Identification of rat and human cytochrome P450 isoforms and a rat serum esterase that metabolize the pyrethroid insecticides deltamethrin and esfenvalerate.


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CYPs and serum esterase which metabolize delta and esfen

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Abstract

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Abbreviations
CEs, carboxylesterases
hCE-1, Human carboxylesterase 1
hCE-2, Human carboxylesterase 2
esfen, esfenvalerate
delta, deltamethrin
CYP – cytochrome P450
Abstract

The metabolism of deltamethrin and esfenvalerate by rat and human liver microsomes differ with respect to the biotransformation pathway (oxidation versus hydrolysis) responsible for their clearance. This study aims to further explore the species differences in the metabolism of these chemicals. Using a parent depletion approach, rat and human CYPs were screened for their ability to eliminate deltamethrin or esfenvalerate during in vitro incubations. Rat CYP isoforms 1A1, 2C6, 2C11, 3A2 and human CYP isoforms 2C8, 2C19, and 3A5 were capable of metabolizing either pyrethroid. Human CYP2C9 metabolized esfenvalerate but not deltamethrin. Rat and human CYPs that metabolize esfenvalerate and deltamethrin do so with similar kinetics. In addition to the liver, a potential site of metabolic elimination of pyrethroids is the blood via serum carboxylesterase (CEs) hydrolysis. The serum of rats, but not humans, contains significant quantities of CEs. Deltamethrin and esfenvalerate were metabolized effectively by rat serum and a purified rat serum CE. In contrast, neither pyrethroid was metabolized by human serum or purified human serum esterases (acetylcholinesterase and butyrylcholinesterase). These studies suggest that the difference in rates of oxidative metabolism of pyrethroids by rat and human hepatic microsomes are dependent on the expression levels of individual CYP isoforms rather than their specific activity. Furthermore, these studies show that the metabolic elimination of deltamethrin and esfenvalerate in blood may be important to their disposition in the rat but not in the human.
Introduction

Pyrethroid pesticides are synthetic analogs of pyrethrins, the natural insecticidal products of *Chrysanthemum cinerariaefolium*. Compared to the pyrethrins, the pyrethroids display enhanced insecticidal activity, greater environmental stability, greater resistance to metabolism and increased mammalian toxicity (Elliot, 1989; Soderlund, 1992). There is no evidence that metabolites of the pyrethrins or pyrethroids induce neurobehavioral changes or other toxic effects. Thus, the neurotoxicity of pyrethrins and pyrethroids is produced by the parent chemical (Narahashi, 1982; Smith et al., 1997). Furthermore, the toxic potency of pyrethroids in mammals is inversely related to their rates of metabolic elimination (Abernathy and Casida, 1973; White et al. 1976).

Pyrethroids are biotransformed by two pathways, CYP-dependent oxidation and esterase-mediated hydrolysis (Soderlund and Casida, 1977). The type II pyrethroids deltamethrin and esfenvalerate are metabolized primarily by CYP-dependent oxidation in mouse and rat liver microsomes (Soderlund and Casida, 1977; Godin et al., 2006). The type II pyrethroids are distinguished from type I pyrethroids by the presence of a cyano group at the alpha carbon of the esterified alcohol. In human liver microsomes, esfenvalerate is metabolized primarily by CYP enzymes, whereas deltamethrin is metabolized mainly by esterase-mediated hydrolysis (Godin et al., 2006). Consistent with this finding, recombinant human carboxylesterases (CEs) display greater enzymatic activity towards deltamethrin than esfenvalerate. The CEs appear to be the major human enzyme responsible for hepatic metabolism of deltamethrin (Godin et al., 2006; Nishi et al., 2006).
The role of specific CYPs in the species difference observed in human and rodent microsomal metabolism of these two chemicals is not clear. Oxidative metabolism of deltamethrin in human liver microsomes is minimal, and while oxidative metabolism of esfenvalerate is relatively efficient compared to deltamethrin, it is still considerably slower than in rat liver microsomes. Pyrethroids that are metabolized rapidly by esterases are typically less toxic than pyrethroids metabolized by slower oxidative pathways (Abernathy and Casida, 1973; Soderlund and Casida, 1977; Soderlund, 1992; Soderlund et al., 2002). Inter-individual variability in the expression or activity of xenobiotic-metabolizing enzymes, which can be caused by genetic polymorphisms, disease state, life stage and environmental exposures (i.e., induction or suppression of CYPs) can lead to altered susceptibility in populations, particularly when a specific enzyme is responsible for the vast majority of a chemical’s clearance. It is therefore important to not only characterize the specific enzymes responsible for the metabolism of pyrethroids, but also to understand the relative flux through each pathway in order to determine which is responsible for metabolic elimination of the pyrethroids.

In addition to the liver, blood is a site of metabolism for pyrethroids in laboratory animals (Anand et al., 2006; Mirfazaelian et al., 2006). Rat serum contains carboxylesterase(s) that are capable of metabolizing pyrethroids (Anand et al., 2006). The activity of serum CEs in the rat may be important in the overall pharmacokinetic disposition of pyrethroids (Anand et al., 2006; Mirfazaelian et al., 2006), particularly since pyrethroids will encounter serum CEs immediately upon absorption from the gut. In contrast to laboratory animals, human serum does not contain carboxylesterase activity (Li et al., 2005). Therefore, while blood may be an important tissue for the metabolic
elimination of pyrethroids in rats it may not be in humans. This is supported by the fact that there are currently no literature reports on the metabolism of pyrethroids in human blood or by human serum esterases.

