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

This study examines the metabolism and interactions of xenobiotic toxicants important in military deployments as inducers, substrates and/or inhibitors of human enzymes including the important isoforms of these enzymes HPLC analytical methods have been brought on line for chlorpyrifos, DEET, permethrin and methods for pyridostigmine bromide and sulfur mustard metabolites are being tested. Five human cytochrome P450 (P450) isoforms metabolizing chlorpyrifos have been identified. They include forms known to be inducible and forms known to be polymorphic. These forms vary through a 50-fold range in their ability to produce chlorpyrifos oxon, the active metabolite, as compared to the production of detoxication products. Both quantitative and qualitative variations between the ability of microsomes from individual human livers to metabolize chlorpyrifos were observed, suggesting that different sensitivities toward organophosphorus toxicants exist in the human population. DEET is shown to be metabolized by human P450 isoforms and, in mice, to be an inducer of P450. These and the remaining studies will permit more confident extrapolation of animal studies to humans and permit identification of interactions not apparent from animal studies. It may also permit identification of human subpopulations at greater risk from specific xenobiotic toxicants and will produce specific analytic methodologies for assessment of future exposures.

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INTRODUCTION

Investigations of deployment-related chemicals carried out in experimental animals assume relevance only when they can be extrapolated with confidence to humans. Since most exogenous chemicals can be substrates, inhibitors and/or inducers of xenobiotic-metabolizing enzymes (XMEs), these enzymes are an important potential locus for interactions. Many XMEs are polymorphic, potentially putting some individuals at greater risk than others. We are examining the role of specific human XMEs on the metabolism of a subset of chemicals important in military deployments. Chlorpyrifos, diethyl toluamide (DEET), permethrin, pyridostigmine bromide as well as sulfur mustard and its degradation products are being tested as substrates and inhibitors of the most important human XMEs, including cytochrome P450s (CYPs), flavin-containing monooxygenases (FMOs), alcohol dehydrogenase and esterases. The potential of the test chemicals to act as XME inducers is being tested in mice. HPLC methods are being adapted or developed to facilitate these studies. The results will enable findings from animal studies to be extrapolated to humans with confidence, permit the identification of populations at risk and provide analytical methods for exposure assessment.

BODY

With some exceptions, the three items outlined in the Statement of Work for Year 1 have been accomplished. In addition, particularly in the case of chlorpyrifos (CPS) and DEET we have gone beyond the Year one tasks and accomplished tasks projected for subsequent years.

Task 1. "Development of new HPLC methods or validation and/or modification of existing methods for analysis of all test compounds and their metabolites.

Chlorpyrifos:

The method developed for the analysis of CPS and its metabolites utilizes a Shimadzu HPLC system (Kyoto, Japan) consisting of 2 pumps (LC-10AT) and an auto injector (SIL-10AD *VP*). The mobile phase for pump A is10% acetonitrile, 89% water and 1% phosphoric acid, for pump B 99% acetonitrile and 1% phosphoric acid. A gradient system is initiated at 20% pump B and increased to 100% pump B in 20 min. A flow rate of 1 ml/min is used. Metabolites are separated on a C18 column (Luna 5 μ, 150 X 3 mm, Phenomenex, Rancho Palos Verdes, CA) and detected by a Waters 486 Tunable Absorbance Detector at 230 nm. Retention times for 3,5,6-trichloro-2-pyridinol (TCP), chlorpyrifos-oxon (CPO) and CPS are 8.5, 12 and 17 min, respectively. Concentrations of metabolites are obtained by extrapolation of peak height from a standard curve and Km_{app} and $Vmax_{app}$ obtained using a Hanes-Woolf plot. *Chlorpyrifos studies were also supported, in part, by the North Carolina Environmental Trust Fund.*

DEET:

