SPECIES DIFFERENCES IN THE METABOLISM OF PYRETHROID PESTICIDES: POTENTIAL IMPLICATIONS FOR HUMAN HEALTH RISK ASSESSMENT

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ABSTRACT

Stephen Joseph Godin: Species differences in the metabolism of pyrethroid pesticides: potential implications for human health risk assessment
(Under the direction of Michael J. DeVito Ph.D.)

Pyrethroids are a class of synthetic pesticides derived from the pyrethrins, the natural insecticidal ingredients of the pyrethrum extract of *Chrysanthemum cinerariaefolium.* In an effort to aid species extrapolation of exposure-dose relationships for pyrethroids, this dissertation aimed to develop appropriate data sets and models to address pharmacokinetic uncertainties for this class of chemicals. Based on experimental data in rats and mice it was previously thought that the rate of metabolism of a pyrethroid directly influenced its toxic potency. Preliminary results indicated that the pyrethroid deltamethrin was metabolized nearly twice as rapidly in human liver microsomes (HLM) as it was in rat liver microsomes (RLM). This is in contrast to esfenvalerate, which was metabolized nearly 3 times slower in HLM compared to RLM. Our hypothesis was that hepatic metabolism of pyrethroids drives blood and brain concentrations thus influencing their toxic potency. Research was conducted to understanding species differences in the hepatic metabolism of deltamethrin and esfenvalerate, and to develop a PBPK model of deltamethrin exposure to examine the influence of hepatic metabolism on exposure-dose relationships between rats and humans.

Results indicate that initial phase I biotransformation (detoxification) of deltamethrin and esfenvalerate occurs via cytochrome P450 oxidation in RLM. This occurs by the same set of P450s for both compounds. In contrast while esfenvalerate is metabolized primarily by P450 oxidation in HLM, deltamethrin is metabolized primarily via esterase hydrolysis in

HLM. Hepatic clearance rates were calculated from microsomal metabolism studies and utilized to parameterize PBPK models of exposure to deltamethrin.

A PBPK model of deltamethrin was developed in rats and evaluated based on literature data. The current PBPK model of deltamethrin displays diffusion limited kinetics in all tissue compartments. This has the effect of limiting the influence of species differences in the hepatic clearance of the pyrethroids. Based on these results it is concluded that diffusion of pyrethroids into and out of the liver and brain are more responsible for the pharmacokinetic influence on toxic potency compared to hepatic metabolism in contrast to what has been previously thought.

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CHAPTER I

GENERAL INTRODUCTION AND LITERATURE REVIEW

GENERAL INTRODUCTION

Pyrethroids are one of the major classes of commercial pesticides along with the organochlorines, organophosphates, carbamates and others. Due to use restriction, and removal of other pesticides from the market, the use of pyrethroids has been increasing. According to a Freedonia Market Research, Inc. report in 2004, the use of pyrethroids has risen from 2.8% of the market share of pesticides in 1993 to an expected 30% in the year 2008. A better understanding of the human health risks associated with exposure to pyrethroids is therefore increasingly important.

Regulatory agencies are responsible for assessing the potential human risk associated with exposure to environmental contaminants such as pesticides. These efforts necessitate dose-response assessment (i.e., how the frequency of adverse effects changes with decreasing dose) and typically involve extrapolations from high to low doses and from a nonhuman species to humans. Typically, this is done with little to no direct human data. Thus, the process of toxicological risk assessment of environmental contaminants is fraught with uncertainties. Regulatory agencies have historically applied default approaches (the use of uncertainty and safety factors) in estimating the potential human risk associated with environmental exposures; this is the case for many pesticide chemicals, including pyrethroids. To improve the scientific basis for risk assessment, the development of sophisticated models, such as physiologically based pharmacokinetic models (PBPK), have been utilized. The goal of these approaches has been to better link what is known about exposure and toxicity, thus reducing the uncertainties in the risk assessment process.