In the present study we examined the ability of specific rat and human CYPs to metabolize deltamethrin and esfenvalerate \textit{in vitro}. Deltamethrin and esfenvalerate were chosen for this study because they are two of the most potent and commonly used pyrethroids. In addition, a clear difference exists in the rates of CYP-mediated metabolism of deltamethrin and esfenvalerate in human liver microsomes but not in rat liver microsomes (Godin et al., 2006). A comparison of the species specific CYP isoforms that can biotransform these pyrethroids is therefore an ideal approach for examining possible determinants of their rates of clearance. A potential species difference in the metabolism of deltamethrin and esfenvalerate in serum was also explored using rat and human sera and purified serum esterases. The results obtained yield a clearer understanding of the differences in the metabolism of deltamethrin and esfenvalerate between rats and humans. Furthermore, improved characterization of the important pathways that metabolize pyrethroids in rats and humans was obtained. The resulting information will be useful for human health risk assessments by decreasing the uncertainty in extrapolating laboratory animal pharmacokinetic data to humans.
Material and Methods

Chemicals

Deltamethrin (98.9% purity) (\((\alpha S)\)-cyano-3-phenoxybenzyl (1R, 3R)-\textit{cis}-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropane carboxylate) was a gift from Bayer Crop Sciences (Research Triangle Park, NC, USA). Esfenvalerate (98.6% purity) (\((\alpha S)\)-cyano-3-phenoxybenzyl 2-(4-chlorophenyl)-3-methylbutyrate) was a gift from Dupont (Johnston, IA, USA). Bifenthrin, used as an internal standard, was obtained from Chem Service Inc (West Chester, PA, USA). These chemicals were used in all CYP assays and serum elimination studies. Deltamethrin (>98%) and esfenvalerate (>98%) used in the carboxylesterase and serum metabolite formation assays were obtained from Chem Service Inc. LC/MS analysis of pyrethroids from the various sources did not reveal any differences in their chemical composition. Chromasolv® acetonitrile and methanol for LC/MS applications were from Riedel-de Haën (Seelze, Germany). Ammonium formate, Trizma-base, \(\beta\)-nicotinamide adenine dinucleotide phosphate reduced form (NADPH), 3-phenoxybenzyl alcohol (3PBAlc), \textit{cis/trans}-3-(2',2'-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid (a 1:1 mixture of \textit{cis} and \textit{trans} isomers) [also called \textit{cis/trans}-dichlorochrysanthemic acid (Cl2CA)], and 3-phenoxybenzaldehyde (3PBCHO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Unless otherwise specified all chemicals were of the highest grade commercially available.

Rat and Human CYPs

Rat CYP 1A1, 1A2, 2A1, 2B1, 2C6, 2C11, 2C12, 2C13, and 3A2 and human CYP 1A2, 2A6, 2B6, 2E1, 2C8, 2C9*1, 2C9*2, 2C19, 2D6*1, 3A4, and 3A5 were
purchased from BD Biosciences (Woburn, USA). The concentration of these enzymes ranged from 1000-2000 pmoles CYP/ml.

**CYP-catalyzed Elimination of Pyrethroids: Screening Assays**

Each rat and human CYP was screened for its ability to oxidize and eliminate deltamethrin and esfenvalerate from the assay mixture. Assay conditions were 0.5 ml of 0.1 M Tris-HCl (pH 7.4), 10 pmoles P450/ml and 1 mg/ml NADPH. The assay mixture containing enzyme and NADPH were preincubated for 10 min at 37 °C. To initiate the reaction, 5 µL of 100 µM stock solutions of deltamethrin or esfenvalerate dissolved in 50% acetonitrile were added for a final concentration of 1 µM pyrethroid (final concentration of acetonitrile in each reaction was 0.5% v/v). The 1µM concentration was previously used in microsomal clearance assays and determined to be below the K_m for microsomal elimination of these pyrethroids (Godin et al., 2006). Each assay was conducted in duplicate. Reactions were carried out at 37 °C over 20 min. At selected time points, 100 µL aliquots were removed and the reaction was terminated by adding 1-ml ice cold 75% acetonitrile containing 1 µg/ml bifenthrin as an internal surrogate of recovery. Samples were vortexed for 10 min and placed into auto-sampler vials for LC/MS analysis. LC/MS identification and quantification of pyrethroids was accomplished as previously described (Godin et al., 2006). Recovery of internal standard was greater than 95%. The duplicate samples were averaged and values are reported as the percentage of pyrethroid eliminated over the first 10 min of incubation, a time interval for which the elimination rate was found to be linear. Control reactions were run in the absence of NADPH to verify that metabolism was enzymatic.
CYP Isozyme Kinetic Assays

The kinetic parameters $K_m$ and $V_{max}$ were determined for the CYPs identified as being metabolically active toward deltamethrin and/or esfenvalerate in the initial screening assay. The assay mixture consisted of 0.5 ml of 0.1 M Tris-HCl (pH 7.4), 10 pmoles P450/ml and 1 mg/ml NADPH. The assay buffer containing enzyme and NADPH was preincubated for 10 min at 37°C. The reaction was initiated by addition of 5 µL of stock solutions of varying concentration of pyrethroid (0.05-2.5 mM) to yield final pyrethroid concentrations of 0.5-25 µM. Assays were carried out for 10 min. Reactions were terminated by the addition of 1 ml of ice cold 75% acetonitrile containing 1 µg/ml bifenthrin. Samples were vortexed for 10 min and LC/MS analysis was carried out as previously described. Each assay was performed in triplicate. Rates of elimination were converted to product formation velocities and plotted versus substrate concentrations. $K_m$ and $V_{max}$ parameters were determined using GraphPad Prism (v 4.0, GraphPad Software, San Diego CA, USA) by fitting the experimental data to the Michaelis-Menten equation by non-linear regression. $V_{max}$ and $K_m$ were unobtainable for CYP3A5 as it displayed linear kinetics in the range of concentrations utilized. Therefore, all data was also analyzed by linear regression of product formation velocities plotted against substrate concentration in the linear range to obtain catalytic efficiencies ($V_{max}/K_m$) from the slope of the regression line.