The method developed for the analysis of DEET and its metabolites (*N*-ethyl-*m*-toluamide (ET) and *N*, *N*-diethyl-*m*-hydroxymethylbenzamide (BALC) utilized the same Shimadzu HPLC system (Kyoto, Japan), and the same column as that used for chlorpyrifos (above). The metabolites were a generous gift from Dr. Wesley Taylor (Saskatoon Research Center, SK Canada). The mobile phase for pump A is 3.5% tetrahydrofuran, 96.5% water and for pump B 100% acetonitrile (ACN). A gradient system was employed in the following manner: 0-3 min (20-10% B), 3-30 min (20-60% B), 30-32 min (60-10% B), and 32-35 min (10% B). Again, detection was accomplished using a Waters 486 Tunable Absorbance Detector at 230 nm. Concentrations of metabolites were obtained by extrapolation from a standard curve.

Permethrin:

The method developed for the analysis of two permethrin isomers (*trans* and *cis*) and their metabolites (phenoxybenzoic acid and phenoxy benzyl alcohol) utilizes a Shimadzu HPLC system (Kyoto, Japan) consisting of a pump (LC-10AT VP), a solvent proportioning valve (FCV-10AL VP), a degasser (DGU-14A), a controller (SCL-10A VP) and an auto injector (SIL-10AD VP). A binary gradient elution scheme using H_2O (pH 3.0) and acetonitrile is used. The gradient system (linear increase) is initiated with 30% H_2O and 70% acetonitrile reaching 99% acetonitrile at 6 minutes. Ninety nine percent acetonitrile is maintained for 5 minutes and then H_2O concentration increased to 30% at 15 minutes. A flow rate of 1ml/min is used. Permethrins and metabolites are separated on a Phenomenex (Rnacho Palos Verdes, CA) Synergy column (Max RP 80A, 4μm, 150 X 4. 6mm) and detected by a Shimadzu APD-10AV VP UV-VIS detector at 230 nm. Retention times for phenoxybenzyl alcohol, phenoxybenzoic acid, *trans*-permethrin and *cis*-permethrin were 3.68, 4.23, 10.27 and 10.61 minute, respectively. Data integration and standard curve extrapolation are conducted using a Shimadzu Class VP Chromatography Data System software (version 4.3).

Pyridostigmine bromide:

A reliable, sensitive HPLC method for the separation and quantitation of pyridostigmine bromide and its possible metabolite was developed by Leo (1996). The equipment described above is compatible with this method and its use in the current studies will require little, if any, adaptation.

Sulfur Mustard and its Degradation Products:

These compounds have always presented an analytical challenge. Their hydrophobic nature and low extinction coefficients in the UV/visible region make them difficult to separate and detect by commonly used techniques.

Micellar electrokinetic chromatography (MEKC), capillary electrophoresis using a Beckman

P/ACE System 5500 instrument (Cheicante et al. J. Chromatog. A. 711:347 (1995) using UV absorption at 214 nm is being investigated as a method for monitoring thiodiglycol and its oxidation products. MECK provides sufficient separation for the analysis of samples containing thiodiglycol and its sulfoxide. If this method is found to be sufficiently sensitive it will be used to screen for metabolism of thiodiglycol by recombinant human P450 isozymes and pooled microsomes. Under current conditions the detection limits for thiodiglycol and thiodiglycol sulfoxide are 1 μg/ml and 7 μg/ml, respectively. Achieving additional sensitivity through the production of fluorescent derivatives and the use of a Lasar Induced Fluorescence detector is being investigated.

An HPLC method has also been developed using a chromatographic system from Waters Corp. (Milford, MA) consisting of a pump (Model 510) a tunable absorbance detector (Model 486) a column heater and an integrator/ data module (Model 746) which allows baseline separation of thiodiglycol, thiodiglycol sulfoxide and thiodiglycolic acid sufficient to permit its use in the quantification of thiodiglycol oxidation products from metabolism by P450 and alcohol dehydrogenase. The system uses a 300 mm Shodex RSpac KC-811 column (Showa Denko, Tokyo) which consists of a rigid sulfonated styrene-divinyl benzene copolymer ion-exchange resin. The column is operated isocratically at a 1 ml/min flow rate and 50^0 C with 0.1% H_3PO_4 as a mobile phase. Retention times under these conditions are: thiodiglycol sulfoxide 10.5 min, thiodiglycolic acid 12 min and thiodiglycol 13.5 min. Sensitivity is in the micromolar range monitoring at a wavelength of 200 nm.

