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In vitro metabolism and interactions of pyridostigmine bromide, *N*,*N*-diethyl-*m*-toluamide, and permethrin in human plasma and liver microsomal enzymes

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Abstract

1. The *in vitro* human plasma activity and liver microsomal metabolism of pyridostigmine bromide (PB), a prophylactic treatment against organophosphate nerve agent attack, *N*,*N*-diethyl-*m*-toluamide (DEET), an insect repellent, and permethrin, a pyrethroid insecticide, either alone or in combination were investigated.

2. The three chemicals disappeared from plasma in the following order: permethrin > PB > DEET. The combined incubation of DEET with either permethrin or PB had no effect on permethrin or PB. Binary incubation with permethrin decreased the metabolism of PB and its disappearance from plasma and binary incubation with PB decreased the metabolism of permethrin and its clearance from plasma. Incubation with PB and/or permethrin shortened the DEET terminal half-life in plasma. These agents behaved similarly when studied in liver microsomal assays. The combined incubation of DEET with PB or permethrin (alone or in combination) diminished DEET metabolism in microsomal systems.

3. The present study evidences that PB and permethrin are metabolized by both human plasma and liver microsomal enzymes and that DEET is mainly metabolized by liver oxidase enzymes. Combined exposure to test chemicals increases their neurotoxicity by impeding the body's ability to eliminate them because of the competition for detoxifying enzymes.

Keywords: *Pyridostgimine bromide, permethrin, N,N-diethyl-m-toluamide (DEET), human plasma, human liver, microsomal enzymes, Gulf War*

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Introduction

Pyridostigmine bromide (PB) (Figure 1) that has been used for the treatment of myasthenia gravis patients (Anquilloins & Hartvig 1980) has also been used as a prophylactic agent against potential nerve gas attack during the Persian Gulf War in 1990–91 (Abou-Donia et al. 1996a, b; Young & Evans 1998). Permethrin is a pyrethroid insecticide effective against mites and head lice (Fraser 1994) and *N*,*N*-diethyl-*m*-toluamide (DEET) is an insect repellent applied against mosquitoes and biting insects (Brown & Hebert 1997). These chemicals were used by military personnel during the Gulf War (Young & Evans 1998; Riviere et al. 2002). Combined exposure to PB, DEET and permethrin enhanced their neurotoxicity in hens (Abou-Donia et al. 1996a, b), and rats (Abdel-Rahman et al. 2001, 2004a, b), increased mortality in rats (McCain et al. 1997), and caused neurobehavioral alterations in male rats (Hoy et al. 2000; Abou-Donia et al. 2004). In addition, the toxicity of DEET to German cockroaches was increased following combined exposure with permethrin or PB (Moss 1996). Furthermore, concurrent administration of PB and DEET caused seizure in rats (Chaney et al. 1999). In addition, following *in vitro* incubation of DEET and PB with *Escherichia coli*, DEET significantly inhibited P-glycoprotein (P-gp), PB uptake and

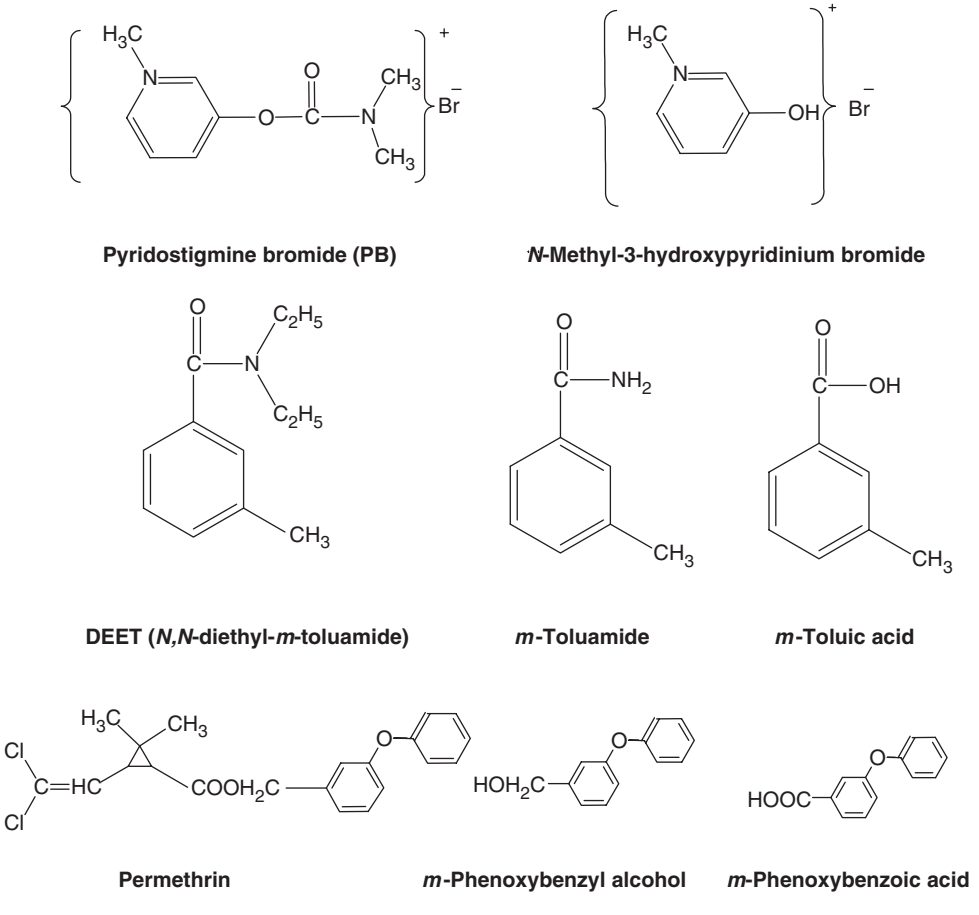

Figure 1. Chemical structures of PB, DEET, and permethrin and their metabolites.

decreased resistance against PB (El-Masry & Abou-Donia 2006). Published reports implicated combined exposure to PB, DEET and permethrin in the development of Gulf War Illnesses (Abou-Donia et al. 1996a, b; Haley & Kurt 1997; Shen 1998; Riviere et al. 2002; Hodgson & Rose 2005). Moreover, other studies reported potential interactions between organophosphorus insecticides such as chlorpyrifos or malathion and the pyrethroid insecticide, permethrin and the insect repellent, DEET on the inhibition certain enzymes including trans-permethrin hydrolysis in human liver (Choi et al. 2004; Hodgson & Rose 2005), alteration of dermal disposition of permethrin by PB (Baynes et al. 2002), and development of neurotoxicity following combined exposure (Abdel-Rahman et al. 2004b). PB is metabolized, mostly via hydrolysis to *N*-methyl-3-hydroxypyridinium bromide (Taylor et al. 1991; Abu-Qare & Abou-Donia 2000). Metabolism and disposition of DEET *in vivo* were rapid following application in human volunteers (Selim et al. 1995), rats (Schoenig et al. 1996), and mice (Blomquist & Thorsell 1977). DEET is metabolized *in vitro* via cytochrome P450-mediated oxidation of the aromatic methyl group and by *N*-deethylation following incubation with rat liver microsomes (Taylor 1986; Constantino & Iley 1999) Metabolism of permethrin has been examined following oral and intravenous dose in rats (Anadon et al. 1991). Furthermore, Vulule et al. (1999) reported that oxidases and esterases catalysed permethrin metabolism.

