
| | Isolated Perfused Porcine Skin Flaps (n=4 / Treatment) |
|---|---|
| 1 | 75%DEET:25%EtOH |
| 2 | 75%DEET:25%EtOH:Permethrin |
| 3 | 75%DEET:25%EtOH:Pyridostigmine-IA |
| 4 | 75%DEET:25%EtOH:Permethrin:Pyridostigmine-IA |
| 5 | 75%DEET:15%EtOH:10%Water |
| 6 | 75%DEET:15%EtOH:10%Water:Permethrin |
| 7 | 75%DEET:15%EtOH:10%Water:Pyridostigmine-IA |
| 8 | 75%DEET:15%EtOH:10%Water:Permethrin:Pyridostigmine-IA |
| 9 | 75%DEET:15%EtOH:10%Water:Permethrin:Pyridostigmine+DFP-IA |
| 10 | 7.5%DEET:55.5%EtOH:37%Water:Permethrin |
| 11 | 75%DEET:25%EtOH:Permethrin:HD |
| 12 | 75%DEET:25%EtOH:Permethrin:HD:Pyridostigmine-IA |
| 13 | 75%DEET:25%EtOH:Permethrin:HD:DFP-IA |
| 14 | 75%DEET:25%EtOH:Permethrin:HD:Pyridostigmine+DFP-IA |
| 15 | 75%DEET:15%EtOH:10%Water:Permethrin:Pyridostigmine+DFP-IA:Cellophane-Occluded |
| 16 | 75%DEET:15%EtOH:10%Water:Permethrin:Pyridostigmine+DFP-IA:Fabric-Occluded |

| | Porcine Skin Flow-Through Diffusion Cells (n=5 / Treatment) |
|---|---|
| 1 | 75%DEET:25%EtOH |
| 2 | 75%DEET:25%EtOH:Permethrin |
| 3 | 75%DEET:15%EtOH:10%Water |
| 4 | 75%DEET:15%EtOH:10%Water:Permethrin |

| | Silastic Membrane Flow-Through Diffusion Cells (n=5 / Treatment) |
|---|---|
| 1 | 75%DEET:25%EtOH |
| 2 | 75%DEET:25%EtOH:Permethrin |
| 3 | 75%DEET:15%EtOH:10%Water |
| 4 | 75%DEET:15%EtOH:10%Water:Permethrin |

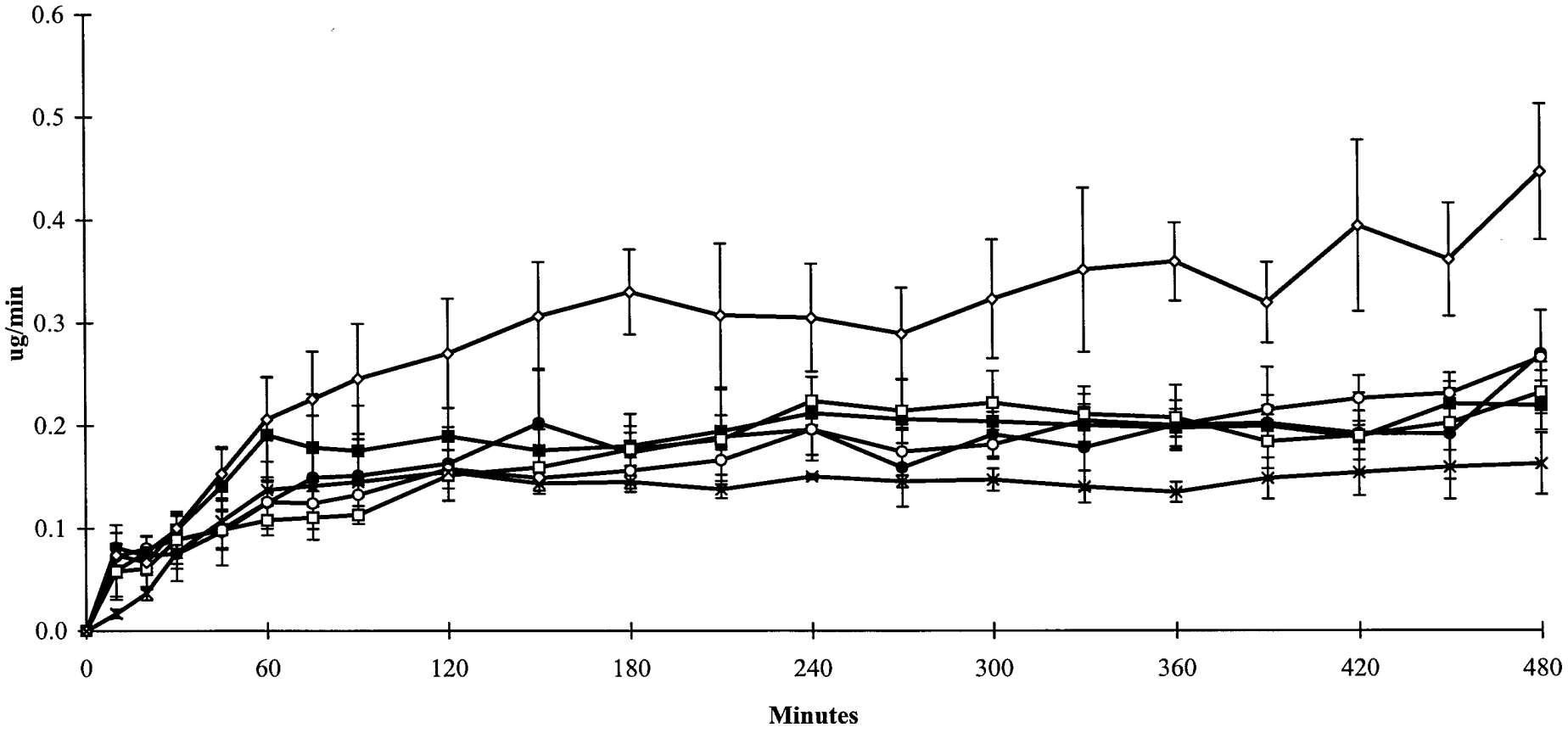
**Table 2. Flux, Permeability, and Diffusivity of DEET Following Topical Doses of 75% DEET in Porcine and Silastic Flow Through Diffusion Cells.**

| **Pig skin** | Ethanol (n=5) | Permethrin:Ethanol (n=5) | Permethrin:Ethanol Water (n=5) | Ethanol:Water (n=5) |
|---|---|---|---|---|
| Flux ($\mu g/cm^2/hr$) | 10.5 (3.7) | 10.4 (2.8) | 11.2 (7.6) | 18.5 (10.4) |
| Permeability($cm/hr \times 10^{-5}$) | 1.4 (0.5) | 1.4 (0.4) | 1.5 (1.0) | 2.5 (1.4) |
| Diffusivity ($cm^2/hr \times 10^{-5}$) | 24.2 (1.5) | 25.4 (2.2) | 155 (70) | 114 (50) |
| $K_{sv} \times 10^{-3}$ | 2.90 (0.95) | 2.73 (0.72) | 0.49 (0.19) | 1.26 (0.81) |
| **Silastic** | (n=5) | (n=5) | (n=4) | (n=5) |
| Flux ($\mu g/cm^2/hr$) | 1336 (483) | 1836 (291) | 988 (35) | 1757 (60) |
| Permeability($cm/hr \times 10^{-5}$) | 179 (65) | 246 (39) | 132 (5) | 235 (8) |
| Diffusivity ($cm^2/hr \times 10^{-5}$) | 23.2 (9.5) | 53.4 (22.0) | 10.8 (0.7) | 13.9 (3.9) |
| $K_{sv} \times 10^{-3}$ | 197 (35) | 130 (53) | 308 (25) | 450 (122) |



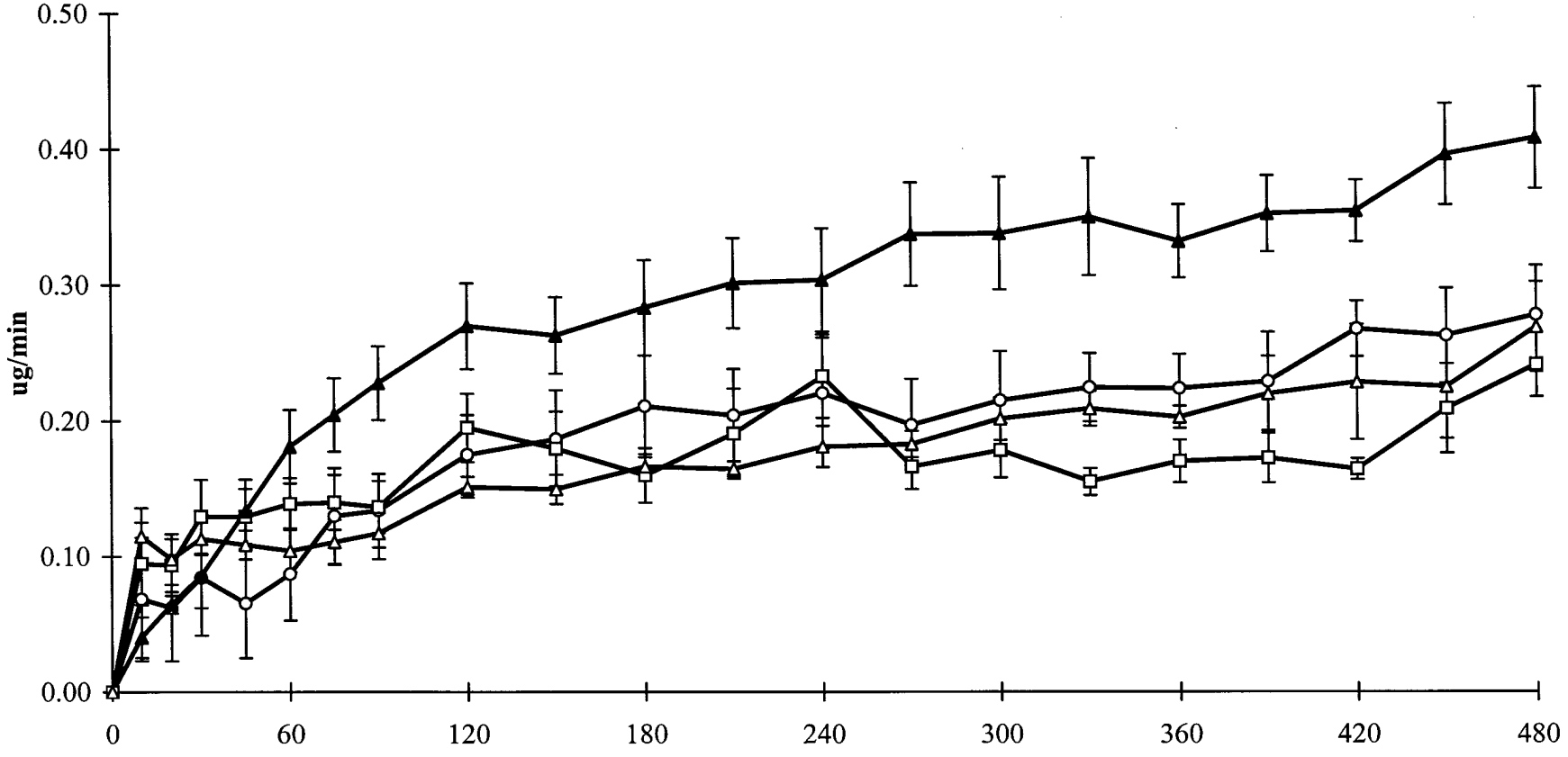

| | 75%DEET 25%EtOH | 75%DEET 25%EtOH Permethrin | Pyridostigmine 75%DEET 25%EtOH | Pyridostigmine 75%DEET 25%EtOH Permethrin |
|---|---|---|---|---|
| Absorption (ug) | 69 (17) [a] | 81 (14) [a] | 75 (20) [a] | 81 (10) [a] |
| AUC (ug-min/ml) | 77 (13) [a] | 92 (13) [a] | 85 (19) [a] | 82 (9) [a] |
| Peak flux (ug/min) | 0.22 (0.12) [a] | 0.27 (0.05) [a] | 0.23 (0.05) [a] | 0.24 (0.03) [a] |