An additional aspect of the work outlined under this cooperative agreement was investigation of the participation of isozymes human alcohol dehydrogenase (ADH) in the metabolism of the environmental breakdown products of sulfur mustard. TDG has been documented as a substrate for mammalian ADHs. However, the same challenges applied to an evaluation of its fate under the influence of ADH as those that have hampered the studies using CYPs and FMOs, namely it presents analytical difficulties. Recent advances in instrumentation and techniques using NMR have enabled us to establish the fate of TDG as it is acted upon by horse liver ADH (Henehan and Oppenheimer, 1993) These techniques allowed us to identify ADH as the source of a new oxidative metabolite of TDG, 2-hydroxyethyl thioacetic acid and to identify this compound as the end product of this oxidation pathway. Kinetic studies and the investigation of recombinant human isozymes (a gift from Dr. Gary W. Winston) are in preparation. This work has been carried out on a 600 mega Hz instrument made by Varian Instruments (Palo Alto, CA)

Task 2. Determination of substrate specificity of human CYP and FMO isoforms relative to the test compounds.

Chlorpyrifos:

Protein concentrations and incubation times used in the assays were determined in preliminary experiments to be in the linear ranges. No metabolites were detected when

incubation of microsomes with CPS was carried out in the absence of an NADPH generating system.

Rat liver microsomes (RLM) displayed a greater affinity (i.e., lower Km_{app}) and reaction velocity toward CPS for both desulfuration and dearylation than human liver microisomes (HLM). Compared to HLM, mouse liver microsomes (MLM) showed similar affinities but a greater reaction velocity toward CPS (Table 1).

Table 1. Dearylation and Desulfuration of Chlorpyrifos by Pooled Human, Rat and Mouse Liver Microsomes

Enzyme	Km_{app} (μM)	$Vmax_{app}$ (nmol/mg protein/min)	$Vmax_{app}/Km_{app}$
		Dearylation	
HLM	14.64±0.74	0.71±0.03	0.048
RLM	6.79±1.62*	2.10±0.37*	0.347
MLM	14.62±1.61	2.45±0.18**	0.170
		Desulfuration	
HLM	11.02±1.18	0.46±0.05	0.041
RLM	4.76±0.92*	1.08±0.03**	0.248
MLM	12.27±1.95	0.78±0.07*	0.068

Activities are express as the mean±SEM (n=3). Means in HLM that different significanly from RLM or MLM are indicated by *($P \leq 0.05$) or **($P \leq 0.01$).

CYP1A2, 2B6, 2C9, 2C19 and 3A4 were identified as the CYP isoforms involved in CPS metabolism (Table 2). Desulfuration and dearylation activities were greatest for CYP2B6 and CYP2C19, respectively, followed by CYP3A4. Changes in metabolic activity toward CPS were observed in different polymorphic forms of CYP2C19 (Table 3).

Table 2. Metabolic Activities[1] Toward Chlorpyrifos In Human Lymphoblast-Expressed Cytochrome P450 Isoforms

	Dearylation[2]	Desulfuration[2]
1A2	0.17±0.01 [a]	0.68±0.03 [a]
2B6	0.21±0.02 [a]	1.71±0.14 [b]
2C9	0.43±0.08 [a]	0.31±0.31 [a]
2C19	1.60±0.43 [b]	0.46±0.46 [a]
3A4	0.44±0.17 [a]	1.13±0.06 [ab]

[1] Activities are expressed as nmol product/nmol P450/min ± SEM (n=3).
[2] Means in the same column followed by the same letter are not significantly different ($P<0.05$).