Rat and Human Serum Elimination Assays

Whole blood was collected from 30 adult male Long-Evans rats (approximately 90 day old) (Charles River, Raleigh, NC, USA). The blood was allowed to clot 1 hr
before being centrifuged for 20 min at 2,000 x g to enable serum collection. Three pools of rat serum were generated \((n=3 \text{ samples})\), each from 10 different animals. Pooled human serum (10 donors per pool) was purchased from Bioreclamation (Hicksville, NY, USA) (Lot #BRH88162, Lot #BRH88163) and Innovative Research (Southfield, MI, USA) (Lot #IR05-044). Rat and human sera were diluted to 50\% with 0.1 M Tris-HCl (pH 7.4) buffer. One ml of the 50\% serum was preincubated at 37°C for 10 min prior to pyrethroid addition. Ten µL of 100 µM stocks of deltamethrin or esfenvalerate were added for a final concentration of 1 µM. Serial aliquots (100 µL) were removed at 0, 20, 40 and 60 min and placed in 2 ml of ice-cold hexane containing bifenthrin as an internal surrogate of recovery. Samples were vortexed and centrifuged for 10 min. The supernatant was removed and the extraction was repeated twice more with 2 ml of hexane. The combined extracts were evaporated to dryness under a stream of N₂. Samples were reconstituted in 1 ml of 75:25 (v/v) methanol:water and placed in autosampler vials for LC/MS analysis. Assays were conducted in triplicate. LC/MS analysis was carried out as previously described (Godin et al., 2006). The concentration of pyrethroid was determined over the time course of the assay and plotted versus time. The slope of the linear regression represents the rate of elimination of pyrethroid. Values were scaled to per ml of serum.

**Human and Rat Serum Hydrolysis Assays**

Blood was collected from five adult male Sprague-Dawley rats (70–110 day) (Sprague-Dawley rats were obtained from an in-house colony at Mississippi State University). The blood was allowed to stand for one hr to clot and was subsequently
centrifuged at 2,000 x g for 20 min to enable serum collection. The sera were then pooled together to form a single pool of rat serum. Human serum obtained from a pool of adult male donors was purchased from Sigma-Aldrich (St. Louis, MO, USA). Pyrethroid hydrolysis reactions in human or rat sera were conducted as follows. The pyrethroids were pre-incubated in 200–225 µL of 50 mM Tris-HCl buffer (pH 7.4) for 5 min before adding 25 µL of pooled rat serum or 50 µL of pooled human serum to each sample. For specific activity assays the final concentration of pyrethroid in each sample was 50 µM. When variable pyrethroid concentrations were added to serum incubations, pyrethroid concentrations ranged from 5–100 µM. The samples were incubated at 37 °C for 30 min before quenching with an equal volume of cold acetonitrile. Following centrifugation, the hydrolysis products in the supernatant were analyzed by HPLC-UV on a Surveyor LC system (Thermo Electron, San Jose, CA) using a reversed-phase HPLC column (2.1 mm x 100 mm, C18, Thermo Electron) as previously described for trans-permethrin (Ross et al., 2006).

**Purified rat serum carboxylesterase**

Rat serum CE protein was purified to homogeneity as described by Crow et al. (2007). Hydrolysis reactions catalyzed by purified rat serum CE were performed in 100-µL volumes at 37 °C. Varying amounts of pyrethroid (5–100 µM) were pre-incubated for 5 min in 50 mM Tris-HCl buffer (pH 7.4) at 37°C. The hydrolytic reactions were initiated by addition of the pure CE (2.5 µg protein per reaction). After 30 min of incubation the reactions were quenched by the addition of an equal volume of ice-cold acetonitrile. The samples were centrifuged and an aliquot of the supernatant was
analyzed by HPLC to quantify the hydrolysis products. Rates of hydrolysis reactions catalyzed by pure CEs have been demonstrated to be linear up to 60 min (Ross et al., 2006). Non-enzymatic controls were also included and found to have negligible rates. Serum CE reactions at each substrate concentration were performed in duplicate.

**Human Serum Esterases**

Human butyrylcholinesterases (BChE) and acetylcholinesterases (AChE) were purchased from Sigma Aldrich (St. Louis, MO USA). Incubation of pyrethroid with BChE or AChE was done in the same manner as the reactions catalyzed by rat serum CE.