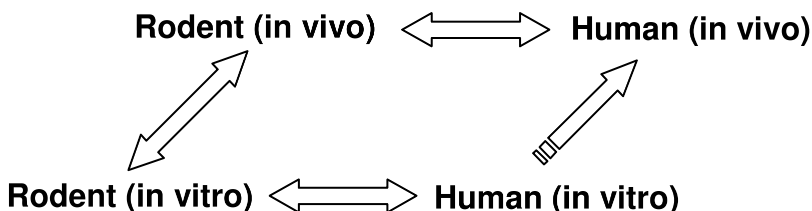
Pyrethroids and other pesticides have been regulated on a single chemical basis utilizing laboratory animal data and default uncertainty factors. Based on the Food Quality

Protection Act of 1996 (FQPA), which amended the Federal Insecticide Fungicide and Rodenticide Act and the Federal Food, Drug and Cosmetic Act regarding regulation of pesticides on food products, the pyrethroids are currently undergoing assessment for a possible cumulative risk assessment by the USEPA Office of Pesticide Programs. Thus, research efforts in the field of pyrethroids have recently been focused on current data gaps to aid a possible risk assessment. A major uncertainty in estimating human risk associated with exposure to pyrethroids is the extrapolation of laboratory animal toxicology data to human beings.

Species extrapolation of toxicology data is complicated by species differences in both the pharmacokinetics (PK) and pharmacodynamics (PD) of a toxicant. In the absence of direct human data, an ideal way to conduct an extrapolation is through a parallelogram approach (Fig. 1.1). Incorporation of a PBPK/PD model enables comparison of exposure-dose relationship at a target tissue to a toxic response (Andersen, 2003). The steps of the parallelogram approach include: (1) developing a hypothesis based model that includes descriptions of the proposed PK and PD determinants in a surrogate mammalian species (2) Determine the influence of the PK and PD determinants in the surrogate species, these are understood through the PBPK/PD model in the surrogate species. (3) *in vitro* determinants are compared with one another in the human and surrogate mammalian system. (4) Human *in vitro* data is then incorporated in the PBPK/PD model with the pertinent human anatomical/physiological data. Empirical adjustments for *in vitro* to *in vivo* scaling derived from the mammalian surrogate are applied if necessary, establishing the human PBPK/PD model. To conduct a risk assessment, population distributions of exposure, either measured or modeled can then be interpreted by the human PBPK/PD model rendering population

distributions of a relevant dose-metric for the toxicological outcome. The potential risk from these exposures is extrapolated from the dose metric based on dose response relationships understood in the rodent system.

Figure 1.1. Parallelogram approach for extrapolation of pharmacokinetics



The research presented in this dissertation attempts to address uncertainties in the species extrapolation of exposure-dose relationships for pyrethroid pesticides. This will be done utilizing the parallelogram approach to develop rat and human PBPK models of exposure to a pyrethroid and address uncertainties in extrapolating pyrethroid pharmacokinetics. Four primary objectives have been identified from the parallelogram approach; (1) formulation of a hypothesis driven model for pyrethroid tissue dosimetry in a mammalian surrogate (the rat); (2) explore the PK determinants of pyrethroid disposition in the rat and human beings; (3) develop a PBPK model of exposure to a pyrethroid in the rat, and; (4) extrapolate the rat PBPK model to a human PBPK model of exposure to a pyrethroid.

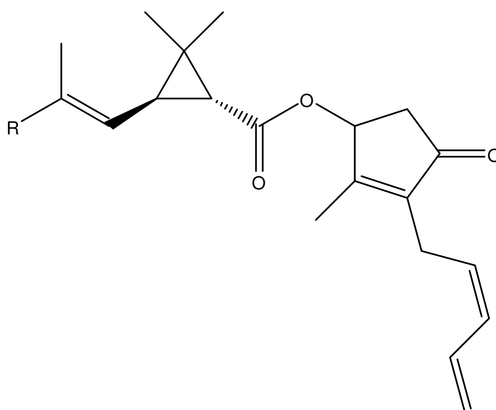
The following is a general literature review of the current state of knowledge in the field of pyrethroid pesticides. Based on current knowledge, formulation of an initial hypothesis driven model will be described, data gaps will be enumerated, and the needs for

development of rat and human PBPK models will be discussed. Within this framework the specific aims of this dissertation will be detailed.

THE PYRETHROIDS

Pyrethroids are synthetic pesticides which have been developed through an iterative process of changes to the chemical structures of the natural pyrethrins (Fig 1.2). Pyrethrins are the insecticidal components of the pyrethrum extract which is derived from the flowers of *Chrysanthemum cinerariaefolium* and *Chrysanthemum cineum*. While the pyrethrins make excellent natural pesticides and have low mammalian toxicity, they are labile in light, air, and moisture making them a less than ideal product for commercial use (Ueda et al. 1974). The pyrethroids were therefore developed to yield more environmentally stable and commercially viable pesticides.