Table 3. Metabolic Activities[1] Toward Chlorpyrifos In CYP2C19 Expressed in *E. Coli*

	Dearylation[2] (nmol product/nmol P450)	Desulfuration[2] (nmol product/nmol P450)
2C19*1B	6.08±0.26[A]	1.26±0.02
2C19*8	1.36±0.15[B]	Not detected
2C19*5	0.27±0.10[BC]	Not detected
2C19*6	0.25±0.15[C]	Not detected

[1] Activities are expressed as nmol product/nmol P450/min ± SEM (n=3).
[2] Means in the same column followed by the same letter are not significantly different ($P<0.01$).

Pooled female HLM showed significantly higher metabolic activity toward CPS than pooled male HLM (Table 4).

Table 4. Metabolic Activities[1] Toward Chlorpyrifos In Pooled Male and Female Human Liver Microsomes (HLM)

	Dearylation		Desulfuration	
	nmol/mg protein/min	nmol/nmol P450/min	nmol/mg protein/min	nmol/nmol P450/min
Male	0.43±0.02	0.96±0.04	0.35±0.01	0.77±0.01
Female	0.76±0.03**	1.20±0.04*	0.56±0.02**	0.89±0.03*

[1] Activities are expressed as mean ± SEM (n=3). Means in female HLM that are significantly different from HLM are indicated by * ($P<0.05$) or **($P<0.01$). Protein concentration and cytochrome P450 content in microsomes are provided by supplier (XenoTech, LLC, Kansas City, KS).

In an attempt to determine the relative importance of CYP2B6, 2C19 and 3A4 in the activation and detoxication of chlorpyrifos, liver microsomes from four individuals

possessing varying levels of these isoforms were examined with respect to their ability to metabolize chlorpyrifos (Table 5). The dearylation pathway was more predominant in the individual with high CYP2C19 but low 3A4 levels. Individuals possessing high levels of CYP2B6 and 3A4 had the greatest desulfuration activities (Table 5).

Table 5. Metabolic Activities[1] Toward Chlorpyrifos In Pooled Human Liver Microsomes (HLM), Rat Liver Microsomes (RLM), Mouse Liver Microsomes (MLM) or Individual HLM

	Dearylation [2] (nmol/mg protein/min)	Desulfuration [2] (nmol/mg protein/min)
HLM [3]	0.69±0.02^{bc}	0.44±0.04^{c}
RLM	2.01±0.34^{a}	1.07±0.04^{a}
MLM	2.17±0.14^{a}	0.71±0.08^{b}
HG006 [4]	0.32±0.02^{c}	0.16±0.02^{d}
HG042 [4]	0.72±0.07^{bc}	0.76±0.09^{b}
HG043 [4]	0.66±0.05^{bc}	0.17±0.02^{d}
HG112 [4]	0.98±0.08^{b}	0.70±0.06^{b}

[1] Activities are expressed as mean ± SEM (n=3-4).
[2] Means in the same column followed by the same letter are not significantly different ($P<0.05$).
[3] Pooled human liver microsomes (from 10 donors, protein concentration 20 mg/ml).
[4] Individual human liver microsomes (protein concentration 20 mg/ml). CYP2B6, 2C19 and 3A4 activities (pmol/mg protein/min), represented by (S)-mephenytoin N-demethylase, (S)-mephenytoin 4'-hydroxylase, and testosterone 6β-hydroxylase catalytic activities, respectively, are 3.1, 36.0, and 2990 for HG006 (16-year-old male), 140.0, 3.5, and 14530 for HG042 48-year-old female), 7.4, 212.0, and 3408 for HG043 (23-year-old female), and 59.1, 260.0, and 17519 for HG112 (2-year-old female) (data were provided by Gentest, Woburn, MA).