Fig 1




| | 75%DEET 15%EtOH 10%Water | 75%DEET 15%EtOH 10%Water Permethrin | Pyridostigmine 75%DEET 15%EtOH 10%Water | Pyridostigmine 75%DEET 15%EtOH 10%Water Permethrin | Pyridostigmine+DFP 75%DEET 15%EtOH 10%Water Permethrin | 7.5%DEET 15%EtOH 10%Water Permethrin |
|---|---|---|---|---|---|---|
| Absorption (ug) | 67 (10) [b] | 66 (8) [b] | 82 (11) [b] | 81 (6) [b] | 148 (22) [a] | 61 (2) [b] |
| AUC (ug-min/ml) | 83 (16) [b] | 83 (7) [b] | 89 (7) [b] | 83 (7) [b] | 141 (21) [a] | 66 (4) [b] |
| Peak flux (ug/min) | 0.27 (0.06) [b] | 0.27 (0.03) [b] | 0.22 (0.06) [b] | 0.23 (0.03) [b] | 0.45 (0.08) [a] | 0.16 (0.03) [b] |

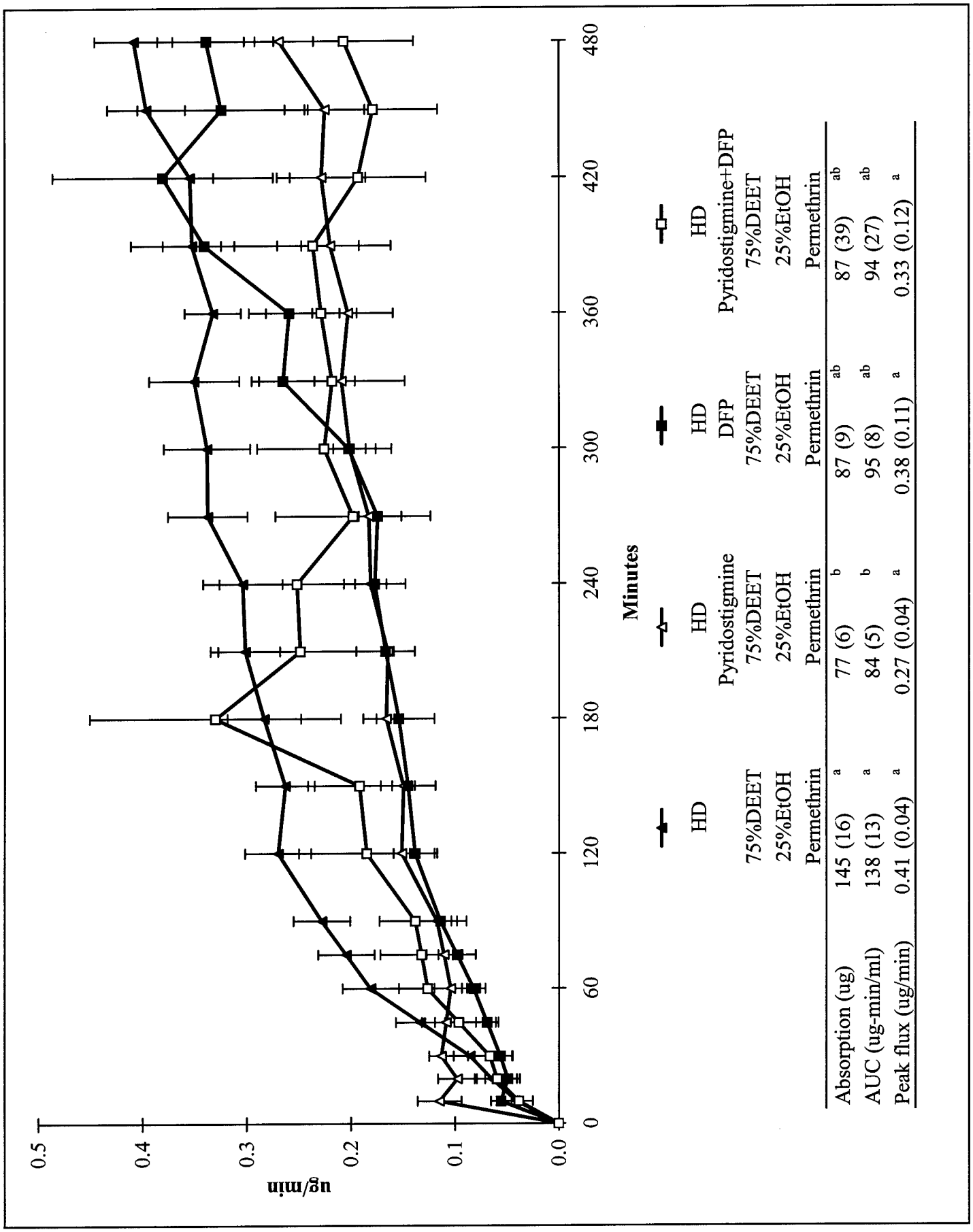
Fig 2



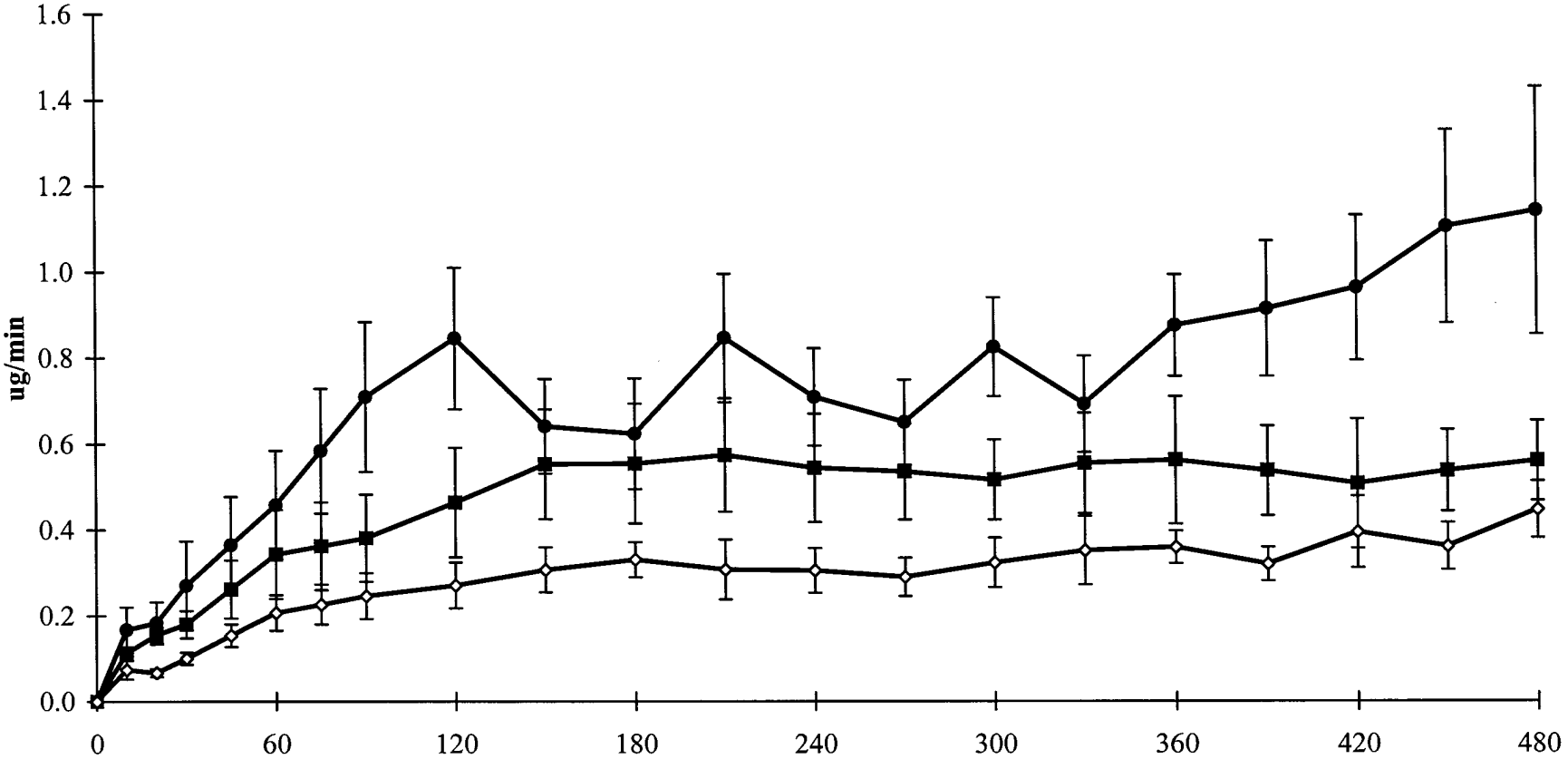

| | 75%DEET 25%EtOH Permethrin | Pyridostigmine 75%DEET 25%EtOH Permethrin | HD 75%DEET 25%EtOH Permethrin | HD Pyridostigmine 75%DEET 25%EtOH Permethrin |
|---|---|---|---|---|
| Absorption (ug) | 81 (14) [b] | 81 (10) [b] | 145 (16) [a] | 77 (6) [b] |
| AUC (ug-min/ml) | 92 (13) [b] | 82 (9) [b] | 138 (13) [a] | 84 (5) [b] |
| Peak flux (ug/min) | 0.27 (0.05) [b] | 0.24 (0.03) [b] | 0.41 (0.04) [a] | 0.27 (0.04) [b] |