Alteration of pharmacokinetics and metabolism is considered a possible mechanism of interaction between chemicals following combined exposure that could change bioavailability and concentration at the toxicity targets leading to enhanced toxicity in chemical mixtures (Abou-Donia et al. 1996a, b, 2004; Moss 1996; Ito et al. 1998; Hodgson & Rose 2005). *In vitro* interactions between PB, DEET, and permethrin have been previously reported (Baynes et al. 2002; Usmani et al. 2002).

Because combined exposure to the three test compounds resulted in neurological deficits among US military personnel during the Persian Gulf War, the main objective of the present study was to investigate whether the metabolic interactions between them could explain their enhanced neurotoxicity following combined exposure. Therefore, the study was designed to do the following:

- Examine and compare the disappearance and the metabolic pathways of PB, DEET and permethrin when tested alone and in combination in human plasma and liver microsomal incubates.
- Identify specific enzyme(s) involved in the metabolism of PB, DEET and permethrin.
- Correlate the findings to an enhanced toxicity following *in vivo* combined exposure to these chemicals.

Materials and methods

Chemicals

Pyridostigmine bromide (3-dimethylaminocarbonyloxy-*N*-methylpyridinium bromide, PB; ≥99%) (Figure 1), *m*-phenoxybenzyl alcohol, *m*-phenoxybenzoic acid, coumarin, tetra-isopropyl pyrophosphoroamide (iso-OMPA), *β*-nicotinamide adenine dinucleotide phosphate (NADPH), glucose 6-phosphate, and glucose 6-phosphate dehydrogenase were obtained from Sigma Chemical Co., Inc. (St Louis, MO, USA). Permethrin (93.6%, ± *cis*/*trans*-3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarboxylic acid (3-phenoxyphenyl)methyl ester) was obtained from Roussel Uclaf Corp. (Pasadena, TX, USA). *m*-Toluamide and *m*-toluic acid were purchased from Fisher Scientific (Pittsburgh,

PA, USA), while DEET (±97%, *N*,*N*-diethyl-*m*-toluamide) was obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI, USA). *N*-methyl-3-hydroxypyridinium bromide was prepared in the authors' laboratory following a method by Somani et al. (1972). Human liver microsomes were obtained from In Vitro Technologies, Inc. (Baltimore, MD, USA).

HPLC system

The analysis was carried out according to the authors' published method (Abu-Qare & Abou-Donia 2000).

Calibration procedures, detection limits, and recovery

Five different calibration standards of a mixture of PB, *N*-methyl-3-hydroxypyridinium bromide, DEET, *m*-toluamide, *m*-toluic acid, permethrin, *m*-phenoxybenzoic acid, and *m*-phenoxybenzyl alcohol were prepared by adding different concentrations of DEET and metabolites, permethrin and metabolites ranged from 100–1000 ng ml^{-1} to 1 ml of human plasma, or 50 µl of (20 µg protein ml^{-1}) of human liver microsomes (without adding an NADPH-generating system). After incubation, the incubation tube was removed to an ice bath. The samples were acidified, extracted and cleaned up as described below before analysis by HPLC. Linear calibration curves were obtained by plotting peak areas of the individual chemicals as a function of concentrations. The standard curves were used to determine recovery of the compounds from plasma and liver microsomal incubation reactions. Limits of detection were determined at the lowest concentration can be detected, taking into consideration a 1:3 baseline noise:calibration point ratio.

Plasma incubations

Concentrations of 20–100 µM of PB, DEET and permethrin, alone and in combination, were incubated with 100 µl of human plasma from 5 to 60 min at 37°C in a shaking water bath. DEET was further incubated for 8 h. The reaction was initiated by the addition of substrate and terminated by the addition of 100 µl of 70% perchloric acid. Plasma butyrylcholinestrase activity was determined according to the method of Ellman et al. (1961). The protein content was determined using the method of Smith et al. (1985).

Liver microsomal incubations

Microsomal incubations were performed by adding 100 µl of stock solution of 20 µg ml^{-1} protein of human liver microsomes to test tube in ice bath then a volume of 640 ml of 100 mM Tris buffer (pH 7.4) was added, followed by a 10 µl of solutions of 100–1000 µM of PB, DEET, and permethrin, both alone and in combination. The total reaction mixture was adjusted to 1 ml by the addition of 250 µl of an NADPH-regenerating system (NRS) in 2% sodium bicarbonate containing 0.5 mg ml^{-1} NADP solution, 2.0 mg ml^{-1} glucose 6-phosphate, and 1.5 unit ml^{-1} glucose 6-phosphate dehydrogenase. The mixture was incubated from 5 to 60 min at 37°C using a shaking metabolic incubator. The reaction was terminated by transferring the incubation tubes into an ice bath and adding 100 µl of 70% perchloric acid. The mixture was centrifuged and the supernatant removed prior to analysis

by HPLC. Control samples were prepared in which substrate (PB, DEET, permethrin), NADPH-regenerating system, or microsomal enzymes were omitted.

Plasma butyrylcholinesterase inhibition

Incubation of PB and permethrin with human plasma was undertaken as described above. Concentrations of 10, 20, 30, 40, 500, 80, and 100 μM of the selective inhibitor butyrylcholinestrase tetra-isopropyl pyrophosphoramide (iso-OMPA) was included and the incubation was carried out for 60 min at 37°C. The reaction was terminated by adding 100 μl of 70% perchloric acid, centrifuged, and the supernatant was removed prior to analysis by HPLC.