Figure 1.2. Structure of the natural pyrethrins.



Pyrethrin I, R = CH_3, Pyrethrin II, R = CO_2CH_3

Pyrethroids are composed of two basic structural moieties, an acid and an alcohol (Elliot et al. 1965; Elliot 1969) (Fig 1.3). For first generation pyrethroids, the acid portion is based on chrysanthemic acid, a cyclopropane ring bonded to a carboxylic acid moiety and a

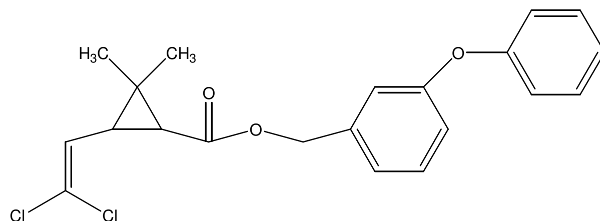
variety of halogenated and non-halogenated substituents. Later developed pyrethroids, such as fenvalerate, do not have a cyclopropane ring. The alcohol portion is either a primary or a secondary alcohol, which is bound to a variety of heterocyclic structures. In addition, several of the pyrethroids have a cyano substituent bound to the α-methylene of the alcohol, which results in enhanced toxicity of the compound. Pyrethroids lacking the α-cyano substituent are termed Type I compounds and the pyrethroids with the α-cyano substituent are termed Type II compounds. Figure 1.3 shows the structures of a Type I pyrethroid, permethrin, and a Type II pyrethroid, cypermethrin.

An important aspect of the chemical and toxicological properties of pyrethroids is their overall stereochemical configuration. The *cis*- and *trans*- designation indicates how a substituent on carbon-3 of the cyclopropane ring is oriented in relation to the carboxylic acid group bound to carbon-1 (Figure 1.4). Pyrethroids with the *trans* configuration and a primary alcohol (e.g., *trans*-permethrin) are hydrolyzed more readily by esterases than those with the *cis* configuration (e.g., *cis*-permethrin, deltamethrin). This may explain in part the reason pyrethroids with the *trans* configuration demonstrate less mammalian toxicity than those with the *cis* configuration (Table 1.1).

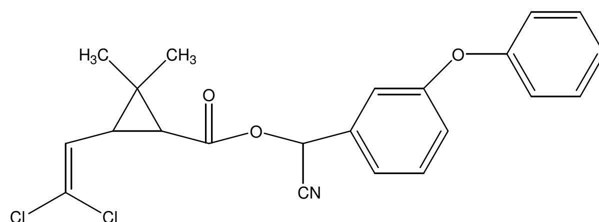
In a review of pyrethroid development and metabolism from early to newer, more potent pyrethroids, Soderlund (1992) describes how changes to the chemical structure of the pyrethrins and the pyrethroids not only affected their environmental stability, but also their metabolic stability. The removal of unsaturated side chains from the pyrethrins (Fig 1.2) decreased chemical oxidation in the environment as well as enzymatic oxidation of the pyrethroids in animals. Addition of the alpha-cyano group resulted in decreased hydrolysis of pyrethroids in the environment and by insect and mammalian esterases. These changes

resulted in more potent and commercially viable pesticides produced. The consequence of which is also more stable environmental contaminants which are more potent to mammalian species.

Figure 1.3. The structures of permethrin and cypermethrin.



Permethrin



Cypermethrin

Figure 1.4. Structure of deltamethrin ((S)-alpha-cyano-3-phenoxybenzyl (1R)-cis-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropane carboxylate).

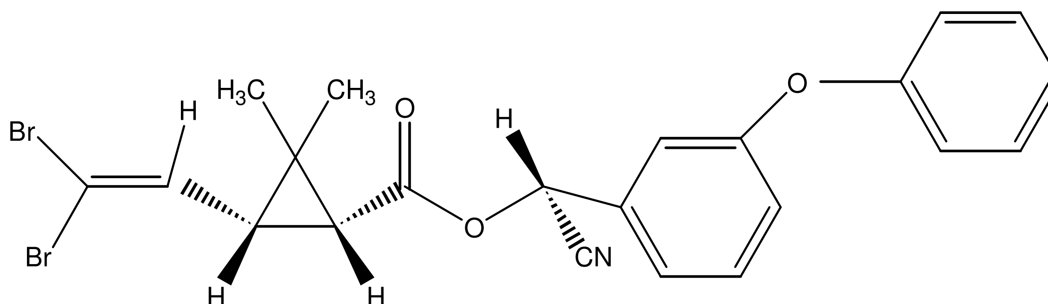


Table 1.1. Acute oral toxicity of permethrin isomers in mice[a].