Fig 3

Fig 4



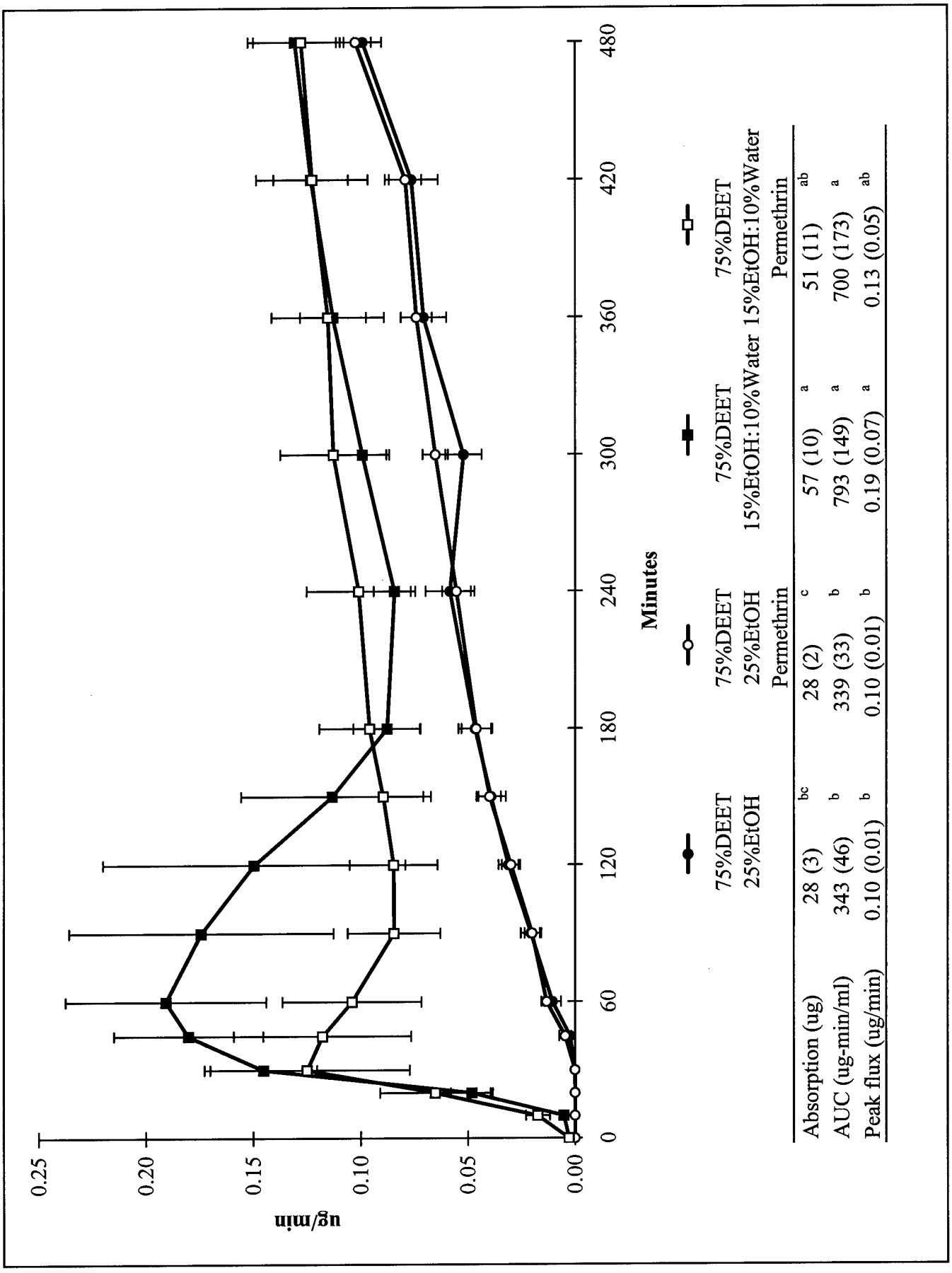

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Absorption (ug) | 145 (16) | a | 77 (6) | b | 87 (9) | ab | 87 (39) | ab |
| AUC (ug-min/ml) | 138 (13) | a | 84 (5) | b | 95 (8) | ab | 94 (27) | ab |
| Peak flux (ug/min) | 0.41 (0.04) | a | 0.27 (0.04) | a | 0.38 (0.11) | a | 0.33 (0.12) | a |



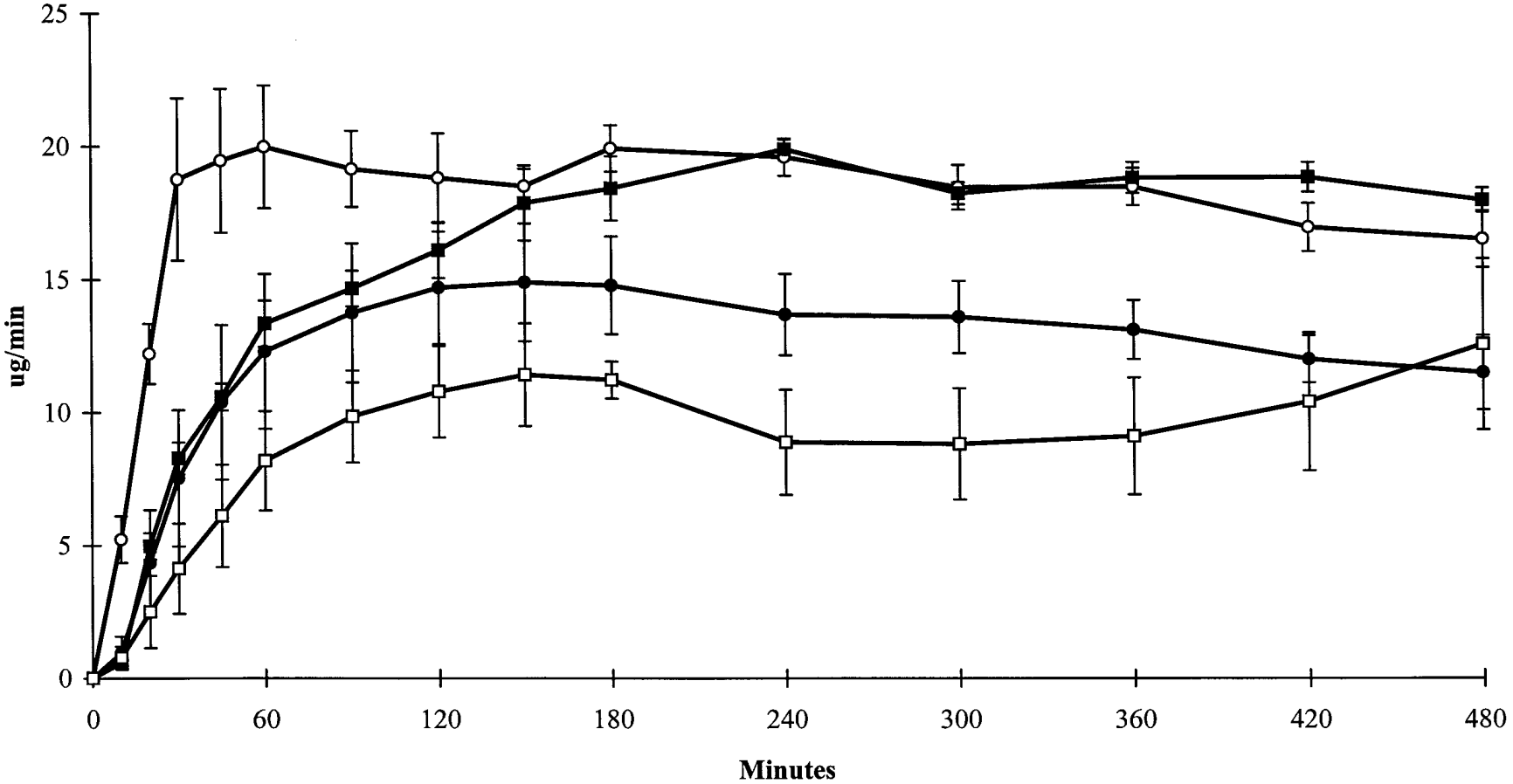

| | Occluded Pyridostigmine+DFP 75%DEET 15%EtOH:10%Water Permethrin | Fabric Occluded Pyridostigmine+DFP 75%DEET 15%EtOH:10%Water Permethrin | Nonoccluded Pyridostigmine+DFP 75%DEET 15%EtOH:10%Water Permethrin |
|---|---|---|---|
| Absorption (ug) | 343 (30) [a] | 256 (70) [ab] | 148 (22) [b] |
| AUC (ug-min/ml) | 352 (26) [a] | 230 (51) [b] | 141 (21) [b] |
| Peak flux (ug/min) | 1.14 (0.29) [a] | 0.57 (0.15) [b] | 0.45 (0.08) [b] |

Fig 5

Fig 6




| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Absorption (ug) | 28 (3) | bc | 28 (2) | c | 57 (10) | a | 51 (11) | ab |
| AUC (ug-min/ml) | 343 (46) | b | 339 (33) | b | 793 (149) | a | 700 (173) | a |
| Peak flux (ug/min) | 0.10 (0.01) | b | 0.10 (0.01) | b | 0.19 (0.07) | a | 0.13 (0.05) | ab |




| | 75%DEET 25%EtOH | | 75%DEET 25%EtOH Permethrin | | 75%DEET 15%EtOH:10%Water | | 75%DEET 15%EtOH:10%Water Permethrin | |
|---|---|---|---|---|---|---|---|---|
| Absorption (ug) | 6,116 (663) | b | 8,749 (248) | a | 8,208 (157) | a | 4,588 (488) | c |
| AUC (ug-min/ml) | 83,741 (9,518) | b | 121,864 (3,730) | a | 114,737 (2,012) | a | 65,209 (3,888) | c |
| Peak flux (ug/min) | 15 (3) | b | 20 (3) | a | 20 (1) | a | 13 (3) | b |

Fig 7

# GULF WAR ILLNESS-RELATED EXPOSURE FACTORS INFLUENCING TOPICAL ABSORPTION OF $^{14}$C-PERMETHRIN

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Abbreviated Title: Topical Absorption of Permethrin

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**ABSTRACT**

Topical exposure to permethrin has often been implicated as a mitigating factor in the Gulf War Illness. These studies were designed to assess the effect of co-exposure to low-level sulfur mustard, JP-8 jet fuel, *N,N*-diethyl-*m*-toluamide (DEET) and occlusion on the percutaneous absorption and skin disposition of topically applied $^{14}$C-permethrin (40 $\mu g/cm^2$) in the isolated perfused porcine skin flap (IPPSF) model. Extent of dermal absorption in vehicle controls in the IPPSF was comparable to literature values for humans. These studies demonstrated an increase in $^{14}$C-permethrin percutaneous absorption and penetration when JP-8 was present, but a decrease in the presence of sulfur mustard. Complete occlusion slightly increased $^{14}$C-permethrin absorption, while occlusion with fabric showed no significant effect. A previously noted effect of DEET to inhibit permethrin absorption was still seen in the presence of sulfur mustard exposure. These studies suggest that co-exposure to JP-8 or sulfur mustard may modulate transdermal flux of $^{14}$C-permethrin. However, the JP-8 increase in absorption and penetration was less than the five-fold increase previously seen with arterial infusion of pyridostigmine bromide and diisopropylfluorphosphate in the IPPSF. The toxicologic significance of this moderate increase in permethrin absorption remains unclear.