**Immunoblotting of pooled human liver microsomes: hCE1 and hCE2 protein levels**

The recombinant human carboxylesterase (CE) proteins (hCE1 and hCE2) were expressed in baculovirus-infected *Spodoptera frugiperda* cells and purified (Morton and Potter, 2000). Polyclonal antibodies against hCE-1 and hCE-2 were kindly provided by Dr. M. Hosokawa (Chiba University, Japan) and Dr. P. Potter (St. Jude Children’s Research Hospital, Memphis, TN, USA), respectively. Pooled human liver microsomes from four different vendors, CellzDirect (Phoenix, AZ USA) (Lot# HMMC-PL020), Cedra (Austin, TX USA) (Lot#821-1), Xenotech (Lenexa, KS USA) (Lot#0310241) and BD Biosciences (San Jose, CA USA) (Lot # 26738), were subjected to SDS-PAGE using standard protocols (Ross and Borazjani, 2007). After electrophoresis, the proteins were transferred to polyvinylidifluoride membranes and probed with either anti-hCE-1 (1:4000, v/v) or anti-hCE-2 (1:5000, v/v) polyclonal antibody in Tris-buffered saline/5% milk. Immuno-complexes were localized on the membrane with a horse radish peroxidase-
conjugated goat anti-rabbit secondary antibody and the SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL, USA). The chemiluminescent signal was captured using a digital camera (Alpha Innotech gel documentation system). Bands on the digital images were quantified using NIH Image J software (v.1.33u). Known quantities of recombinant hCE1 and hCE2 proteins were loaded on the same gels to establish calibration curves.

**Kinetic analysis and statistics for carboxylesterase studies**

Non-linear regression of substrate concentration versus reaction velocity plots were analyzed using SigmaPlot v. 8.02 software (San Jose, CA, USA) by fitting experimental data to the Michaelis-Menten equation. Each substrate concentration in the kinetic experiments was evaluated in duplicate. The specific activity data obtained using pooled rat serum (n=5 animals/pool) are reported as the mean (± S.D.) of three replicates.
Results

Rat CYP screening assays

The ability of rat CYP isoforms to metabolize deltamethrin and esfenvalerate were studied by evaluating the elimination of 1 μM pyrethroid. Rat CYPs 1A1, 2C6, 2C11, and 3A2 metabolized both deltamethrin and esfenvalerate (Fig. 1). However, rat CYPs 1A2, 2A1, 2B1, 2C12, and 2C13 did not metabolize either compound (Fig. 1). Rat CYP2C6 eliminated the greatest percentage of deltamethrin followed by 1A1>2C11>3A2 (Fig. 1). Rat CYP2C6 also eliminated the greatest percentage of esfenvalerate followed by 2C11>3A2>1A1 (Fig. 1). CYP2C11 eliminated a similar percentage of both pyrethroids. CYPs 2C6 and 1A1 eliminated a significantly greater of deltamethrin than esfenvalerate, while CYP3A2 eliminated a greater percentage of esfenvalerate than deltamethrin (Fig. 1). These CYP isoforms were chosen for this study based on their relatively high expression in rat liver microsomes (e.g., 2C6, 2C11, 3A2; Guengerich et al., 1982) and because previous studies indicated they may be involved in pyrethroid metabolism (e.g., 1A1, 1A2, 2B1) (Anand et al., 2006; Dyal et al., 2001). CYPs 2C12 and 2C13 were chosen due to their sex specific expression, thus allowing the potential influence of gender on the metabolism of deltamethrin and esfenvalerate to be determined.

Human CYP screening assays

The ability of human CYP isoforms to metabolize deltamethrin and esfenvalerate were also studied by evaluating the elimination of 1 μM pyrethroid. Deltamethrin was
metabolized by human CYPs 2C8, 2C19, 3A4 and 3A5 (Fig. 2). Esfenvalerate was metabolized by human CYPs 2C8, 2C9*1, 2C9*2, 2C19, 3A4 and 3A5 (Fig. 2). However, human CYPs 1A2, 2A6, 2B6, 2E1, and 2D6*1 did not metabolize either deltamethrin or esfenvalerate (Fig. 2). CYP2C19 eliminated the greatest percentage of deltamethrin followed by 2C8 > 3A5 > 3A4 (Fig. 2). CYP2C19 also eliminated the greatest percentage of esfenvalerate followed by 2C8 > 2C9*1 > 3A5 = 2C9*2 > 3A4. CYPs 2C8, 2C19, 3A4, and 3A5 eliminated a similar percentage of esfenvalerate and deltamethrin (Fig. 2), while CYPs 2C9*1 and 2C9*2 metabolized esfenvalerate but not deltamethrin (Fig. 2). These CYP isoymes were chosen for study based on their relatively high expression in human liver microsomes and their known contributions to xenobiotic metabolism (Rodrgiues 1999).

**Kinetic analysis of deltamethrin metabolism by rat and human CYPs**

The kinetic parameters of deltamethrin metabolism by rat CYPs 2C6, 2C11, 3A2, and human CYP 2C8 and 2C19 were examined because they appear to contribute significantly to pyrethroid metabolic clearance. Rat CYP2C6 and 2C11 had the highest K_m and V_max values among rat CYPs examined for deltamethrin metabolism (Table 1). Rat CYP3A2 eliminated deltamethrin at a significantly slower rate than CYPs 2C6 and 2C11; however, it also exhibited a lower K_m value (Table 1).

Human CYP2C8 and 2C19 have similar K_m and V_max values for deltamethrin (Table 1). K_m and V_max values were unobtainable for CYP 3A5 using non-linear regression as the data appeared to display linear kinetics across the range of pyrethroid concentrations utilized in these experiments. Catalytic efficiencies (V_max/K_m) for each
enzyme were therefore obtained from the slopes of the linear region of the substrate-velocity plots and used to compare to the catalytic efficiency for CYP 3A5 (Table 1). The catalytic efficiency of CYP 3A5 was found to be greater than both 2C8 and 2C19 for deltamethrin.

\[ K_m, V_{\text{max}}, \text{and } V_{\text{max}}/K_m \text{ parameters for deltamethrin were similar for rat and human CYPs. The large standard error associated with the estimates of } K_m \text{ and } V_{\text{max}} \text{ for some enzymes is likely due to the inability to accurately determine } V_{\text{max}} \text{ because of the solubility limits of the pyrethroids under the experimental conditions utilized.} \]

Kinetic analysis of rat CYP 1A1 was not attempted due to its very low constitutive expression in the mammalian liver (Nebert et al., 2004). In addition, since the induction of CYP1A1 is minimal in human livers (Xu et al., 2000; Silkworth et al., 2005), and deltamethrin is predominately metabolized by esterases in humans, CYP1A1 metabolism is less interesting for the purposes of this study. Due to minimal metabolism of both deltamethrin and esfenvalerate by human CYP 3A4, kinetic parameters were not determined for this CYP. It is, therefore, unlikely that human CYP 3A4 plays any role in the metabolism of these pyrethroids.