Kinetics analysis

IC_{50} values were also determined by plotting the inhibitor concentration versus concentration of the metabolite. The kinetic analysis of test compounds in plasma and microsomal incubates was performed by plotting the log concentration as a function of time, and fitting the curve using linear regression. The terminal half-life of each test compound was calculated from the apparent first-order disappearance rate constant, K, that was obtained by linear regression of the terminal linear decline in test compound concentration using the formula:

$$t_{1/2} = \frac{0.693}{K}.$$

Statistical analysis

The mean values for each of five assays were calculated separately for each metabolism incubation reaction with single compounds or combinations. The mean values between different reactions were compared separately for each of the test compounds, alone and in combinations using a one-way analysis of covariance (ANOVA) with a Student–Newman–Keuls multiple comparison *post-hoc* test. A p-value < 0.05 was considered as being statistically significant.

Results

Recovery and detection limits of PB, DEET, permethrin, and metabolites

PB, DEET, permethrin, and their metabolites were analysed in human plasma and liver microsomal homogenates using HPLC, according to the present authors' published method (Abu-Qare & Abou-Donia 2000). Average percentage recoveries of PB, *N*-methyl-3-hydroxypyridinium bromide, DEET, *m*-toluamide, *m*-toluic acid, permethrin, *m*-phenoxybenzoic acid, and *m*-phenoxybenzyl alcohol from human plasma were 75, 78, 71, 73, 80, 84, 85 and 77%, respectively. Recoveries from human liver microsomal incubates were 77, 76, 75, 79, 68, 83, 89, and 90%, respectively. Detection limits were 100 ng ml^{-1} for PB, *N*-methyl-3-hydroxypyridinium bromide and DEET; 50 ng ml^{-1} for *m*-toluamide, *m*-toluic acid, *m*-phenoxybenzyl alcohol, and *m*-phenoxy benzoic acid; and 20 ng ml^{-1} for permethrin, respectively.

Kinetics of test compounds disappearance

In vitro human plasma incubation. Substrate disappearance data for PB, DEET, and permethrin following incubation with human plasma, alone or in combination are shown in Figures 2, 3, and 4, respectively. The apparent first-order disappearance rate constants (K, min^{-1}) and the half-lives (min) for each test compound, alone or in combination, are presented in Table I. Following incubation with human plasma, the three chemicals disappeared at different rates in the following order: permethrin > PB >> DEET. Permethrin disappeared at rates of 1.5 and 26 times faster than that of PB and DEET, respectively. The apparent first-order disappearance constants (K, min^{-1}) and half-lives (min) were: (0.026, 26.6), (0.017, 40.2), and (0.001, 665) for permethrin, PB and DEET, respectively. Following binary incubations, permethrin greatly affected the rate of disappearance of PB. The addition of permethrin reduced the rate of PB disappearance by 3.3-fold, whereas the addition of DEET had no effect on the disappearance rate of PB. Thus, binary incubation of PB with permethrin or tertiary incubation with both permethrin and DEET equally diminished the metabolism of PB with K (min^{-1}) and half-life (min) of: 0.005, 131; and 0.005, 127, respectively. Binary combinations of permethrin with PB decreased the disappearance of permethrin by 5.5-fold, in contrast to DEET that had no effect on the metabolism of permethrin. In addition, permethrin metabolism was greatly diminished in the presence of both PB and DEET by 11.2-fold. Binary incubation of PB or permethrin with DEET greatly enhanced the metabolism of DEET and decreased its half-life to 0.32- and 0.39-fold of DEET alone, respectively. Furthermore, combined incubation of DEET with PB and permethrin decreased its half-life to 0.35-fold of that of DEET alone. Although DEET was the most stable test compound in plasma after incubation for 1 or 8 h, combined incubation with PB, permethrin or both PB and permethrin accelerated its disappearance, with K (min^{-1}) and half-life (min) values of: 0.003, 212; 0026, 258; and 003, 230, respectively.

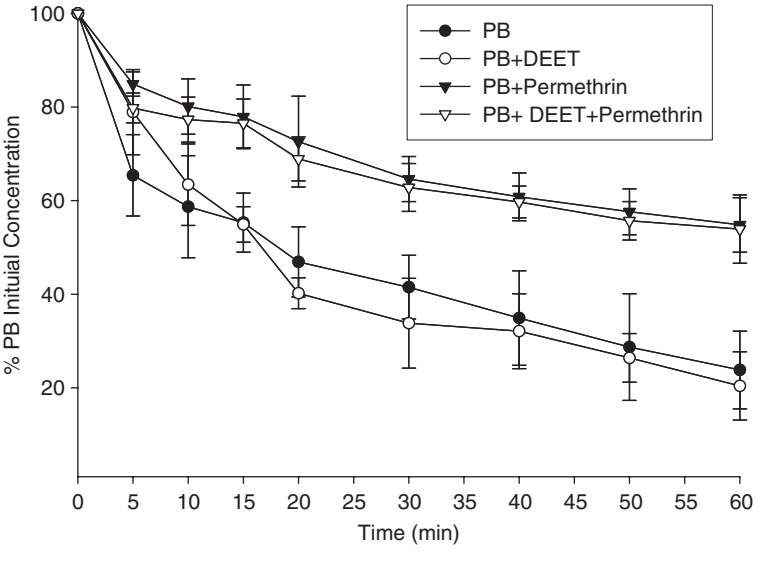

Figure 2. Disappearance of PB alone and in combination with DEET and permethrin following *in vitro* incubation with human plasma.

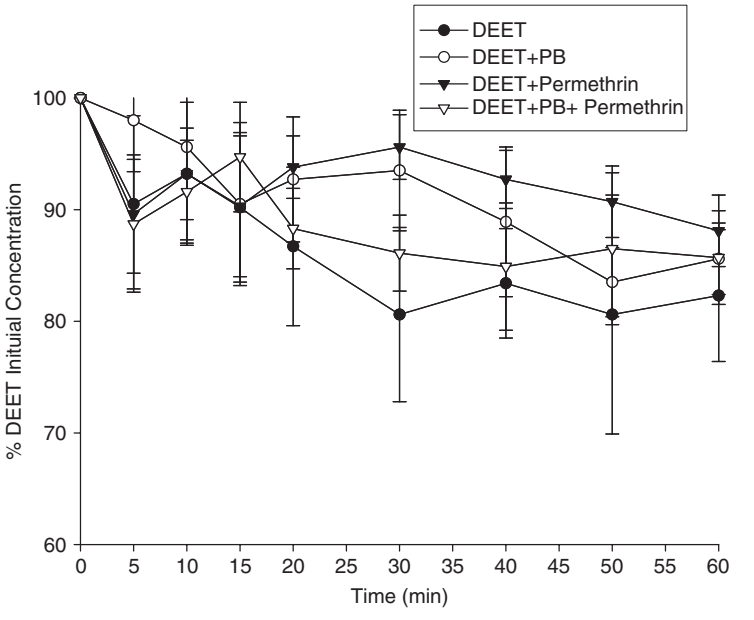

Figure 3. Disappearance of DEET alone and in combination with PB and permethrin following *in vitro* incubation with human plasma.