**Key Words:** Permethrin, Gulf War, JP-8, Jet Fuel, Sulfur Mustard, skin, absorption

## INTRODUCTION

Topical exposure to drugs and chemicals often results in unwanted chemical absorption across the skin barrier into the systemic circulation. It is axiomatic that percutaneous absorption must occur before systemic effects secondary to direct chemical toxicity is seen (Bronaugh and Maibach, 1999; Roberts and Walters, 1998). The potential role of topical chemicals in the purported Gulf War Illness has been extensively studied and reviewed (Abou-Donia et al., 1996; McCain et al., 1997; Hoy et al., 2000). However, it is still not known if other factors not related to chemical exposure, such as stress, vaccinations, and environment may instead play a dominant role in its pathogenesis; if the syndrome even exists (Fulco et al., 2000; Jagannathan et al., 2000; Wessely, 2001). The chemicals identified as potentially being involved in the Gulf War Illness include the pyrethroid pesticide permethrin, as well as the chemical insect repellent *N,N*-diethyl-*m*-toluamide (DEET). A synergistic toxicologic interaction between topical permethrin and DEET, coupled with systemic exposure to the oral prophylactic nerve agent drug pyridostigmine bromide, has been suggested by some as a causative factor in the Gulf War Illness (Abou-Donia et al., 1996). Previously, our laboratory has investigated these interactions (Baynes et al., 1997, 2002) and demonstrated that systemic pyridostigmine may enhance the absorption of topically applied $^{14}$C-permethrin activity, while DEET tended to retard permethrin penetration. In addition, pyridostigmine bromide inhibited the release of the pro-inflammatory cytokine, interleukin 8, from intact skin and keratinocyte cell cultures which was seen after identical exposure to topical chemical mixtures in the absence of pyridostigmine (Monteiro-Riviere et al., 2002). These studies suggest that simultaneous exposure to these chemicals do modulate the response seen

after a single chemical exposure, which affects the role for these chemicals in a complex syndrome such as the Gulf War Illness.

The exposure "environment" is actually far more complex than simply these two chemicals. Additional chemical exposures and environmental factors that would be expected to directly interact with the skin, and could be potentially encountered in a military theater, may further modulate permethrin absorption. The potential co-exposure to organophosphate nerve agents were previously examined (Baynes et al., 2002) using the simulant diisopropylfluorphosphate (DFP). Topical and/or systemic DFP slightly modulated $^{14}$C-permethrin dermal absorption. Three additional factors were investigated in this manuscript. These included the effect of fabric and occlusion, as well as simultaneous exposure to jet fuel or low levels of sulfur mustard. Covering exposed skin sites with fabric was used to model the effects of a uniform (Snodgrass, 1992; NRC, 1994) on subsequent dermal permethrin absorption. Topical exposure to jet fuels is a potential dermatotoxicologic hazard in itself, and has been shown to result in both dermal deposition and percutaneous absorption of hydrocarbon components (Riviere et al., 1999; Baynes et al., 2000, 2001), as well as result in direct toxicity to the skin (Monteiro-Riviere at al., 2001). Similarly, topical exposure to the low dose vesicating chemical warfare agent sulfur mustard would be expected to modulate percutaneous absorption of co-administered chemicals. Sub-vesicating doses of sulfur mustard were employed in the present experiments to model exposure at a level that would not be easily detectable, since overt mustard-induced vesication was not reported to have occurred in the Gulf War.

These experiments were conducted in the isolated perfused porcine skin flap (IPPSF) model. In all experiments, both penetration of topically applied chemical into the skin, as well as percutaneous absorption across the skin were assessed. Porcine skin is widely accepted to be an appropriate animal model for assessing chemical absorption in humans (Monteiro-Riviere, 1991, Monteiro-Riviere and Riviere, 1996; Wester and Maibach, 1993). The IPPSF allows for chemical-chemical, chemical-skin, as well as chemical-vascular interactions, or the effects of chemical-induced inflammatory mediator release, to be detected without the confounding factors often seen in intact animal studies (Riviere and Monteiro-Riviere, 1991). Previously, the IPPSF has been demonstrated to predict drug and chemical absorption in humans (Riviere and Monteiro-Riviere, 1991; Riviere at al., 1992; Wester et al., 1998). The focus of these studies was thus to assess whether simultaneous exposure to jet fuel, low-level sulfur mustard, or fabric modulated the percutaneous absorption or dermal disposition of topically dosed $^{14}$C-permethrin.

## MATERIAL AND METHODS

**Chemicals and Doses**: Radiolabeled $^{14}$C-permethrin (specific activity = 8.1 mCi/mmol) was obtained from Sigma Chemical (St. Louis. MO) and used at a surface concentration of 40 μg/cm$^2$ in all studies. *N,N*-Diethyl-*m*-toluamide (DEET) (98% pure) was purchased from Chem Service Company (West Chester, PA) and applied in 75% solutions to model that used by Gulf War veterans. Pyridostigmine bromide (Mestinol®) was obtained from ICN Biomedicals Inc., Costa Mesa, CA and was added to the perfusion media at a concentration (50 ng/ml) to simulate the highest pyridostigmine blood concentrations seen in soldiers taking this drug as prophylaxis against nerve agent exposure (Marion et al., 1998). Diisopropylfluorophosphate (DFP), employed as a chemical warfare nerve agent simulant in these studies, was obtained from Sigma Chemical (St. Louis, MO) and was added to perfusate (30 ng/ml) to mimic previously modeled DFP concentrations in the IPPSF (Carver et al., 1989). Stock surety sulfur mustard (HD) in ethanol was obtained from the U.S. Army Medical Research and Development Command, and was dosed at a surface concentration of 40 μg/cm$^2$, an amount previously determined to be below that required to form blisters in the IPPSF (Monteiro-Riviere and Inman, 1995, 1997). A sub-vesicating dose was selected since such an exposure at this level would go undetected and thus could potentially modify permethrin absorption. In contrast, a blister producing exposure to sulfur mustard would be readily detected and decontaminated, removing this factor as a potential modulator of permethrin absorption. JP-8 jet fuel was kindly supplied by Major T. Miller from Wright Patterson Air Force Base. All other chemicals used in these experiments were HPLC grade and obtained from Sigma Chemicals. Vehicles

consisted of ethanol or ethanol/water (3:2 fixed v/v ratio), the latter to assess effects of hydration conditions as would be seen with perspiration.

**Isolated Perfused Porcine Skin Flaps**: IPPSFs were prepared and perfused as described in detail elsewhere (Riviere et al., 1986; Monteiro-Riviere, 1990; Bowman et al., 1991; Riviere and Monteiro, 1991). Skin flaps were perfused in a non-recirculating perfusion chamber maintained at 37°C and 50-60% relative humidity, and perfused at 1 ml/min with an oxygenated (95% $O_2$ / 5% $CO_2$) Krebs-Ringer bicarbonate buffer solution, maintained between pH 7.4-7.5, and spiked with dextrose (0.12%) and bovine serum albumin (4.5%). In some treatments, either pyridostigmine bromide and/or DFP were included in the perfusate to simulate systemic exposure conditions. After one hour of equillibration, a flexible 1 cm x 5 cm dosing template (Stomahesive®, ConvaTec-Squibb, Princeton, NJ) was affixed to the skin flap surface with Skin Bond® (Pfizer Hospital Products, Largo, FL).

Test solutions (100 µl), containing chemicals at the doses stated above, were applied to the 5 $cm^2$ dosing sites according to the experimental design protocols (n=4 replicates) in **Table 1**. These experimental treatment selections were based on previous research (Baynes et al., 2002) that determined vehicle and DFP/pyridostigmine exposure parameters that significantly modulated $^{14}C$-permethrin absorption or skin deposition. The effects of JP-8 were limited to assessing vehicle effects since occupational exposure would most likely be in Air Force personnel not exposed to pyridostigmine or Army mandated insect control programs which included DEET (Young and Evans, 1998). Additionally, formulating exposure solutions that contained significant quantities of both JP-8 and 75% DEET was problematic. In contrast, the exposure to HD would be

expected to be seen in Army personnel, and thus co-exposure to pyridostigmine, DFP and DEET were investigated. Water was not included due to the susceptibility of HD to hydrolysis. The final group selected used a "worse case" co-exposure conditions (DEET, ethanol, water, DFP, pyridostigmine) to assess occlusive effects of cellophane or 100% cotton fabric. A double thickness Stomahesive® patch was used and occlusive material secured to this. Fabric applied over the dose site was used to simulate covering of exposed sites by a uniform.

Glucose utilization and vascular resistance were monitored hourly to insure viable skin flap preparations. Perfusate samples (3 ml) were collected at 0, 10, 20, 30, 45, 60, 75, and 90 minutes, and every ½ hour until termination at 8 hrs. The dose area was swabbed with cotton gauze soaked in an Ivory® detergent solution. Stratum corneum tape strips (12) obtained using cellophane tape (3M Corporation, Minneapolis, MN), skin and fat tissues were collected from the dosing site and surrounding areas. Tissue, stratum corneum tape strips, swabs, and perfusate samples were digested in Soluene® (Packard Chemical Co., Downers Grove, IL), combusted in a Packard Model 387 Tissue Oxidizer, analyzed in a Packard Model 1900TR Liquid Scintillation Counter and the observed DPMs converted to CPMs, and finally to mass of permethrin absorbed, using the specific activity. All permethrin data thus reflects $^{14}$C-permethrin activity in tissue and perfusate.

**Calculation and Statistics:** A full discussion and description of the IPPSF absorption analysis is presented elsewhere (Riviere et al., 1999). Briefly, absorption is defined as the percentage of applied dose detected in the perfusate over the 8-hr experimental period. Penetration is defined as the amount detected in the perfusate (absorption), as well as that detected in all skin and fat tissue, but not stratum corneum. Penetration is the quantity of

drug that could potentially be mobilized for local or systemic effects in experiments of longer duration, as the amount penetrated is required to be taken into consideration when IPPSF data is extrapolated from the 8 hr experiment to six day in vivo human absorption (Wester et al., 1998, Williams et al., 1990). Surface residue was that quantity of label removed by the cotton gauze. Stratum Corneum was the quantity of label recovered in the cellophane tape strips. This is not total stratum corneum, as a significant portion of stratum corneum is actually in the dosed skin that is tabulated under penetration. Absorption was further analyzed by determining the area under the curve (AUC) of the perfusate flux profiles. All parameters are expressed as mean $\pm$ standard error of $^{14}C$-permethrin activity. Statistical significance ($p \leq 0.05$) between treatments were determined by analysis of variance (ANOVA) with multiple comparison tests performed using the LSD method (SAS 8.1 for Windows, SAS Institute Inc., Cary, NC).

## RESULTS

The results of these exposures are presented in graphical and tabulated forms. The effects are best compared by examining the tabulated data where statistical comparisons are presented. The figures in turn illustrate the effect that additives have on the shape of the $^{14}$C-permethrin activity flux profiles. In examining these data, one must take into consideration that the vehicle in which $^{14}$C-permethrin is dosed, ethanol and ethanol/water (aqueous ethanol), also affects deposition making comparisons of JP-8 effects within vehicles necessary.

**Effects of Jet Fuel:** Table 2 and Figure One depict the effects of JP-8 exposure on the absorption and skin deposition of $^{14}$C-permethrin in the two solvent systems. Statistically significant effects of JP-8 on an increase in $^{14}$C-permethrin absorption (AUC) and penetration were seen in the ethanol vehicle, as well as an increase in absorption in the ethanol/water exposure. In contrast, the surface residue of $^{14}$C-permethrin was generally lower in the JP-8 exposures. Peak $^{14}$C-permethrin perfusate fluxes in Figure One are seen at 120 minutes in the 75% JP-8 : 15% ethanol : 10% water treatment group. When all JP-8 versus vehicle treatments are compared; AUC, absorption, penetration and stratum corneum residues are consistently elevated compared to vehicle controls, while surface residues are reduced. In summary, absorption is enhanced almost two-fold, and skin penetration is increased almost three fold when JP-8 exposure occurs.