In summary of the results to date, HLM and human CYP isoforms utilize the same pathways as RLM and MLM to metabolize CPS. Pooled HLM show lower activities in CPS metabolism than RLM and MLM, suggesting that the human population in general is not as active as rodents in CPS metabolism. Human lymphoblast-expressed CYP1A2, 2B6, 2C9*1, 2C19 and 3A4 show oxidation activities toward CPS. While CYP2B6 and CYP2C19 display the greatest desulfuration and dearylation activities, respectively, CYP3A4 is the major isoform responsible for CPS metabolism in HLM. Pooled female HLM display a higher activity in CPS metabolism than pooled male HLM. Variation in human population results in

at least a 3- to 4-fold difference among individuals in CPS metabolism. *Chlorpyrifos studies were also supported, in part, by the North Carolina Environmental Trust Fund.*

DEET:

HLM have metabolic activity toward DEET resulting in the production of both the ring hydroxymethyl metabolite, BALC, and the N-deethylated metabolite, ET, although the activities are somewhat lower than in rodents, RLM and MLM. (Table 6)

Table 6. Microsomal oxidation of DEET by Pooled Human, Rat and Mouse Liver Microsomes

Enzyme	Km_{app} (μM)	$Vmax_{app}$ (nmol/mg protein/min)	$Vmax_{app}/Km_{app}$
Ring Methyl Oxidation			
HLM	67.6±4.2	12.9±1.6	191.5
RLM	25.8±4.7	17.3±1.1	672.1
MLM	43.4±0.6	6.8±1.4	156.8
MLM*	42.6±13.6	16.4±3.4	385.1
N-Deethylation			
HLM	842.5±49.9	20.5±3.4	24.4
RLM	214.3±26.1	19.2±2.8	89.5
MLM	660.6±59.5	14.5±2.9	21.7
MLM*	630.9±128.0	22.9±2.9	38.3

*From DEET treated animals (200 mg/kg/day)

Among the human CYP isoforms screened, CYPs 1A2, 2B6, 2D6*1 (Val374), and 2E1 all displayed detectable activity for the production of BALC, with CYPs 1A2, 2B6, 2D6*1 (Val374) showing significantly higher activities than CYP2E1. Formation of ET metabolite, on the other hand, depended on an entirely different set of CYPs, including 3A4, 3A5, 2A6, and 2C19.

Permethrin:

Initial studies indicate that permethrin is not oxidized by HLM, RLM or MLM but that both cis- and trans-permethrin are hydrolyzed by microsomal esterases in all three species. These studies will be expanded to include possible secondary oxidative metabolism of the products of esterase action and the role of esterases in cellular compartments in addition to microsomes.

Pyridostigmine bromide: An earlier study by Leo (1996) provided evidence that pyridostigmine bromide is not metabolized in humans and specifically that it is not a substrate for human CYPs 1A1, 2C9, 2E1, 2D6 and 3A4. If these findings are confirmed studies to be initiated will concentrate on pyridostigmine as an inhibitor of XMEs, including P450s, FMOs and esterases.

Sulfur Mustard and its Degradation Products:

Initial studies with rat and mouse liver microsomes indicate that in these species TDG is not oxidized in either the the P450 or the FMO systems. Early results with the flavin monooxygenases had a similar outcome. Whether this is a biochemical fact or an analytical shortcoming awaits the application of our capillary electrophoresis method and/or the new HPLC method using the Shodex KC-811 column. Initial work with human CYP 2E1 (Gentest Laboratories, Woburn, MA) did not provide indications of metabolism with TDG as a substrate. However, the compound was an inhibitor of *p*-nitrophenol hydroxylase (catechol production) by CYP 2E1 at the millimolar level.

Task 3. Initiate induction experiments with mice.

Initial induction studies were carried out with adult male CD-1 mice, 28-30 grams treated with daily doses DEET (low, 2 mg/kg medium, 20 mg/kg, and high, 200 mg/kg), phenobarbital (PB), and 3-methylcholanthrene (3-MC) administered intraperitoneally for 3 days. Microsomes were prepared from livers on the fourth day.

The following substrates were used as indicators of the activities of the following isozymes: ethoxyresorufin O-deethylation (EROD) for CYP1A1 (3), methoxyresorufin O-demethylation (MROD) for CYP1A2 (4), pentoxyresorufin O-dealkylation (PROD) for CYP2B10 (5), and benzyloxyresorufin O-dealkyaltion (BROD) for CYP2B6. Mice treated with DEET exhibited significantly higher activities toward dealkylation of BROD and PROD indicating induction of CYP2B6 and 2B10 although the dose required was in the medium to high range.