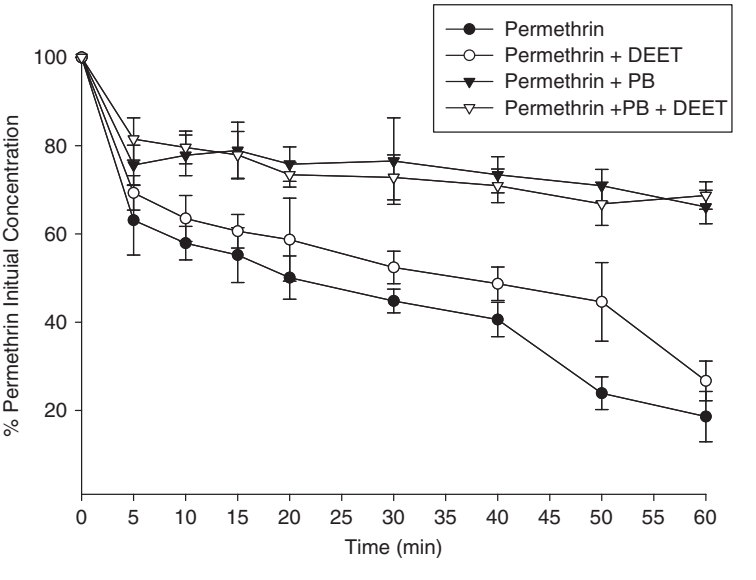

Figure 4. Disappearance of permethrin alone and in combination with PB and DEET following *in vitro* incubation with human plasma.

In vitro human liver microsomal incubation. Disappearance data for PB, DEET, and permethrin following human liver microsomal incubation with an NADPH-generating system, alone or in combination, are shown in Figures 5, 6, and 7, respectively, and Table II. The results show that the three test compounds had remarkably similar disappearance rates,

Table I. Apparent first-order disappearance rate constants (K) and half-lives of elimination for PB, DEET, and permethrin, alone and in combination following *in vitro* incubation with human plasma (mean of five experiments).

K (min^{-1}), half-life (min), half-life of combined/parent											
PB				DEET				Permethrin			
PB	PB + DEET	PB + permethrin	PB + DEET + permethrin	DEET	DEET + PB	DEET + permethrin	DEET + PB + permethrin	Permethrin	Permethrin + PB	Permethrin + DEET	Permethrin + PB + DEET
0.017	0.017	0.005	0.005	0.001	0.003	0.0026	0.003	0.026	0.005	0.021	0.002
40.2	40.5	131[a]	127[a]	665	212[b]	258[b]	230[b]	26.6	147[c]	32.8	297[c]
1.00	1.01	3.26	3.16	1.00	0.32	0.39	0.35	1.00	5.53	1.23	11.17

Notes:
[a]Significantly different than that of PB alone, $p < 0.05$.
[b] Significantly different than that of DEET alone, $p < 0.05$.
[c] Significantly different than that of Permethrin alone, $p < 0.05$.

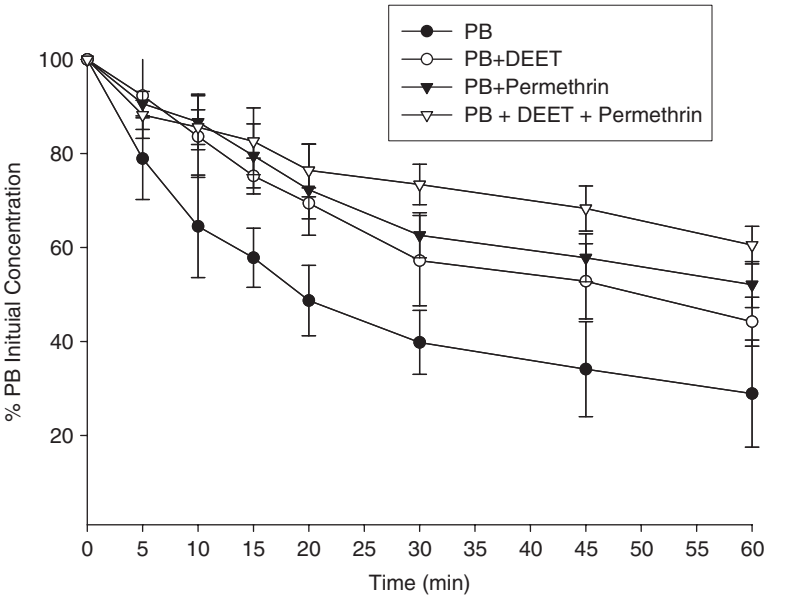

Figure 5. Disappearance of PB alone and in combination with DEET and permethrin following *in vitro* incubation with human liver microsomes.

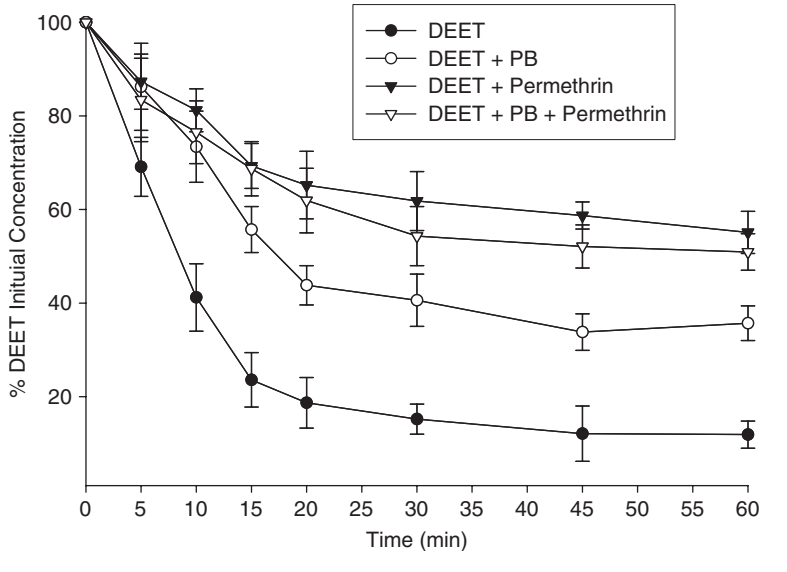

Figure 6. Dissipation of DEET alone and in combination with PB and permethrin following *in vitro* incubation with human liver microsomes.