**Effects of Sulfur Mustard:** Table 3 and Figure Two depict the effects of sulfur mustard on $^{14}$C-permethrin absorption and deposition. Complex vehicle effects are evident in these treatments which preclude easy categorization. There are two levels of comparison that must be made, the first between HD and non-HD treatment combinations

(*statistically compared vertically within columns for each parameter across the two datasets*), and the second across all HD exposures in different vehicle and chemical co-exposures (*statistically compared using lower case letters across rows for each parameter*). When HD exposure is compared to controls, HD decreases several of the absorption and penetration parameters in three of four vehicles (not 100 % ethanol). This is clearly seen in Table 3 by examining the effect of HD in the second column (pyridostigmine / 100% ethanol) where the AUC with HD (0.08 %Dose-min / ml) is three-fold less than its control (0.24 %Dose-min / ml). Surface residues are decreased by HD in two groups and stratum corneum residues are decreased in three groups. When all of these effects are compared, HD tends to decrease absorption and penetration parameters while also decreasing surface residues. The two lowest flux profiles in Figure Two are those with HD co-treatment. Across HD treatments, $^{14}$C-permethrin penetration and stratum corneum deposition was significantly higher when DEET was not present. This can be clearly seen in the top of Table 3 when penetration in the second (0.63% – no DEET) and fourth (0.22% - DEET) columns are compared. Absorption was not as affected.

**Effects of Occlusion and Fabric:** Occlusion effects can be seen in Table 4. Complete occlusion with cellophane statistically increased $^{14}$C-permethrin absorption, while fabric occlusion did not alter deposition. Surface residues also tended to be lower in this cellophane occluded group.

**Summary of Results:** The principle findings from these studies were that an *increase* in $^{14}$C-permethrin percutaneous absorption and skin penetration when JP-8 was present. In this exposure scenario, recovered surface residue was reduced. The primary effect of HD

exposure was to *decrease* $^{14}$C-permethrin absorption and penetration, while decreasing surface residue recovery. Previously, we noted the effect that DEET retarded $^{14}$C-permethrin absorption (Baynes et al., 2002) was still evident. Finally, complete occlusion slightly increased $^{14}$C-permethrin absorption, although not to the extent of co-exposure with JP-8.

## DISCUSSION

There are a number of observations of factors governing dermal absorption of permethrin that can be made based on the above results. These must be viewed in the context of the focus of this research, which is on chemical factors potentially involved in the Gulf War Illness. Specifically, could there be exposure scenarios that significantly increased dermal absorption, and or penetration, of topical permethrin to the extent that systemic exposure to permethrin would be great enough to be involved in a systemic toxicologic effect? All experimental studies on the potential role of permethrin in the Gulf War Illness (Abou-Donia et al., 1996; McCain et al., 1997; Hoy et al., 2000), assume that significant dermal absorption occurs to the extent that clinically significant concentrations of permethrin can be achieved in the systemic circulation. Unfortunately, sufficient controlled dose-response studies do not exist that could precisely define the extent of systemic exposure that is required for modulating the Gulf War Illness. The focus of this research was to probe the possible exposure variables that could enhance permethrin percutaneous absorption to an extent that permethrin could play a role in systemic toxicity.

Permethrin is poorly absorbed in humans (Wester et al., 1994; Franz et al., 1996), with an estimate of only 2% of applied dose absorbed into the systemic circulation per 24 hrs (Bartelt and Hubbel, 1987). This level produces an internal dose well below any acute or subchronic "no-observed-effect-levels" (NRC, 1994). As can be seen from the vehicle control exposures in the present study, percutaneous absorption and penetration of $^{14}C$-permethrin activity is also very low. The amount penetrated in an 8 hr IPPSF experiment has been shown to closely correlate to longer-term in vivo human exposures (Wester et

al., 1999). Importantly, the amount penetrated of 1.5 to 1.6 % over 8 hrs from vehicle controls in the IPPSF is consistent with the known in vivo human absorption of 2% over 24 hrs (Bartelt and Hubbel, 1987). This suggests that the results reported herein are relevant to in vivo human exposure.

Our previous research (Baynes et al., 2002) identified that co-exposure of pyridostigmine bromide and DFP, in the absence of DEET, resulted in $^{14}$C-permethrin absorption of 1%, with a penetration of 6% of the applied dose, a five fold increase over vehicle controls. In this scenario, surface residues did not decrease suggesting that an increase in permeability of the epidermal barrier was not the primary mechanism of action. This was greater than any conditions investigated in the present work. JP-8 was the only condition that began to approach this extent of enhancement. Although occlusion significantly increases $^{14}$C-permethrin absorption and penetration, the extent of the enhancement was still less than that seen with JP-8.

JP-8 significantly increased topical $^{14}$C-permethrin absorption to 0.5%, and penetration to 5% of the applied dose. This effect of JP-8 is not surprising as JP-8 is an organic solvent that would be expected to significantly alter the stratum corneum permeability, and thus enhance absorption. Previous work has demonstrated that hydrocarbon components of JP-8 penetrate the skin and prolonged exposure results in cutaneous toxicity (Riviere et al., 1999; McDougal et al., 2000; Baynes et al., 2000, 2001; Kabbur et al., 2001; Monteiro-Riviere et al., 2001). JP-8 has been shown to increase epidermal permeability by increasing transepidermal water loss (Kanikkannan et al., 2001; Monteiro-Riviere et al., 2001). The finding of reduced $^{14}$C-permethrin surface

residues in the present study is consistent with an increase in the permeability of the epidermal barrier.

A clear-cut result of this study is the effect of sulfur mustard to decrease dermal absorption and penetration of $^{14}$C-permethrin. Previous studies in our laboratory (unpublished observations) have demonstrated that sulfur mustard co-exposure to DEET actually enhanced DEET absorption, the opposite effect to that seen with $^{14}$C-permethrin in this study. This effect on DEET was believed to be secondary to sulfur mustard damage to the epidermal barrier. The sulfur mustard effect of reducing $^{14}$C-permethrin absorption could be explained by its ability to decrease enzymatic biotransformation of $^{14}$C-permethrin. All previous data is consistent with $^{14}$C-permethrin absorption being a metabolite and not parent drug. Dermal absorption of $^{14}$C-permethrin activity is restricted by both a diffusional as well as a metabolic barrier. Previosuly, sulfur mustard has been shown to decrease cutaneous enzyme activity using enzyme histochemistry techniques (Monteiro-Riviere and Inman, 2000). Thus, sulfur mustard damage to cutaneous enzymes responsible for the metabolism of permethrin would result in a decrease in transdermal flux, but not necessarily a decrease in surface residues. Alternatively, sulfur mustard or one of its reactive metabolites, could have reacted directly with permethrin reducing both its dermal absorption and surface residues.

These mechanisms are in contrast to a modulator that decreases absorption by decreasing epidermal permeability. In that scenario, absorption and penetration would decrease, while surface residues would increase. This permeability modulating effect is identical, although in an opposite direction, to the effect discussed earlier of JP-8 increasing $^{14}$C-permethrin absorption and penetration with a resulting decrease in

available surface residues. A similar effect was observed with an increase in absorption and a decrease in surface residues with the cellophane occlusion method of exposure.

The effect of DEET blocking $^{14}$C-permethrin within the sulfur mustard treatment groups is consistent with the DEET effects previously observed across many vehicle combinations. This phenomenon could be secondary to a direct effect of DEET on reducing permethrin's log stratum corneum / vehicle partition coefficient (Baynes et al., 1997, Baynes et al., 2002). Similarly, DEET has been shown to reduce carbaryl absorption (Baynes et al., 1997). Co-exposure to sulfur mustard did not seem to alter this DEET effect on blunting $^{14}$C-permethrin absorption.

In conclusion, these studies suggest that co-exposure of permethrin to JP-8 increases, while co-exposure to low level sulfur mustard decreases, $^{14}$C-permethrin absorption and penetration in isolated perfused porcine skin. Occlusion with fabric, which would be similar to the effect of a uniform covering an application site, did not significantly increase absorption. Complete occlusion with cellophane only modestly increased absorption. It is unlikely that any of these scenarios could significantly increase permethrin absorption to the extent that toxicologic manifestations would result.

## ACKOWLEDGMENTS

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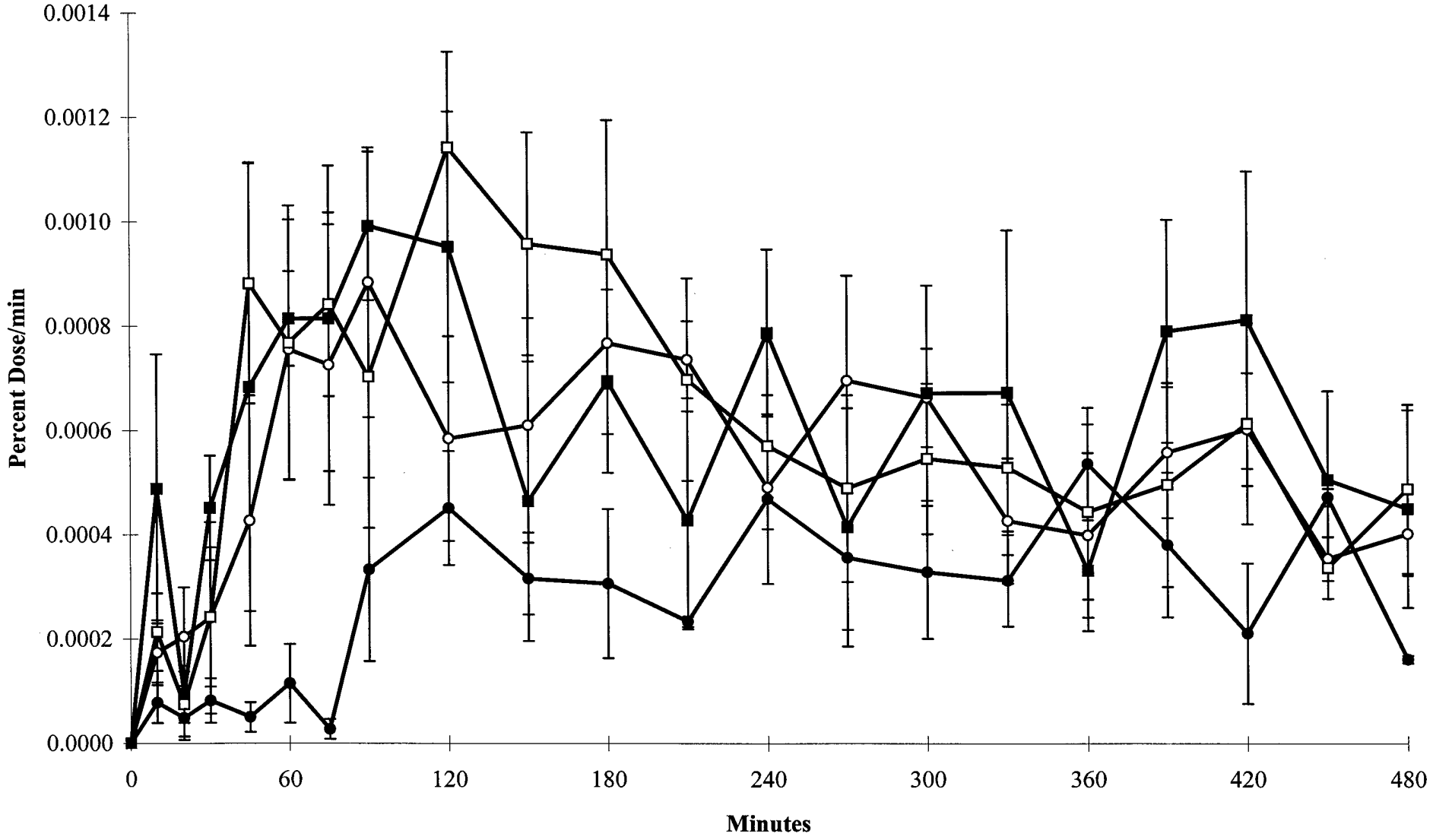
## FIGURE CAPTIONS