**Kinetic analysis of esfenvalerate metabolism by rat and human CYPs**

The kinetic parameters of esfenvalerate metabolism by rat CYPs 2C6, 2C11, and 3A2 and human CYPs 2C8, 2C9*1, and 2C19 were examined. Of the rat enzymes that metabolized esfenvalerate, CYPs 2C6 and 2C11 had the highest \( K_m \) and \( V_{\text{max}} \) values, and were similar to the values for deltamethrin (Table 1). Rat CYP3A2 had lower \( K_m \) and \( V_{\text{max}} \) values then 2C6 and 2C11 (Table 1). Of the human CYPs examined each had
similar $V_{\text{max}}$ values (Table 1). However, the $K_m$ for CYP2C19 was ~5-6-fold lower than the $K_m$ for 2C8 and 2C9*1. As with deltamethrin, $K_m$ and $V_{\text{max}}$ values were unobtainable for human CYP 3A5 since the data displayed linear kinetics for esfenvalerate oxidation. The catalytic efficiency was therefore estimated from the slope of the linear regression and, as with deltamethrin, CYP 3A5 had a higher value than the other human CYPs (Table 1). As previously noted, kinetic parameters for human CYP 3A4 were not determined due to a lack of significant metabolism of esfenvalerate.

Elimination of deltamethrin and esfenvalerate by pooled rat and human sera

The ability of rat and human sera to metabolize deltamethrin and esfenvalerate was examined by incubating 1 $\mu$M pyrethroid in 50% rat or human sera. In rat serum deltamethrin and esfenvalerate were eliminated at rates of 15.33 ± 3.24 (mean ± SD) and 9.97 ± 2.94 pmoles/min/ml serum respectively. Neither deltamethrin nor esfenvalerate were eliminated during incubation in human serum.

Hydrolysis of Pyrethroids

When the prototypical type I pyrethroid, trans-permethrin, is hydrolyzed by purified rat or human CEs the two products formed are 3-phenoxybenzyl alcohol (3PBAIc) and trans-dichlorochrysanthemic acid (Cl$_2$CA). Both metabolites can be analyzed by HPLC (Fig. 3). Hydrolysis of the type II pyrethroid, deltamethrin, by purified CEs liberates cis-dibromochrysanthemic acid (Br$_2$CA), which is a stable metabolite, and a cyanohydrin that spontaneously converts to 3-phenoxybenzaldehyde (3PBCHO) at pH>7 (see scheme in Fig. 4). Br$_2$CA and 3PBCHO are also conveniently
quantified by HPLC analysis (Fig. 3) and thus product formation rates can be determined.
The hydrolysis of esfenvalerate also liberates the same cyanohydrin that spontaneously
yields 3PBCCHO. When deltamethrin is incubated with hepatic microsomes, 3PBCCHO
can undergo redox reactions to produce 3PBAlc and 3-phenoxybenzoic acid
(3PBCOOH) (Fig. 4), which are likely catalyzed by alcohol and aldehyde
dehydrogenases, respectively, present in the heterogeneous protein mixtures (Choi et al.,
2002).

**Rates of deltamethrin and esfenvalerate hydrolysis: Pooled rat serum and purified
rat serum carboxylesterase.**

The hydrolysis of deltamethrin and esfenvalerate by pooled rat serum and a
purified rat serum CE was examined. The pooled rat serum sample and the purified rat
serum CE hydrolyzed both pyrethroids, but at different rates (Fig. 5A and B). The
specific activity of deltamethrin hydrolysis by the purified CE was nearly 2-fold greater
than the activity for esfenvalerate (Fig. 5B). This compares well with the greater specific
activity of the pooled rat serum with deltamethrin than with esfenvalerate (Fig. 5A).
Concentration-velocity plots were analyzed for deltamethrin using both the pooled rat
serum and the purified CE. In the pooled serum sample, deltamethrin displayed linear
kinetics (Fig. 5C). In contrast, deltamethrin displayed hyperbolic kinetics with the
purified CE (Fig. 5D). The estimated \( k_{\text{cat}} \) and \( K_m \) values for the rat serum CE-catalyzed
deltamethrin hydrolysis (Fig. 5D) were 0.48 min\(^{-1}\) and 12.6 \( \mu \)M, respectively, and the
calculated \( k_{\text{cat}}/K_m \) was 38 min\(^{-1}\)mM\(^{-1}\). Furthermore, the hydrolysis of deltamethrin and
esfenvalerate by two human esterases, BChE and AChE, present in human serum was
also examined. No evidence of hydrolysis was detected (data not shown), which is consistent with the lack of pyrethroid elimination in human serum (see above).