with K (min^{-1}) and half-life (min) values of: 0.011, 65.0; 0.01, 60.0; and 0.011, 63 (Table II). DEET had no effect on the metabolism and disappearance of PB from microsomal incubate, whereas permethrin moderately decreased disappearance of PB and prolonged its half-life by 1.74-fold. Tertiary incubation of both DEET and permethrin with

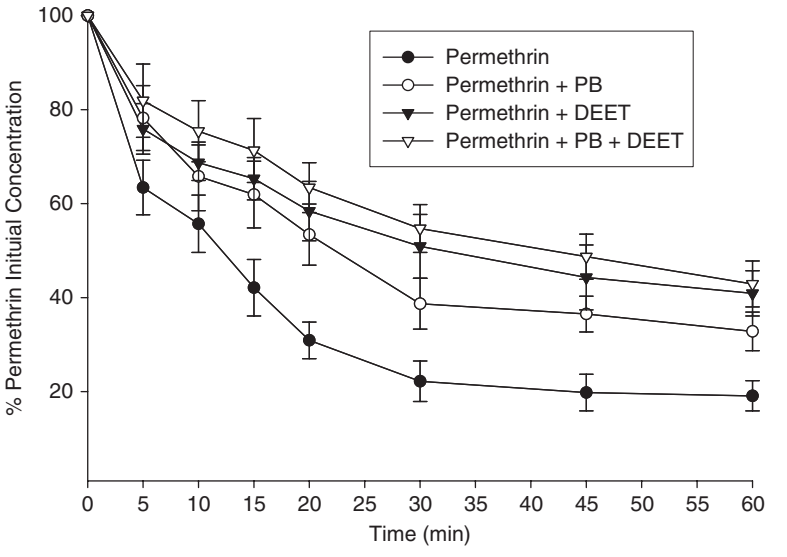

Figure 7. Disappearance of permethrin alone and in combination with PB and DEET following *in vitro* incubation with human liver microsomes.

PB moderately decreased PB metabolism and prolonged its half-life by 1.66-fold. Furthermore, binary incubation of permethrin with PB or DEET, and tertiary incubation with both PB and DEET did not significantly affect the metabolism and disappearance rate of permethrin. In contrast, combined incubation of DEET with PB or permethrin alone greatly decreased the metabolism of DEET, slowed its disappearance, and prolonged its half-life by 2.64- and 3.27-fold, respectively. Furthermore, the metabolism of DEET was further diminished by combined incubation with both PB and permethrin and its half-life was prolonged by 5.31-fold.

Metabolic biotransformation of test compounds

Plasma esterases. *N*-methyl-3-hydroxy pyridinium bromide was detected as the hydrolysis metabolite of permethrin in plasma (Figure 8). The rapid disappearance of permethrin following incubation with human plasma was accompanied by the appearance of its hydrolytic metabolite *m*-phenoxybenzyl alcohol (Figure 9).

Liver microsomal oxidases. The PB metabolite, *N*-methyl-3-hydroxypyridinium bromide, and the permethrin metabolite *m*-phenoxybenzoic acid were detected after the incubation of parent compounds with human liver microsomal incubations. *m*-Toluamide was also identified as metabolites of DEET in human liver microsomal incubates, with K_m and V_{max} values of 62 μM and 112 pmol $min^{-1} mg^{-1}$ of protein, respectively. Figures 10, 11 and 12 show the rate of formation of the PB metabolite, *N*-methyl-3-hydroxypyridinium bromide, the DEET metabolite *m*-toluamide, and the permethrin metabolite *m*-phenoxybenzyl alcohol with human liver microsomal enzymes.

Table II. Apparent first-order disappearance rate constants (K) and half-lives of elimination of PB, DEET, and permethrin alone and in combination following *in vitro* incubation with human liver microsomal oxidases (mean of five experiments).

K (min^{-1}), half-life (min), half-life of combined/parent											
PB				DEET				Permethrin			
PB	PB + DEET	PB + permethrin	PB + DEET + permethrin	DEET	DEET + PB	DEET + permethrin	DEET + PB + permethrin	Permethrin	Permethrin + PB	Permethrin + DEET	Permethrin + PB + DEET
0.011	0.010	0.006	0.006	0.010	0.004	0.0035	0.002	0.011	0.011	0.009	0.009
65.0	66.3	113[a]	108[a]	60.6	162[b]	198[b]	322[b,c]	63.0	64.2	78.7	73.6
1.00	1.02	1.74	1.66	1.00	2.64	3.27	5.31	1.00	1.02	1.25	1.13

Notes:
[a]Significantly different than that of PB alone, $p < 0.05$.
[b] Significantly different than that of DEET alone, $p < 0.05$.
[c] Significantly different than that of DEET + permethrin, $p < 0.05$.

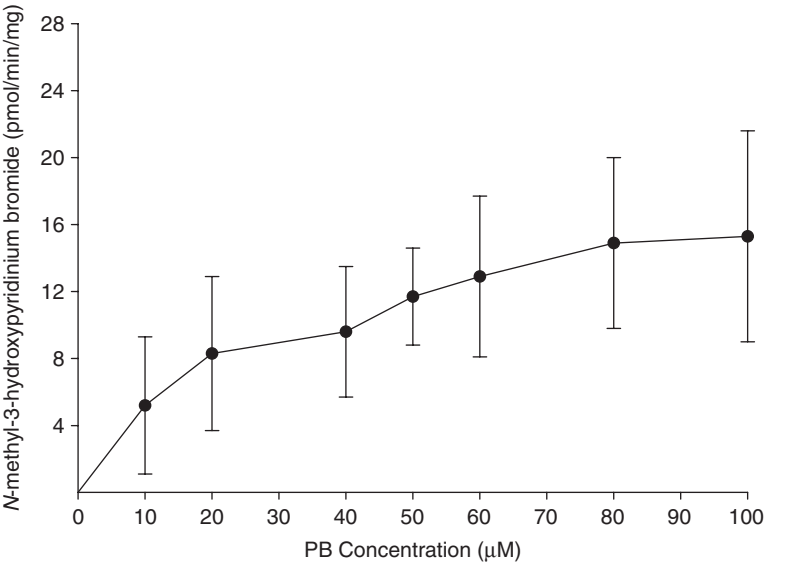

Figure 8. Representative plot for the formation of *N*-methyl-3-hydroxypyridinium bromide following PB incubation in human plasma.