The metabolism of chlorpyrifos (CPS) to chlorpyrifos-oxon (CPO), and trichloropyridinyl (TCP) by MLM from treated mice was also determined. The activity assays were performed by the methods described above under task 1. It is interest, in consideration of possible

interactions between the test chemicals, that DEET treated MLM metabolized chlorpyrifos more easily to chlorpyrifos-oxon, a potent anticholinesterase, than did control MLM.

KEY RESEARCH OUTCOMES

- Analytical methods for the test compounds, chlorpyrifos, DEET, permethrin, pyridostigmine bromide and sulfur mustard metabolites have been modified and are available for all of the proposed experiments.

- The most complete description to date of the human metabolism of an organophosphorus toxicant has been developed. CYP isoforms involved in detoxication and activation of chlorpyrifos, the possible effects of poymorphisms and variation between individuals are all part of this model.

- The first description of DEET metabolism by human enzymes has been accomplished, with identification of the CYP isoforms involved in the production of the two principal metabolites.

- Studies of induction by DEET in mice raise the possibility of an interaction between DEET and chlorpyrifos since induction by DEET increased the capacity of mouse liver microsomes to metabolize chlorpyrifos.

REPORTABLE OUTCOMES

Presentations given and submitted:
International Society for the Study of Xenobiotics (ISSX), 2000
Tang, J., Y. Cao, S. Coleman, R. L. Rose, J. A. Goldstein and E. Hodgson. In vitro metabolism of chlorpyrifos by human liver microsomes and human cytochrome P450 isoforms.

Conference on Illnesses among Gulf War Veterans: A decade of scientific research", 2001
Tang, J., Y. Cao, R. L. Rose, A. A. Brimfield, J. A. Goldstein, and E. Hodgson. Differential activities of chlorpyrifos metabolism in human liver microsomes and cytochrome P450 isoforms.
Usmani, K. A., R. L. Rose, J. A. Goldstein and E. Hodgson. Metabolism of diethyl toluimide (DEET) by pooled human liver microsomes and human cytochrome P450 isoforms.

Society of Toxicology, 2001
Tang, J., Y. Cao, R. L. Rose, A. A. Brimfield, J. A. Goldstein and E. Hodgson, Metabolic activity of human cytochrome P450 isoforms toward chlorpyrifos.

CONCLUSIONS

HPLC analytical methods have been brought on line for chlorpyrifos, DEET, permethrin and methods for pyridostigmine bromide and sulfur mustard metabolites are being tested. Capillary electrophoresis is being applied to sulfur mustard metabolites. Five human cytochrome P450 isoforms metabolizing chlorpyrifos have been identified. They include forms known to be inducible and forms known to be polymorphic. These forms vary through a 50-fold range in their ability to produce chlorpyrifos oxon, the active metabolite, as compared to the production of detoxication products. Both quantitative and qualitative variations between the ability of microsomes from individual human livers to metabolize chlorpyrifos were observed, suggesting that different sensitivities toward organophosphorus toxicants exist in the human population. DEET has also been shown to be metabolized by human cytcohrome P450 isoforms and, in mice, to be an inducer of cytochrome P450.

These studies and the successful completion of the remaining studies will permit more confident extrapolation of past and future animal studies to humans and permit identification of interactions not apparent from animal studies. It may also permit identification of human subpopulations at greater risk from specific xenobiotic toxicants and will produce specific analytic methodologies for assessment of future exposures.

It is becoming increasing apparent that induction studies previously requiring the use of experimental animals may be accomplished directly with human materials. We are exploring the possibility that emerging techniques that maintain the capacity for induction in cultured human hepatocytes combined with microarray techniques for determination of gene expression and repression may substitute for the proposed animal studies. If this proves to be a viable approach, a modified proposal will be submitted.

REFERENCES

Three references are added to those listed in the original proposal.

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APPENDICES: None