**Figure One.** $^{14}$C-permethrin absorption flux profiles following topical doses of permethrin mixtures with and without JP-8. (Mean $\pm$ SEM)

**Figure Two.** $^{14}$C-absorption flux profiles following topical doses of permethrin mixtures with and without sulfur mustard (HD). (Mean $\pm$ SEM)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Minutes
[14C]-Permethrin:75%JP-8:25%EtOH (n=4)
[14C]-Permethrin:100%EtOH (n=4)
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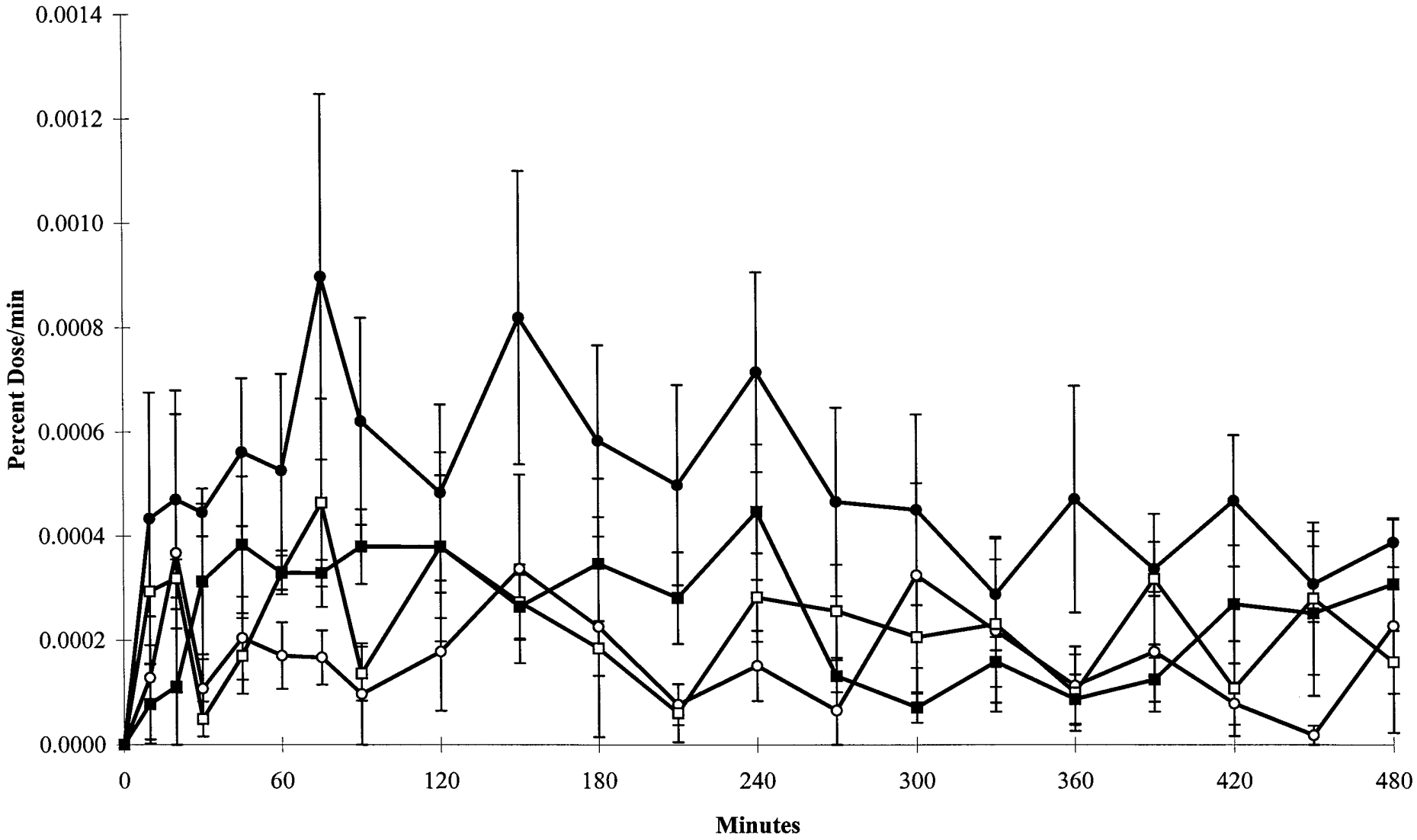
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[14C]-Permethrin:HD:100%EtOH:Pyridostigmine-IA (n=4)
[14C]-Permethrin:100%EtOH:Pyridostigmine-IA (n=4)
[14C]-Permethrin:HD:75%DEET:25%EtOH:Pyridostigmine-IA (n=4)
[14C]-Permethrin:75%DEET:25%EtOH:Pyridostigmine-IA (n=4)

## Table 1. $^{14}$C-Permethrin Dosing Protocols (n=4 each)

| | |
|---|---|
| | **Effects of JP-8** |
| 1 | $^{14}$C-Permethrin:75%JP-8:25%EtOH |
| 2 | $^{14}$C-Permethrin:75%JP-8:15%EtOH:10%Water |
| | **Effects of Sulfur Mustard (HD)** |
| 1 | $^{14}$C-Permethrin:HD:100%EtOH |
| 2 | $^{14}$C-Permethrin:HD:100%EtOH:Pyridostigmine-IA |
| 3 | $^{14}$C-Permethrin:HD:75%DEET:25%EtOH |
| 4 | $^{14}$C-Permethrin:HD:75%DEET:25%EtOH:Pyridostigmine-IA |
| 5 | $^{14}$C-Permethrin:HD:75%DEET:25%EtOH:DFP-IA |
| 6 | $^{14}$C-Permethrin:HD:75%DEET:25%EtOH:Pyridostigmine+DFP-IA |
| | **Effects of Occlusion and Fabric** |
| 1 | $^{14}$C-Permethrin:75%DEET:15%EtOH:10%Water:Pyridostigmine+DFP-IA:Occluded |
| 2 | $^{14}$C-Permethrin:75%DEET:15%EtOH:10%Water:Pyridostigmine+DFP-IA:Fabric-Occluded |
| 3 | $^{14}$C-Permethrin:75%DEET:15%EtOH:10%Water:Pyridostigmine+DFP-IA:Nonoccluded |

## Table 2. Mean (SEM) Percent Dose $^{14}$C-Permethrin Residues Following Topical Doses of $^{14}$C-Permethrin Mixtures - Effects of JP-8

| | 75%JP-8 25%EtOH $^{14}$C-Permethrin | | 75%JP-8 15%EtOH:10%Water $^{14}$C-Permethrin | | 100%EtOH $^{14}$C-Permethrin | | 60%EtOH:40%Water $^{14}$C-Permethrin | |
|---|---|---|---|---|---|---|---|---|
| AUC (%D-min/ml) | 0.27 (0.04) | a | 0.30 (0.04) | a | 0.10 (0.02) | b | 0.19 (0.05) | ab |
| Absorption (%D) | 0.27 (0.07) | ab | 0.44 (0.18) | a | 0.13 (0.04) | b | 0.19 (0.06) | b |
| Penetration (%D) | 4.95 (1.53) | a | 3.92 (1.73) | ab | 1.62 (0.30) | b | 1.51 (0.28) | b |
| Surface Residue (%D) | 21.75 (3.05) | b | 25.74 (4.13) | b | 38.25 (3.97) | ab | 46.15 (6.13) | a |
| Stratum Corneum (%D) | 12.01 (2.22) | a | 8.58 (3.50) | ab | 6.59 (2.13) | ab | 2.73 (0.80) | b |

Means with the same letters are not significantly different.

## Table 3. Mean (SEM) Percent Dose $^{14}$C-Permethrin Residues Following Topical Doses of $^{14}$C-Permethrin Mixtures - Effects of HD

| | HD<br>100%EtOH<br>$^{14}$C-Permethrin | HD<br>Pyridostigmine<br>100%EtOH<br>$^{14}$C-Permethrin | HD<br>75%DEET<br>25%EtOH<br>$^{14}$C-Permethrin | HD<br>Pyridostigmine<br>75%DEET<br>25%EtOH<br>$^{14}$C-Permethrin | HD<br>DFP<br>75%DEET<br>25%EtOH<br>$^{14}$C-Permethrin | HD<br>Pyridostigmine+DFP<br>75%DEET<br>25%EtOH<br>$^{14}$C-Permethrin |
|---|---|---|---|---|---|---|
| AUC (%D-min/ml) | 0.16 (0.06) $^{a}$ | 0.08 (0.02) $^{aB}$ | 0.10 (0.02) $^{a}$ | 0.11 (0.03) $^{a}$ | 0.09 (0.01) $^{a}$ | 0.16 (0.05) $^{a}$ |
| Absorption (%D) | 0.16 (0.07) $^{a}$ | 0.17 (0.11) $^{a}$ | 0.04 (0.03) $^{aB}$ | 0.07 (0.04) $^{a}$ | 0.13 (0.04) $^{a}$ | 0.06 (0.03) $^{a}$ |
| Penetration (%D) | 0.85 (0.33) $^{a}$ | 0.63 (0.29) $^{abB}$ | 0.20 (0.06) $^{cB}$ | 0.22 (0.11) $^{cB}$ | 0.49 (0.16) $^{abc}$ | 0.34 (0.14) $^{bc}$ |
| Surface Residue (%D) | 14.58 (1.51) $^{bB}$ | 16.14 (2.22) $^{bB}$ | 10.91 (1.36) $^{b}$ | 36.67 (7.35) $^{a}$ | 13.22 (1.28) $^{b}$ | 15.74 (2.36) $^{b}$ |
| Stratum Corneum (%D) | 1.23 (0.49) $^{a}$ | 0.36 (0.23) $^{bB}$ | 0.20 (0.07) $^{b}$ | 0.14 (0.03) $^{bB}$ | 0.23 (0.05) $^{b}$ | 0.22 (0.10) $^{b}$ |

| | 100%EtOH<br>$^{14}$C-Permethrin | Pyridostigmine<br>100%EtOH<br>$^{14}$C-Permethrin | 75%DEET<br>25%EtOH<br>$^{14}$C-Permethrin | Pyridostigmine<br>75%DEET<br>25%EtOH<br>$^{14}$C-Permethrin |
|---|---|---|---|---|
| AUC (%D-min/ml) | 0.10 (0.02) $^{b}$ | 0.24 (0.06) $^{aA}$ | 0.14 (0.06) $^{ab}$ | 0.12 (0.02) $^{ab}$ |
| Absorption (%D) | 0.13 (0.04) $^{a}$ | 0.23 (0.07) $^{a}$ | 0.27 (0.01) $^{aA}$ | 0.17 (0.08) $^{a}$ |
| Penetration (%D) | 1.62 (0.30) $^{b}$ | 1.64 (0.19) $^{bA}$ | 2.86 (0.64) $^{aA}$ | 1.32 (0.24) $^{bA}$ |
| Surface Residue (%D) | 38.25 (3.97) $^{abA}$ | 23.64 (2.11) $^{cA}$ | 25.79 (6.32) $^{bc}$ | 40.92 (3.28) $^{a}$ |
| Stratum Corneum (%D) | 6.59 (2.13) $^{ab}$ | 8.59 (2.05) $^{aA}$ | 2.54 (0.95) $^{b}$ | 2.88 (0.69) $^{abA}$ |

Means with the same letters are not significantly different.