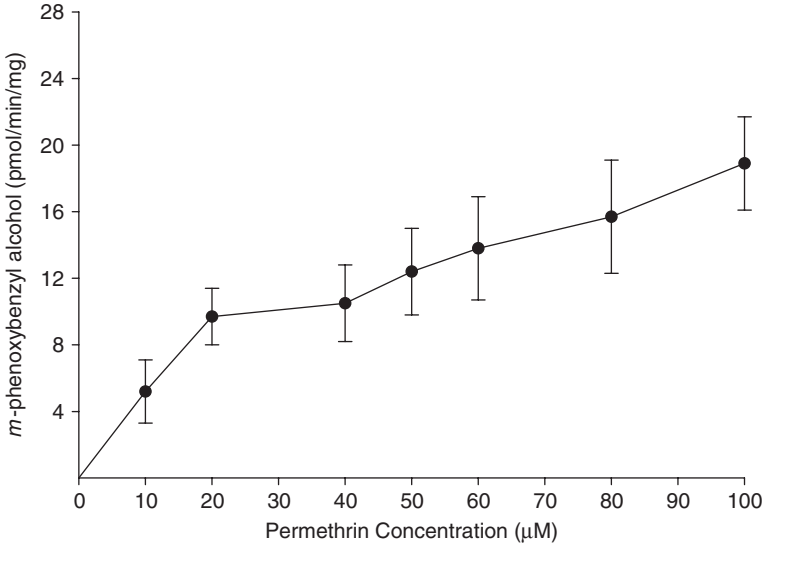

Figure 9. Representative plot for the formation of *m*-phenoxybenzyl alcohol following permethrin incubation in human plasma.

Butyrylcholinestrase inhibition studies

The selective inhibitor of butyrylcholinestrase, tetra-isopropyl pyrophosphoramide (iso-OMPA) inhibited PB and permethrin disappearance in human plasma (Figures 13 and 14), indicating that their metabolism is primarily mediated via aliesterases such as

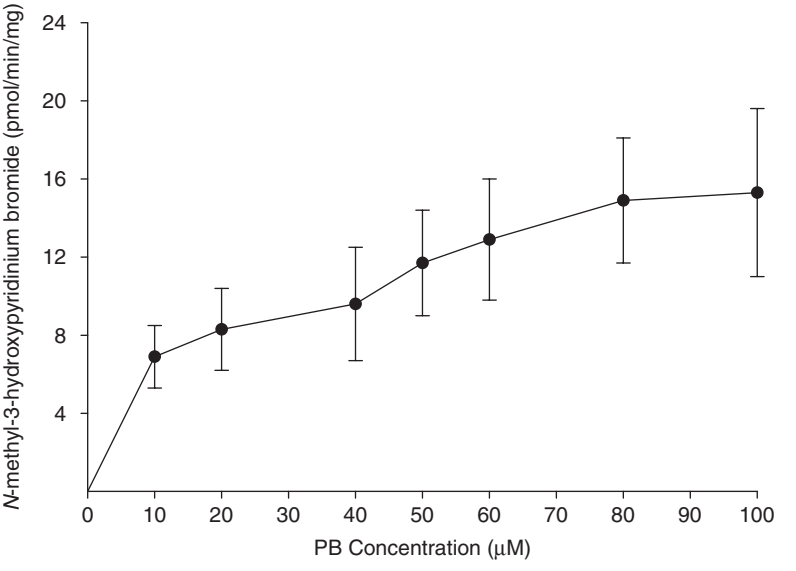

Figure 10. Representative plot for the formation of *N*-methyl-3-hydroxypyridinium bromide following PB incubation in human liver microsomes.

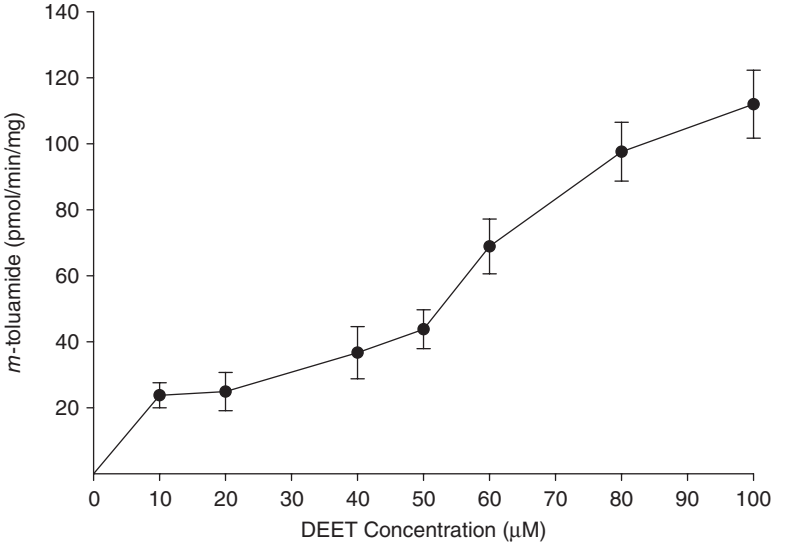

Figure 11. Representative plot for the formation of *m*-toluamide following FEET incubation in human liver microsomes.

butyrylcholinesterase enzyme The extent of iso-OMPA-dependent inhibition as reflected by the IC_{50} values was determined to be 42 and 56 μM following incubation with PB and permethrin, respectively. The results show that BChE plays a major role in the metabolism of PB and permethrin. The results also show that permethrin is moderately more efficiently metabolized by BChE than PB.

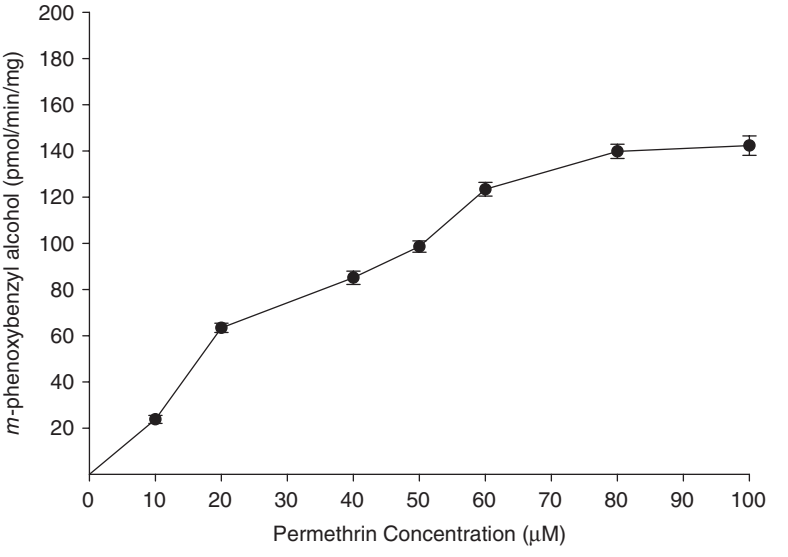

Figure 12. Representative plot for the formation of *m*-phenoxybenzyl alcohol following permethrin incubation in human liver microsomes.