Compound	Male	Female
Racemic Permethrin	490[b]	490
cis permethrin	107	85
trans permethrin	3100	3200

[a]Data from Miyamoto (1976)
[b]LD_{50}, mg/kg

MAMMALIAN TOXICITY OF PYRETHROIDS

The pyrethroids are a class of excitatory neurotoxic chemicals whose insecticidal and mammalian effects are thought to be primarily the result of modulation of nerve axon voltage gated sodium channels by the parent chemical. Numerous studies exist and multiple reviews have been written on the subject (Narahashi 1982 and 1985; Vijverberg and Van Den Bercken 1982 and 1990). Recently, Smith et al. (1997) demonstrated that point mutations in a house fly sodium channel result in loss of susceptibility to pyrethroids. The interaction of the parent chemical with the sodium channel alters the normal gating kinetics of the sodium channel causing varied firing patterns in the nervous system leading to neurobehavioral alterations. The neurotoxic behaviors elicited by Type I pyrethroids in laboratory animals are aggression, hyperexcitability, fine tremor, prostration with coarse whole body tremor, increased body temperature, coma and death. These neurobehavioral responses are termed the T-syndrome because of the fine tremors induced by the Type I pyrethroids (Gammon et al. 1981) (Lawrence and Casida 1982). For Type II pyrethroids, the neurotoxic behaviors include pawing and burrowing, salivation, hyperexcitability, abnormal hind limb movements, coarse whole body tremor, sinuous writing or choreoathotosis, coma and death. These neurobehavioral responses are termed the CS-syndrome for the choreoathotosis and salivation observed in laboratory animals (Gammon et al. 1981) (Lawrence and Casida

1982). Some pyrethroids such as fenpropathrin and cyphenothrin may elicit mixed behaviors of the T- and CS-syndromes. The T-and CS-syndromes are considered to be acute responses to exposure to pyrethroids and are dose-dependent.

While there is a substantial body of evidence suggesting a prominent role of the sodium channels in the toxicity of Type I and II pyrethroids, uncertainties remain surrounding their mechanism(s) of toxicity particularly since there is also evidence that alterations of chloride, calcium and other channels by pyrethroids may also play a role in the toxicity of these chemicals. Moreover, the pyrethroid sodium channel binding site has not been identified. Type I and II pyrethroids are believed to slow the activation (opening) and the inactivation (closing) of the sodium channels. These delays in opening and closing of the sodium channel prolong the sodium current. The length of this current is dependent on whether the chemical is a Type I or Type II pyrethroid. The Type I pyrethroids open the channel just long enough to cause a repetitive firing of the neuron. The Type II pyrethroids hold the channel open long enough so that the neuron becomes depolarized and no longer fires (Soderlund et al., 2002; Ray and Fry, 2006).

The induction of neurotoxic effects of pyrethroids in laboratory animals is largely dependent on the distribution of the pyrethroid into the CNS. White et al. (1976) examined brain concentrations of cismethrin and bioresmethrin after an oral dose and reported threshold concentrations in the brain necessary to induce signs of pyrethroid toxicity. Rickard and Brodie (1985) utilized IP injection of deltamethrin, and extensive tissue concentration time profiles in the brain and found a correlation between the onset of the different symptoms of pyrethroid intoxication and brain concentrations. Threshold concentrations of deltamethrin required for each symptom were identified and symptoms

persisted as long as the threshold brain concentration was maintained (Rickard and Brodie 1985). With direct injection of deltamethrin and cismethrin into the central nervous system the characteristic symptoms of pyrethroid intoxication are evident (Gray and Rickard (1982a). Based on these data it is thought that the actions of the pyrethroid parent chemical within the central nervous system are the principal mode of action for pyrethroid toxicity.