Lower case letters are statistical comparisons within groups across rows.

Upper case letters are statistical comparisons between HD and non-HD counterparts down columns.

## Table 4. Mean (SEM) Percent Dose $^{14}$C-Permethrin Residues Following Topical Doses of $^{14}$C-Permethrin Mixtures - Effects of Occlusion

| | Occluded Pyridostigmine+DFP 75%DEET 15%EtOH:10%Water $^{14}$C-Permethrin | Fabric-Occluded Pyridostigmine+DFP 75%DEET 15%EtOH:10%Water $^{14}$C-Permethrin | Nonoccluded Pyridostigmine+DFP 75%DEET 15%EtOH:10%Water $^{14}$C-Permethrin |
|---|---|---|---|
| AUC (%D-min/ml) | 0.09 (0.03) [a] | 0.04 (0.01) [a] | 0.04 (0.01) [a] |
| Absorption (%D) | 0.13 (0.03) [a] | 0.03 (0.01) [b] | 0.04 (0.01) [b] |
| Penetration (%D) | 0.46 (0.11) [a] | 0.32 (0.07) [a] | 0.22 (0.03) [a] |
| Surface Residue (%D) | 20.46 (5.22) [a] | 35.85 (4.96) [a] | 33.52 (10.94) [a] |
| Stratum Corneum (%D) | 0.32 (0.23) [a] | 0.85 (0.45) [a] | 0.03 (0.01) [a] |

Means with the same letters are not significantly different.

# Analysis of DEET absorption in porcine skin perfusates using solid phase extraction discs and reversed-phase high-performance liquid chromatography

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## 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 cell system. The technical improvement involved the use of disc solid phased 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 for this method ranged from 93.3 – 117%, and the relative standard deviation (RSD) ranged from 3.1 – 12.0%. 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 *in vitro* was $1.61 \times 10^{-5}$ cm/hr.

## 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 drugs and pesticides [1,2].

In light of these findings, there has been an urgent 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 biological matrices however, can be a challenge as many endogenous compounds may interfere with UV detection at low wavelengths (200 – 220) where UV absorption is optimum for DEET. Previous analytical methods entailed the use of traditional packed bed solid phase extraction (SPE) [3,4,5] and others have used liquid-liquid extraction (LLE) [6] in separating DEET from the biological matrix. The focus of this research was to evaluate the application of disc SPE extraction technology (Ansys Technologies, Inc.) 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.

Previous investigators have successfully applied this disc SPE technology to drug screening in urine [7] and extraction of polycyclic aromatic hydrocarbons (PAHs) in water [8]. However, none of the SPE methods have taken advantage of the disc SPE technology for DEET analysis and furthermore applied it to a complex biological matrix such as bovine serum albumin

(BSA). Traditional packed-bed SPE can result in non-uniform flow through the extraction bed which results in channeling and poor reproducibility. The discs used in our research are really 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 tradition packed bed SPE.

This paper describes an important improvement to the traditional SPE method as it was applied to the porcine skin flow through cell, and this improved method uses significantly less solvent for sample extraction/preparation saving time and money when a large number of samples are to be analyzed. BSA or perfusion media that imitates blood is frequently used to perfuse tissue or whole organs [9]. However most of the previous *in vitro* transdermal research with DEET [10,11] utilized Franz cells and phosphate buffered solution (PBS) which did not require extraction prior to HPLC analysis. PBS does not contain the many proteins normally associated with many biological matrices such as blood, and 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, and should mimic partitioning and diffusion processes observed in human skin *in vivo*. Finally, DEET has an octanol-water partition coefficient of 2.0, therefore DEET should partition more readily into BSA than PBS.

## 2. Conditions

### *2.1. Chemicals*

N,N-Diethyl-*m*-toluamide (DEET) was purchased from Chem Service (West Chester, PA). HPLC grade acetonitrile, reagent grade ammonium acetate, and glacial acetic acid was purchased from Fisher Scientific (Fair Lawn, NJ). All water was purified with an ultra high purity water filtration system (Dracor Water Systems, Durham, NC).

### *2.2 Apparatus*

The HPLC system was equipped with a Waters model 60F solvent pumping system, Waters 600 controller, Waters 717plus 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 porcine skin flow through cell system is described later in this section. The 24-port vacuum manifold was obtained from VWR Scientific Products (So. Plainfield, NJ, USA). The solid-phase extraction discs were obtained from ANSYS Technologies Inc. (Lake Forest, CA, USA).

*2.3. HPLC conditions*

The mobile phase composition was 36% acetonitrile and 64% ammonium acetate buffer, 0.03M. The pH of the 0.03M ammonium acetate buffer was adjusted to pH 4.5 with glacial acetic acid. The guard and analytical columns temperature was 35±0.5°C.

The detector wavelength was 220 nm. The flow rate and injection volume was 1.0 ml/min. and 10µL respectively. The amount of DEET in samples was 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 (2,740 µg/ml) with acetonitrile. The 100 µg/ml DEET working solution was prepared by diluting approximately 1.825 ml of the DEET stock solution (2,740 µg/ml) 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 (2,740 µg/ml) 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 media. For example, approximately 4ml of the solid-phase extraction (SPE) elution solvent was added to each of five 16 x 125mm borosilicate glass tubes. The SPE elution solvent consisted of 40% acetonitrile and 60% ammonium acetate buffer (pH 4.5, 0.03M). 8 µl of the 50 µg/ml DEET working solution was added to the first tube. 40 µl of the 50 µg/ml DEET working solution was added to the second tube. 40 µl of the

100 µg/ml DEET working solution was added to the third tube. 7.3 µl 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, respectively. These DEET concentrations covered the range of expected levels of DEET extracted from real, non-spiked, samples. All solutions were vortexed briefly. The spiked samples were prepared in the same manner using nondosed bovine serum albumin media (BSA) instead of SPE elution solvent. The preparation of the bovine serum albumin media (BSA) was previously described. [12]

*2.5.Sample Preparation*

In order to determine the amount of DEET absorbed through the porcine skin, it was necessary to extract the perfusate prior to analysis by HPLC. The perfusate from the porcine skin flow-through experiments consisted of bovine serum albumin media (BSA). The perfusate was extracted by placing one milliliter of each sample into 16 x 125-mm glass culture tubes. Each sample was then diluted with three milliliters of HPLC grade water. The solid phase extraction discs (Ansys SPEC PLUS 3ml C18 15mg), housed inside 3ml plastic syringe barrels, were placed onto the vacuum manifold (Burdick & Jackson, 24-port) then preconditioned with 500 µl of acetonitrile followed by 500 µl of water. It was important to use a slow flow rate during the preconditioning and sample loading steps. If necessary, a low vacuum (2 inches Hg) was applied to the discs not to exceed a flow rate of one to two drops per second during the preconditioning step. Care was taken not to allow the discs to dry out during the preconditioning or sample loading steps. The samples were loaded onto the discs using glass disposable Pasteur pipettes. The samples were allowed to drain through the discs very slowly (about one drop per

second) using low vacuum or gravity. When each sample meniscus reached the top of the discs, 500 µl of the wash solvent (10:90 Acetonitrile: pH 4.5 ammonium acetate buffer 0.03M) was added to the discs. Once the sample wash drained through the discs, a strong vacuum (15 in. Hg) was applied to dry out the discs before the final elution step. New clean glass tubes were placed in the vacuum manifold. The samples were then eluted with 1 ml of the elution solvent (40:60 acetonitrile: pH 4.5 ammonium acetate buffer 0.03M) and placed directly into HPLC vials for analysis.

*2.6.Method Validation*

Recoveries and blanks were run with every batch of samples as part of the quality control. 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 samples were taken at smaller time intervals, it was necessary to use 0.5ml samples with 0.5ml elution volumes for samples taken up to one hour. For example, the first sample was taken prior to dosing. Immediately after dosing, samples were taken at ten, twenty, thirty, forty-five, and sixty minutes. After sixty minutes, the sampling time interval was at least thirty minutes. Therefore, the earliest samples up to one hour did not contain 1.0 ml of sample. As a result, the inter-day precision and accuracy was also measured for a sample volume of 0.5 ml with 0.5 ml elution volume at four different concentration levels on six different days. The elution volumes were always kept the same as the sample volumes so that there was no dilution factor.

*2.7.Flux and Permeability Determination of DEET in porcine skin*

Fresh porcine skin sections (n=4) was carefully mounted into the flow-through cell system and perfused with bovine serum albumin (BSA) as previously described [2,12]. The diffusion cell temperatures were kept constant at 37 ± 1°C through out the course of the experiments. These four cells were dosed with 10 µl 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 four ml per hour. The BSA effluent from each receiver compartment was sampled at various time intervals up until 8 hours, and immediately frozen at –80°C until extraction and analysis by disc SPE and HPLC-UV. The following equation was used to calculate the apparent permeability constants from these *in vitro* porcine flow-through cell experiments:

$$K_p = J/\Delta C$$

Where $K_p$ is the apparent permeability constant, 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 7,470µg DEET in the 10 µl of dosing solution and applied to a surface area of 0.32 $cm^2$. The steady state flux, J (µg/$cm^2$/hr), was estimated by calculating the slope of the plot of cumulative amount of DEET per unit surface area (µg/$cm^2$) versus time (hrs). The following equation was used to calculate DEET diffusivity (D) 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*

Figure 1A shows a typical chromatogram for a sample containing 1.0 μg/ml of DEET extracted from BSA media. The blank (Figure 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 and endogenous compounds associated with skin. DEET typically eluted at 9.5 minutes under the conditions specified. Any variation in the retention time of DEET was monitored with external standards in every sample table. External standards were injected at the beginning, in the middle and at the end of each set of samples. Figure 1 also further demonstrates that the good chromatography often associated with packed-bed SPE was not compromised with the disc SPE method.

### *3.2 Linearity*

As shown in Figure 2, the average slope of the external standard curves, covering a DEET concentration range of 0.1 to 5.0 μg/ml, was 24698 ± 362 (average 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 an average correlation coefficient, $R^2$, of 0.9998 ± 0.0001 (average $R^2$ ± SD, n=6).

### *3.3 Precision and accuracy*

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

The results for the 1.0ml sample and elution volumes showed that the lowest spiked concentration of 0.10 μg/ml DEET gave an average calculated concentration of 0.09 ± 0.01 μg/ml with a relative standard deviation of 11.0% and an average percent recovery of 93.3 ± 10.2 %. The highest spiked concentration of 5.0 μg/ml DEET gave an average calculated concentration of 5.14 ± 0.23 μg/ml with a relative standard deviation of 4.5% and an average percent recovery of 102.9 ± 4.7%.