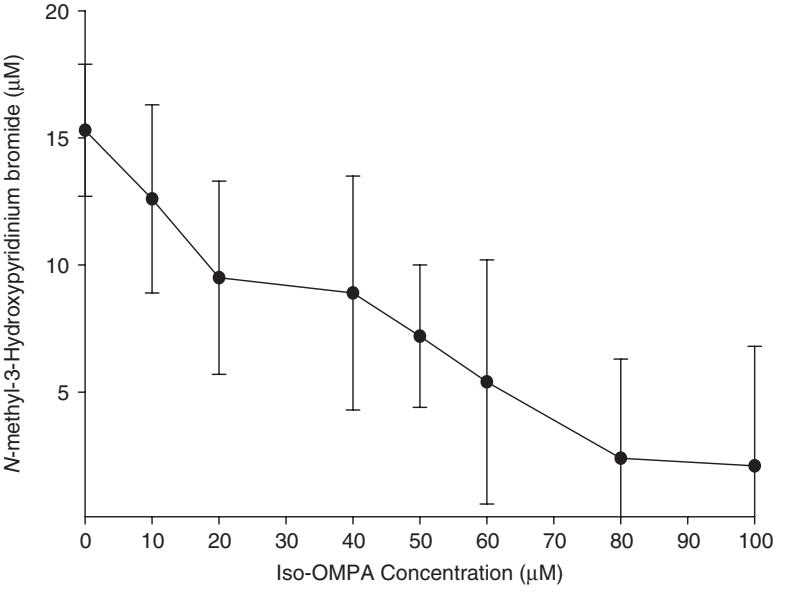

Figure 13. Inhibition of PB metabolism by iso-OMPA.

Discussion

This study presents the profile of *in vitro* human plasma and hepatic metabolism and kinetic disappearance of PB, DEET and permethrin, alone and in combination, and an attempt to identify specific enzymes involved in their metabolic pathways. The results indicate that PB

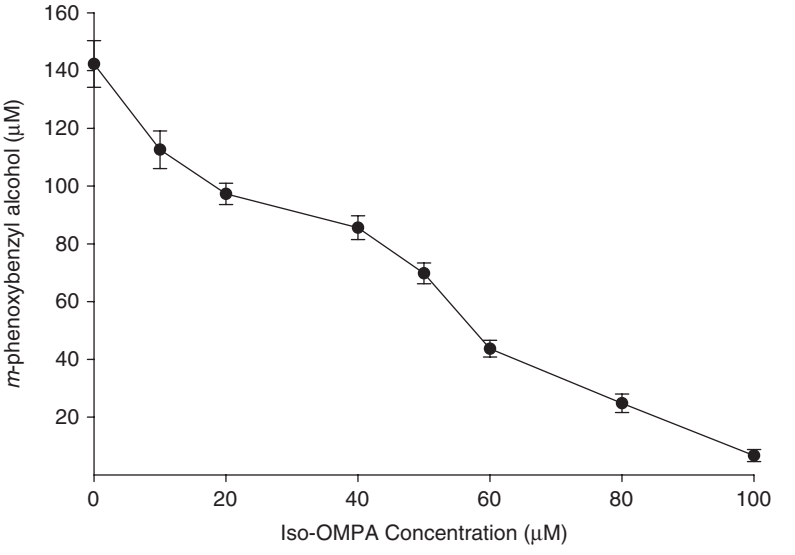

Figure 14. Inhibition of permethrin metabolism by iso-OMPA.

and permethrin are metabolized by both human plasma and liver microsomal enzymes and that DEET is mainly metabolized by liver cytochrome oxidase enzymes.

Incubation of test compounds with plasma showed the following:

- PB and permethrin were efficiently metabolized by human plasma. However, DEET metabolism was very slow even after longer incubation.
- Binary incubation with permethrin decreased the metabolism of PB and its disappearance from plasma, but it also seemed to enhance the metabolism of DEET in plasma.
- Binary incubation with PB greatly decreased the metabolism of permethrin and its disappearance rate, but it increased the metabolism of DEET in plasma.
- Binary incubation of DEET had no effect on metabolism of either PB or permethrin in plasma.
- Tertiary incubation with PB and DEET greatly decreased the metabolism of permethrin and its disappearance from plasma.
- Tertiary incubation of DEET and permethrin decreased the metabolism of PB and delayed its disappearance.
- Tertiary incubation with PB and permethrin increased the metabolism of DEET in plasma.

PB, a carbamate, and permethrin, an ester, rapidly disappeared following 60-min incubation with plasma as the result of hydrolysis via esterase enzymes to yield the hydrolysis products *N*-methyl-3-hydroxypyridinium bromide and *m*-phenoxybenzyl alcohol, respectively. These results are in agreement with previous studies that reported the formation of such metabolites *in vivo*: the *m*-phenoxybenzyl alcohol metabolite of permethrin was detected in plasma and in tissues up to 48 h after an oral dose in rats (Anadon et al. 1991). Furthermore, the PB metabolite *N*-methyl-3-hydroxypyridinium bromide has been identified in human and rat plasma and urine (Taylor et al. 1991; Abu-Qare & Abou-Donia 2000).

The results show that permethrin and PB compete for certain metabolism enzymes in human plasma because both compounds diminished the rate of disappearance of the other compound following binary incubations. The effect of permethrin on PB disappearance was more pronounced, indicating that permethrin was a better substrate for human plasma esterases than PB, and effectively competed with it for metabolism. This is consistent with the previous report of Choi et al. (2004) that hydrolysis of permethrin is a main route of detoxification and a potential site for interactions with compounds metabolized through similar pathways. Furthermore, Baynes et al. (2002) reported that PB modulates the dermal disposition of permethrin in isolated perfused porcine skin flap. Further, Hoy et al. (2000) reported an increase in the concentration of permethrin in the serum of male and female rats following combined administration of PB with permethrin compared with permethrin alone. The inhibition of permethrin and PB hydrolysis by the addition of the selective butyrylcholinesterase inhibitor iso-OMPA indicates that butyrylcholinesterase plays a major role in the metabolism of permethrin and PB metabolism, with permethrin being a better substrate than PB.