**Quantitative Immunoblotting: hCE-1 and hCE-2**

Quantitative immunoblotting of samples of pooled human liver microsomes using anti hCE-1 and hCE-2 antibodies demonstrated that the average amount of hCE-1 protein expressed in four separate pools of human liver microsomes was 64.4 ± 16.5 µg hCE-1/mg microsomal protein (mean +/- SD) (Fig. 6). In contrast, the level of hCE-2 protein (1.4 ± 0.2 µg hCE-2/mg microsomal protein) in the same samples of liver microsomes was nearly 50-fold lower than the level of hCE-1 protein (Fig. 6). Thus, hCE-1 is clearly the most abundant CE isozyme in human liver microsomes and is found at much higher concentrations compared to hCE-2.
Discussion

The relative rates of oxidation and hydrolysis of the pyrethroids deltamethrin and esfenvalerate differ between human and rat hepatic microsomes (Godin et al., 2006). The current work examined the role of specific CYP isozymes responsible for deltamethrin and esfenvalerate metabolism in rat and human liver microsomes. The difference between rat and human serum hydrolysis rates and the substrate specificities of a purified rat serum CE were also examined for these pyrethroids.

In rat liver microsomes, both deltamethrin and esfenvalerate are cleared at comparable rates by CYP mediated oxidation (Godin et al., 2006). In agreement with this observation, the elimination of both compounds by rat CYPs 1A1, 2C6, 2C11, and 3A2 in the current study were similar. CYPs 2C6, 2C11, and 3A2 are highly expressed in rat liver (Guengerich et al., 1982) and likely contribute the bulk of the oxidative metabolism of deltamethrin and esfenvalerate in this organ. The kinetics of deltamethrin and esfenvalerate metabolism by CYPs 2C6, 2C11, and 3A2 were very similar (Table 1). Rat CYPs 2C6 and 2C11 displayed higher $K_m$ and $V_{max}$ values than CYP3A2 suggesting they are responsible for the largest proportion of the metabolism of pyrethroids in rat liver at saturating concentrations.

In contrast to rat liver microsomes, human liver microsomes primarily metabolize deltamethrin (hydrolysis) and esfenvalerate (oxidation) by different pathways (Godin et al., 2006). We have previously shown that hCE-1 is likely the principal enzyme responsible for human hepatic microsomal metabolism of deltamethrin (Godin et al., 2006). In contrast, esfenvalerate is not hydrolyzed efficiently by hCE-1 but is primarily metabolized by oxidative processes in human liver microsomes (Godin et al., 2006).
Because of these metabolic pathway differences, it was expected that there would be a considerable variation in the substrate specificity of human CYPs that are capable of metabolizing these pyrethroids. This, however, was generally not the case. Deltamethrin and esfenvalerate were each metabolized at comparable rates by CYPs 2C8, 2C19, and 3A5 (Fig 2). An important exception was their metabolism by the human CYP2C9 isozymes (Fig 2) (Table 1). While esfenvalerate was metabolized effectively by the 2C9 isozymes, deltamethrin was not. Although 2C19 eliminated the greatest percentage of both deltamethrin and esfenvalerate, (Fig 2), CYP 2C9 has the highest expression in human liver (Rodrigues, 1999). CYP2C9 is expressed at approximately 4-fold and 2-fold greater levels than 2C19 and 2C8 respectively and nearly 100 fold greater levels than 3A5 (Rodrigues, 1999). Thus, the higher expression level of CYP2C9 and its ability to oxidize esfenvalerate, but not deltamethrin, may account for the greater rate of oxidative metabolism of esfenvalerate by human liver microsomes (Godin et al., 2006).

In terms of the CYPs that metabolized the pyrethroids investigated in this study, individual rat and human enzymes had comparable $K_m$ and $V_{\text{max}}$ values for deltamethrin and esfenvalerate. However, we had previously observed that the rates of pyrethroid oxidative metabolism were slower in human hepatic microsomes than in rat hepatic microsomes (Godin et al., 2006). This difference is likely due to the levels of CYP expression in rat and human hepatic microsomes. According to Guengerich (1982), expression of rat CYPs 2C6, 2C11, and 3A2 ranges from 300 to >1000 pmoles P450/mg of microsomal protein. In contrast, estimates of average CYP isozyme expression in human liver microsomes are much lower, ranging from 1-100 pmols/mg microsomal protein (Rodrigues, 1999). Thus, the abundance of CYP isozymes in rat liver compared
to human liver, and not the individual enzyme’s activity or specificity, likely accounts for
the difference in oxidation rates of deltamethrin and esfenvalerate that was previously
observed (Godin et al., 2006).

The current study also quantified the expression of the two major CEs in human
liver microsomes, hCE-1 and hCE-2 (Fig. 6). hCE-1 is robustly expressed in human
liver, at >60 µg (1000 pmoles) per mg of microsomal protein. hCE-2 is expressed at
much lower levels, 1.4 µg (23 pmoles) per mg of microsomal protein. If one assumes the
average molecular weight of a CYP is 52 kDa (Lewis, 2001), then the expression of the
major individual human CYPs in the liver ranges from approximately 0.05-5 µg (~1–100
pmoles) of CYP enzyme per mg microsomal protein (Rodrigues, 1999). Thus, the
expression of hCE-1 is approximately 12–1200-fold greater than the levels of individual
CYPs in human liver microsomes. Therefore, the results of these studies suggest that the
relative levels of expression of both hCE-1 and CYP2C9 are important determinants of
the rate and pathway of metabolism of pyrethroids in human liver microsomes.