The results for the 0.5ml sample and elution volume showed that lowest spiked concentration of 0.10 μg/ml DEET gave an average calculated concentration of 0.12 ± 0.01 μg/ml with a relative standard deviation of 9.2% and an average percent recovery of 117.2 ± 10.8%. The highest spiked concentration of 5.0 μg/ml DEET gave an average calculated concentration of 4.97 ± 0.26 μg/ml with a relative standard deviation of 5.3% and an average percent recovery of 99.3 ± 5.2%.

### 3.4 *Limit 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, the LOD was estimated as 0.02 μg/ml DEET. The LOQ was estimated as 0.08 μg/ml DEET.

### 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 minutes. Figure 3 shows the plot of DEET concentration versus time following a topical application of DEET to porcine skin. The average flux was 12.04 ± 1.66 ($\mu g/cm^2/hr$), the lag time was 1.71 ± 0.11 hours, the apparent permeability was $1.61 \times 10^{-5} \pm 0.22 \times 10^{-5}$ cm/hr, and the diffusivity was $2.42 \times 10^{-6} \pm 0.15 \times 10^{-6}$.

Porcine skin is similar anatomically and physiologically to human skin, and dermal absorption of many chemicals in pigs skin is comparable to that in human skin [13]. However our *in vitro* study suggests that DEET permeability and diffusivity in porcine skin was about 100-fold less and 100-fold greater, respectively, than what has been reported for human skin *in vitro* utilizing Franz static cells [10]. In the 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 a complex biological matrix such as BSA which was used in the present study. This work therefore has resulted in development of a validated disc

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.

## 4. Conclusions

We have reported an important improvement in the solid-phase extraction of DEET coupled with HPLC-UV detection utilizing discs SPE rather than packed-bed SPE. This improved method uses approximately 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. We were also able to validate this method using low volumes of the biological matrices. This application may be useful in the screening of small biological sample volumes 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 perfusate, BSA, used in this system consist of many serum proteins as well as antibiotics which could have potentially interfered with the separation and liquid chromatography analysis. However, the disc SPE provided quality chromatograms and reproducible results in the validation and its application in assessing DEET permeation in skin.

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**Figure Captions**

Figure 1. Representative chromatograms of bovine serum albumin (BSA) media spiked with 1.0 μg/ml of DEET (A) and not spiked with DEET (B). Column: Waters SymmetryShield RP18 (4.6x150mm, 3.5μm particle size) at 35°C. Mobile phase: 36:64 acetonitrile:ammonium acetate buffer (pH 4.5, 0.03M). Detection: 220nm.

Figure 2.. A plot of the average of six external standard plots (peak area versus DEET concentration, μg/ml) used for this study with standard error bars. The equation for the straight line is shown on the graph.

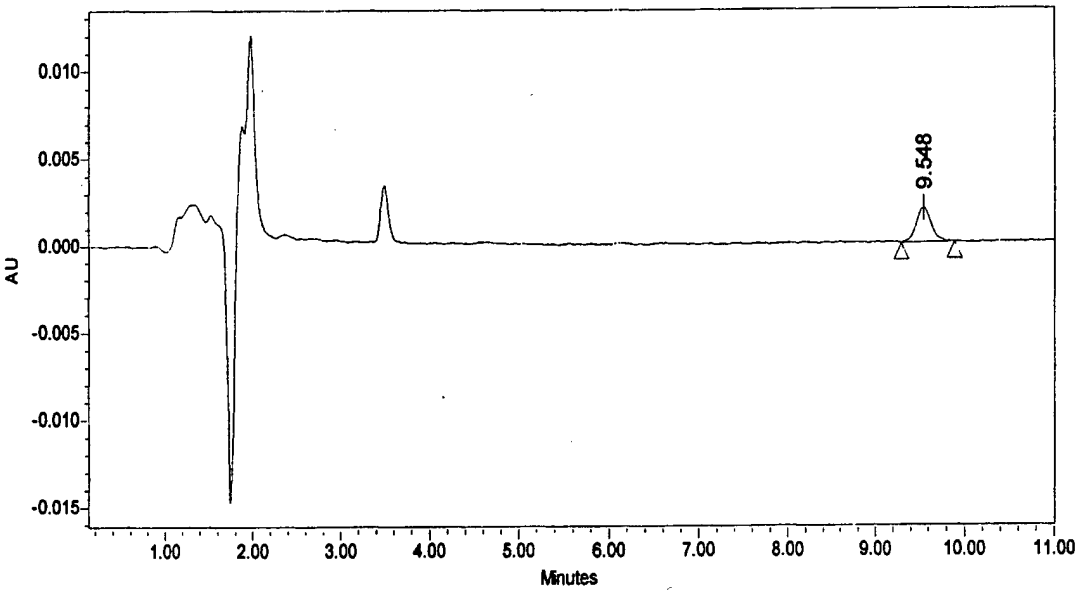
Figure 3. A plot of DEET concentration (μg/ml) versus time (hours) with standard error bars following topical administration of porcine skin *in vi*tro (n=4) with 75% DEET in ethanol and perfused with BSA media. Samples from each perfused skin section and each time point were analyzed in duplicate.

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 | 0.5ml | 1.0 ml | 0.5ml | 1.0 ml | 0.5ml |
| 0.1 | 0.09 ± 0.01 | 0.12 ± 0.01 | 11.0 | 9.21 | 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 |

# Fig 1

A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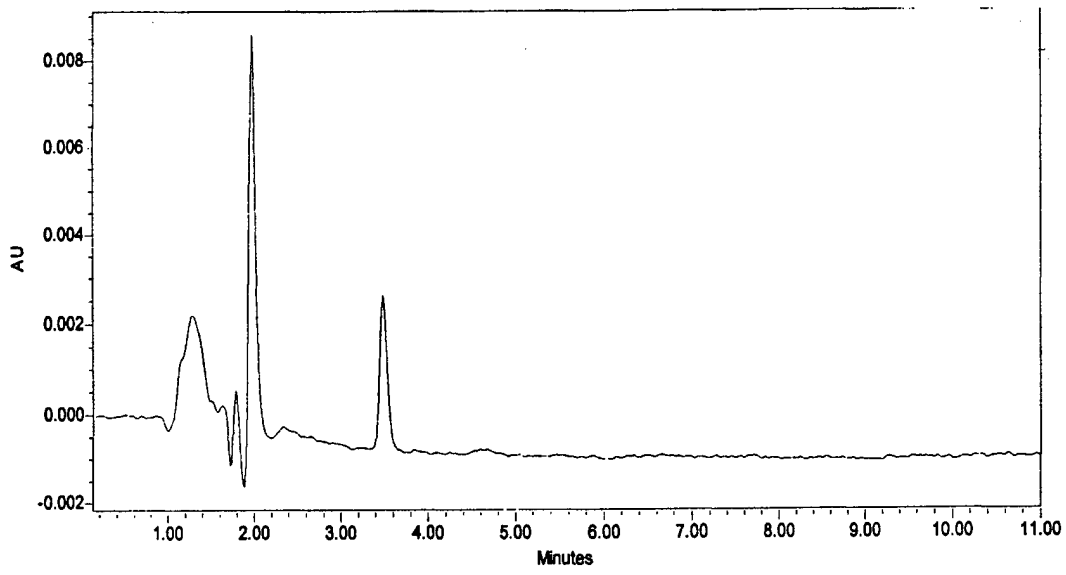
B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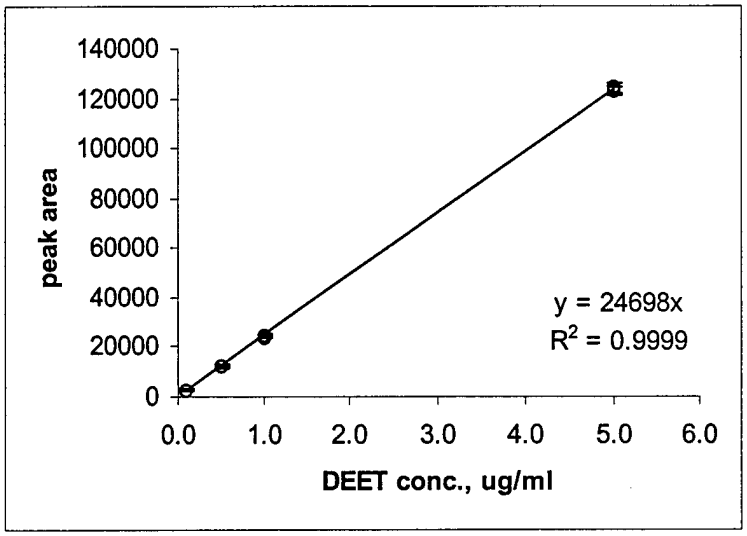
# Fig 2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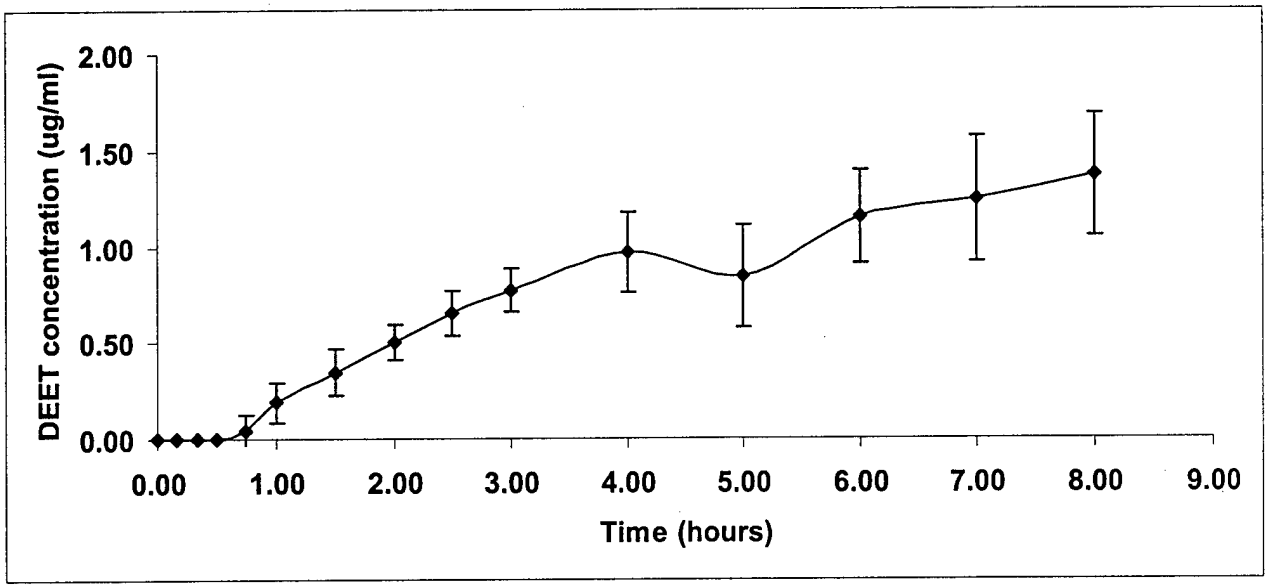
4.0
5.0
6.0
DEET conc., ug/ml

# Fig 3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9.00
Time (hours)