DEET, an amide, did not significantly disappear following 1 or 8 h of incubation with human plasma indicating that DEET was not efficiently metabolized by human plasma esterases *in vitro*. This conclusion is also supported by the results that PB and permethrin that were efficiently hydrolysed by plasma esterases did not compete with DEET for metabolism enzymes. In contrast, either compound alone or in combination increased the metabolism of DEET and enhanced its disappearance from human plasma. PB and permethrin may enhance amidase activity by increasing its expression or its stability by inhibiting specific proteases that breakdown amidases. The present result that DEET did not alter the *in vitro* metabolism of PB or permethrin in human plasma indicates that esterases are not involved in the metabolism of DEET. The lack of inhibition of esterases by DEET is consistent with earlier findings that DEET synergized the toxicity to German cockroaches, of acetyl cholinesterase inhibitors, malathion, carbaryl, and PB, but not of bendiocarb, or chlorpyrifos indicating that DEET has a mechanism other than acetylcholinesterase inhibition responsible for the toxic interactions between DEET and the tested chemicals (Moss 1996). Furthermore, DEET antagonized the toxicity of PB against *Escherichia coli* by inhibiting P-glycoprotein and PB uptake (El-Masry & Abou-Donia 2006).

Incubation of each of the three compounds, alone or in combination with human liver microsomes in the presence of an NADPH-generating system, revealed the following:

- PB, DEET, and permethrin are metabolized to a similar extent with human liver microsomal enzymes.
- Binary incubation with permethrin, moderately decreased PB metabolism, but greatly diminished the metabolism of DEET and prolonged their disappearance from microsomal incubates.
- Binary incubation of PB had no effect on the metabolism of permethrin and its disappearance rate but decreased the metabolism of DEET and delayed its disappearance.
- Binary incubation of DEET had no effect on the metabolism of either PB or permethrin.
- Tertiary incubation with PB and DEET had no significant effect on the metabolism of permethrin and its disappearance from plasma.
- Tertiary incubation of DEET and permethrin moderately decreased the metabolism of PB and delayed its disappearance.
- Tertiary incubation with PB and permethrin significantly decreased the metabolism of DEET.

Thus, whereas combined incubation with DEET did not affect the metabolism of PB or permethrin, both PB and permethrin, alone or in combination, greatly decreased the metabolism DEET and its disappearance via liver microsomal enzymes. These results suggest that liver microsomal enzymes that metabolize DEET might also metabolize PB and permethrin. In contrast, liver microsomal enzymes that predominately metabolize PB and permethrin did not significantly affect metabolism of DEET.

In the present study, the DEET oxidative metabolite *m*-toluamide was identified following incubation with human liver microsomal enzymes indicating that DEET was metabolized through oxidative *N*,*N*-deethylation. Previous reports indicated that DEET was metabolized through *N*-dealkylation, ring hydroxylation and ring dealkylation following incubation with rat liver microsomes (Constantino & Iley 1999). These results are in agreement with previous studies that reported the detection of the DEET metabolite *m*-toluamide following DEET incubation in rat liver microsomes (Taylor 1986; Yeung & Taylor 1988), and following dermal administration of DEET in rats (Schoenig et al. 1996), and in cattle (Taylor et al. 1994). An *in vitro* study using human liver microsomes indicated that DEET was metabolized by the following P450 enzymes: CYPs1A2, 2B6 and 2D6*1 (Val.374), and 2E1 to yield *N*,*N*-diethyl-*m*-hydroxymethylbenzamide, with CYP2B6 being the principal CYP responsible its production (Usmani et al. 2002). On the other hand, CYPs2A6, 3A4, 3A5, and 2C19 produced an *N*-deethylated metabolite, *N*-ethyl-*m*-toluamide, with 2C19 exhibiting the greatest activity.

The addition of PB and permethrin to DEET with human liver microsomes decreased the rate of disappearance of DEET. This is in agreement with Usmani et al. (2002) who reported that pre-incubation of human or rodent microsomes with PB or permethrin can lead to either stimulation or inhibition of DEET metabolism, respectively. The differential metabolic interactions between PB, DEET and permethrin might explain the regional selective neurotoxicity induced by test compounds in rat brains when tested alone or in combination (Abdel-Rahman et al. 2001). Thus, dentate gyrus and hippocampal regions exhibited a significantly greater neuronal cell death from each chemical alone than exposure to combined DEET and permethrin. In contrast, in the Purkinje cell layer of the cerebellar hemisphere, exposure to combined DEET and permethrin caused more injury than exposure to both chemicals when given singly. Decreased neurotoxicity or antagonism might result from increased DEET metabolism via amidases, with combined exposure to PB and permethrin, whereas increase toxicity or synergism might result from competition of the three chemicals for oxidase enzymes.

The permethrin metabolites *m*-phenoxybenzyl alcohol and *m*-phenoxybenzoic acid were identified in the liver microsome incubates, indicating oxidation of permethrin. Previous studies reported the involvement of cytochrome P450 enzymes in permethrin metabolism; Vulule et al. (1999) reported an increased toxicity of permethrin when co-administered with the cytochrome P450 inhibitor piperonyl butoxide. The findings of the present study suggest that *in vitro* combined incubation of PB, DEET and permethrin with human liver microsomes might have significantly altered the rate but not the pattern of their transformation.

In summary, human esterases and oxidases are involved in the metabolism of PB and permethrin *in vitro*. The metabolism of both PB and permethrin is catalysed by butyrylcholinesterase in human plasma as shown from the inhibition of this enzyme by iso-OMPA, with permethrin being more efficiently metabolized than PB. Both of these latter chemicals also undergo oxidative metabolism presumably via the cytochrome P-450 oxidase system. Metabolism of DEET, however, is mainly catalysed by cytochrome P450 enzymes.

The results predict that combined exposure to test compounds results in increased neurotoxicity than when given singly by: competition of PB and permethrin for plasma butyrylcholinesterase; and PB, permethrin, and DEET can also compete for presumably, the cytochrome P450 oxidase system-catalysed metabolism of the three compounds.

In conclusion, this study explains, at least in part, the reported increased neurotoxicity of the compounds examined following their combined exposure by US military personnel during the Persian Gulf War. Combined exposure to test chemicals increases their neurotoxicity by impeding the body's ability to rid itself of them because of the competition for detoxifying enzymes by these chemicals, leading to their persistence in the body and enhancing their delivery to the nervous system and increasing their concentration at neurotoxicity target.

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