The blood is a potential site of pyrethroid metabolism. Rat serum possesses
significant carboxylesterase activity that can hydrolyze pyrethroids (Anand et al., 2006;
Crow et al., 2007), while human serum lacks carboxylesterase activity (Li et al., 2005).
Consistent with these previous findings, deltamethrin and esfenvalerate were hydrolyzed
in rat serum (see Fig. 5A). Neither pyrethroid was eliminated or hydrolyzed following
incubation in human serum or with purified preparations of human AChE and BChE
esterases, consistent with previous results for the pyrethroid trans-permethrin (Ross et al.,
2006). The rate of the hydrolysis catalyzed by purified rat serum CE of deltamethrin
was 2-fold greater than the rate of esfenvalerate (Fig. 5B). Similar results were observed
in rat serum (Fig. 5A). Concentration-velocity plots for deltamethrin in rat serum revealed non-hyperbolic kinetics (Fig. 5C), which is similar to the kinetic plot observed by Anand et al. (2005) up to 100 µM in rat serum. However, when deltamethrin hydrolysis was studied using the purified rat serum CE, we found that it exhibited hyperbolic kinetics characteristic of a classical Michaelis-Menten enzymatic mechanism (Fig. 5D). One possible explanation to account for the discrepancy in kinetics between whole serum and purified serum CE is that deltamethrin may bind non-covalently to serum albumin, thus reducing its effective concentration available for hydrolysis by the serum CE enzyme. This could account for the much higher apparent $K_m$ for deltamethrin when investigated in whole serum.

Hydrolase A is the most abundant rat hepatic CE (Morgan et al., 1994; Sanghani et al., 2002). The $k_{cat}$ value obtained for deltamethrin hydrolysis by pure rat serum CE (this study) was ~2-fold greater than the $k_{cat}$ for Hydrolase A (Godin et al., 2006). However, due to a slightly higher $K_m$ value for the serum CE, the calculated catalytic efficiencies ($k_{cat}/K_m$) are similar. Therefore, the contribution of rat serum CE to deltamethrin elimination is likely to be important, particularly at low serum concentrations. These results highlight a significant species difference between rats and humans with respect to pyrethroid metabolism in the blood since no hydrolysis of deltamethrin or esfenvalerate occurs in human serum.

In vitro metabolism studies using rodent and human tissues have been used to estimate in vivo pharmacokinetic parameters such as half-life and clearance (Iwatsubo et al., 1997). In vitro metabolism parameters do not always directly scale to the in vivo situation and often a correction factor is used (Naritomi et al., 2001; Obach et al., 1997).
The species differences noted in this and previous work (Godin et al. 2006) indicate that the rat may not be a good model for understanding human metabolism of all pyrethroids. As such, any correction factor used for scaling the rodent in vitro data to in vivo may not apply to scaling the human data. However, understanding these species differences provides information on data gaps and uncertainties inherent in these extrapolations.

The results obtained in this study qualitatively and quantitatively provide information on the relative importance of the liver and blood to the metabolic clearance of pyrethroids in rats and humans, which can be used to estimate metabolism parameters in a physiologically based pharmacokinetic model. These results also address potential human variability in pyrethroid metabolism. Identifying and quantifying the role of oxidative and hydrolytic enzymes in the metabolism of pyrethroids in humans can provide insight into how variability in the expression of these enzymes will affect exposure-dose relationships. For example, the expression of both CYPs and esterases can vary greatly in human populations due to genetic polymorphisms, disease states, life stage, and environmental exposures (i.e. induction or suppression of metabolizing enzymes). If a single enzyme is primarily responsible for a chemical’s metabolic elimination, variability in the expression or activity of that enzyme can lead to altered susceptibility within a subpopulation. Significant human variability exists in enzymes such as CYP 2C9 (Stubbins et al. 1996) and hCE-1 (Hosokawa et al., 1995). Populations with decreased 2C9 expression may have slower elimination of esfenvalerate and potentially greater risk associated with those exposures. Similarly, populations with decreased hCE-1 activity could have significantly reduced deltamethrin clearance rates.
References


Sanghani SP, Davis WI, Dumaual NG, Mahrenholz A, and Bosron WF (2002)


Footnotes

This article has been reviewed in accordance with the policy of the National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use. S.J.G. was supported by NHEERL-DESE, EPA CT826513. Research support to M.K.R and J.A.C. was provided by NIH grant P20 RR017661.

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Figure. Legends

Fig. 1. Elimination of deltamethrin and esfenvalerate by rat CYPs.
Elimination of 1 uM deltamethrin (black bars) or esfenvalerate (white bars) by rat CYP isoforms. Assays were run with 10 pmoles P450 isozyme/ml. Results are expressed as the average of the % eliminated over 10 min of duplicate samples.

Fig. 2. Elimination of deltamethrin and esfenvalerate by human CYPs.
Elimination of 1 uM deltamethrin (black bars) or esfenvalerate (white bars) by human CYP isoforms. Assays were run with 10 pmoles P450 isozyme/ml. Results are the average of the % eliminated over 10 min of duplicate samples.

Fig. 3. Comparison of hydrolysis products of trans-permethrin and deltamethrin by esterases.
A, Overlay of HPLC chromatograms of hydrolysis products derived from each pyrethroid catalyzed by human carboxylesterase 1. B and C, UV spectra of the hydrolysis products of trans-permethrin (type I pyrethroid) and deltamethrin (type II pyrethroid).

Fig. 4. Hydrolytic metabolism of deltamethrin.

Fig. 5. Comparison of hydrolysis rates of esfenvalerate and deltamethrin by whole rat serum and purified rat serum CE.
A, Specific hydrolysis activity for each pyrethroid (50 µM) catalyzed by rat serum. B, Specific hydrolysis activity for each pyrethroid (50 µM) catalyzed by pure rat serum CE. C, Substrate concentration-velocity plot of deltamethrin hydrolysis in whole serum. D, Substrate concentration-velocity plot of deltamethrin hydrolysis by pure rat serum CE.

Fig. 6. Quantitative immunoblotting of hCE1 protein (A) and hCE2 protein (B) in pooled human liver microsomes from four sources.
Sources of pooled human liver microsomes: a, Cellz Direct; b, CDR; c, Xenotech; d, BD Biosciences. Equal quantities of microsomal protein were loaded on each individual gel; however, 25-fold less protein was loaded on the gel in A (0.2 µg protein per lane) than in B (5 µg per lane). Membranes were probed with rabbit anti-hCE1 antibody (A) or rabbit anti-hCE2 antibody (B).
Table 1. Kinetic parameters for deltamethrin and esfenvalerate metabolism by rat and human CYPs

<table>
<thead>
<tr>
<th></th>
<th>Km (µM)</th>
<th>Vmax (pmoles/min/pmoleP450)</th>
<th>Vmax/Km&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Vmax/Km&lt;sup&gt;b&lt;/sup&gt;</th>
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<tbody>
<tr>
<td><strong>Deltamethrin</strong></td>
<td></td>
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<tr>
<td><strong>Rat CYPs</strong></td>
<td></td>
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</tr>
<tr>
<td>2C6</td>
<td>21.6 ± 9.4</td>
<td>150.0 ± 36.6</td>
<td>6.9</td>
<td>3.4 ± 0.3</td>
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<tr>
<td>2C11</td>
<td>31.9 ± 25.7</td>
<td>205.8 ± 107.6</td>
<td>6.5</td>
<td>5.2 ± 0.7</td>
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<tr>
<td>3A2</td>
<td>6.4 ± 3.8</td>
<td>25.9 ± 5.8</td>
<td>4.0</td>
<td>2.1 ± 0.2</td>
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<tr>
<td><strong>Human CYPs</strong></td>
<td></td>
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</tr>
<tr>
<td>2C8</td>
<td>10.2 ± 9.5</td>
<td>42.7 ± 16.6</td>
<td>4.2</td>
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<tr>
<td>2C19</td>
<td>9.0 ± 5.6</td>
<td>61.6 ± 17.7</td>
<td>6.8</td>
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</tr>
<tr>
<td>3A5</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>4.8 ± 0.6</td>
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<tr>
<td><strong>Esfenvalerate</strong></td>
<td></td>
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<tr>
<td><strong>Rat CYPs</strong></td>
<td></td>
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<tr>
<td>2C6</td>
<td>38.2 ± 26.2</td>
<td>159.2 ± 72.8</td>
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<td>2C11</td>
<td>33.5 ± 21.8</td>
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<td>3A2</td>
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<td>9.2</td>
<td>2.4 ± 0.5</td>
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<tr>
<td><strong>Human CYPs</strong></td>
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<td>2C8</td>
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<td>2C9*1</td>
<td>24.3 ± 5.6</td>
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<tr>
<td>2C19</td>
<td>4.1 ± 2.2</td>
<td>64.4 ± 12.3</td>
<td>15.7</td>
<td>4.4 ± 0.6</td>
</tr>
<tr>
<td>3A5</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>4.7 ± 0.7</td>
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</table>

Kinetic assays conducted with concentrations ranging from 0.5-25 µM pyrethroid. Data is the mean ± SE (N=3)

<sup>a</sup>Calculated value using kinetic parameters obtained from non-linear regression analysis

<sup>b</sup>Catalytic efficiency estimated from the slope of the linear regression analysis of concentration versus velocity plots
Figure 2

![Graph depicting the percentage of elimination of Deltamethrin and Esfenvalerate by different human P450s.](dmd.aspetjournals.org)
Figure 3

(A) A graph showing the absorbance at 230 nm over time (min) with peaks at 4, 5, 6, 7, 8, 9, 10, 11, 12, and 13 minutes. The absorbance values range from 0 to 40.

(B) A graph showing the absorbance at 230 nm at different wavelengths (nm) with absorbance values ranging from 0 to 25.

(C) A graph showing the absorbance at 230 nm at different wavelengths (nm) with absorbance values ranging from 0 to 16.
Figure 4

deltamethrin

\[ \text{Br}_2\text{CA} \]

\[ \text{3PBAlc} \rightleftharpoons \text{3PBCHO} \]

\[ \text{3PBCOOH} \]
Figure 5

A: Specific activity (nmol/min/mL serum) for esfen and delta.

B: Specific activity (nmol/min/mg CE protein) for esfen and delta.

C: Reaction velocity (nmol/min/mL serum) as a function of Deltamethrin concentration (µM).

D: Reaction velocity (nmol/min/mg serum CE protein) as a function of Deltamethrin concentration (µM).
**Figure 6**

**A**

Pooled HLMs

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<th>a</th>
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<tbody>
<tr>
<td>ng</td>
<td>1</td>
<td>5</td>
<td>10</td>
<td>25</td>
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</table>

Standard curve for hCE1

$y = 83.7x + 404.4$

$R^2 = 0.963$

**B**

Pooled HLMs

<table>
<thead>
<tr>
<th></th>
<th>a</th>
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<tr>
<td>ng</td>
<td>1</td>
<td>5</td>
<td>10</td>
<td>25</td>
</tr>
</tbody>
</table>

Standard curve for hCE2

$y = 110.7x + 155.6$

$R^2 = 0.991$