Human toxicity data is very limited for the pyrethroids. Gotoh et al. (1998) reported on a case of permethrin ingestion. In this study a 59 year-old man ingested approximately 600mL of a 20% permethrin emulsion in an apparent suicide attempt. It was reported that there were no reported signs of clinical neurotoxicity. It is unclear however how much of the ingested permethrin was absorbed into the systemic circulation as a portion of the ingested permethrin was recovered from the stomach of the individual. He et al. (1989) compiled a review of 573 cases of pyrethroid poisoning (229 cases of occupational poisoning and 344 cases of accidental poisoning) reported in the Chinese medical literature from 1983-1989. These cases included 325 deltamethrin, 196 fenvalerate, 45 cypermethrin, and 7 other pyrethroids poisonings. In the most severe cases, ingestion of pyrethroids induced symptoms consistent with central nervous system excitation as disturbances in consciousness, convulsions, and seizures were apparent. In less severe cases following occupational exposures patients presented with abnormal facial sensations (burning, itching, or tingling sensations), muscular fasciculations, myopia, and other symptoms. These symptoms indicate probable excitation of the peripheral nervous system (PNS) suggesting that the PNS is affected by pyrethroids. The clinical effects of acute pyrethroid poisoning in humans describe a similar set of symptoms (He et al. 1989) to those seen in laboratory animals. Similar clinical effects suggest a similar mode of action across species.

The similarities in laboratory animal studies and known human exposures indicate that there is likely a common mode of action across species for the pyrethroids. The potency of individual pyrethroids, however, is determined by a combination of their pharmacodynamic and pharmacokinetic properties. There are therefore numerous uncertainties in extrapolating the toxic potency of pyrethroids from laboratory animals to humans.

There is currently little known about the pharmacodynamic cascade of events following the interaction of the pyrethroid with the nerve axon sodium channel. How these events eventually lead to the symptoms of pyrethroid intoxication is unclear in laboratory animals and humans. A better understanding of the pharmacokinetic determinants of pyrethroid potency, however, is available from studying pyrethroids in laboratory animals.

PYRETHROID METABOLISM AND POTENTIAL IMPACT ON TOXIC POTENCY

The rate of systemic clearance (metabolism) of a xenobiotic, from which toxic effects are the result of the parent compound, may influence its toxic potency. More rapid metabolism can lead to lower target tissue concentrations. Slower metabolism can lead to increased target tissue concentrations due to greater systemic availability of the parent compound.

The potency of some pyrethroid isomers in mammals appears to be inversely related to the rate of metabolic elimination of the parent chemical (Abernathy and Casida 1973; White et al. 1976; Soderlund and Casida 1977). Abernathy and Casida (1973) were the first to report that the rate of metabolism of pyrethroids might be directly correlated to differences in their potency. Bioresmethrin, the trans-isomer of resmethrin, has an LD_{50} 50 times higher

than its cis-isomer, cismethrin, and is metabolized *in vitro* approximately 10-times more rapidly in mice (Abernathy and Casida (1973). Additionally the onset of neurobehavioral symptoms following exposure to cismethrin require brain concentrations only 5-8 times less than bioresmethrin to cause similar neurotoxic effects (White et al. 1976). White et al. (1976) concluded based on these results, and the results of Abernathy and Casida (1973), that both pharmacodynamic and pharmacokinetic properties must play a role in the differential potency of these two pyrethroids, which differ only in their stereochemistry.

The detoxification of a xenobiotic, whose parent form is the primary toxicant, can occur via a number of different pathways including phase I biotransformation pathways and/or phase II conjugation pathways. The initial biotransformation of pyrethroids follows one of two phase I pathways, cytochrome P450 (P450) oxidation, or esterase hydrolysis and takes place primarily in the liver of mammals (Soderlund and Casida 1977). These processes result in the production of numerous metabolites (Cole et al 1982; Crawford et al 1981a and b; Ruzo et al. 1978; Gaughan et al. 1976; Shono et al. 1979). The terminal phase of pyrethroid metabolism is the formation of glucuronide and glycine conjugates (Cole et al. 1982; Ruzo et al. 1978). These pathways appear similar between laboratory animals and humans. Common metabolites are found in the urine of both laboratory animals and humans (Cole et al. 1982; Heudorf and Angerer 2001).

The rate and pathway of phase I biotrasfomation of pyrethroids is in part dependent on their chemical structure (Shono et al. 1979; Soderlund and Casida 1977; Ueda et al. 1975). The stereochemistry around the cyclopropane functional group of the acid moiety of pyrethroids directly impacts the pathways by which the initial biotransformation of a pyrethroid occurs. Trans-isomers are more rapidly metabolized by hydrolytic (esterase)

pathways while cis-isomers are preferentially metabolized by slower oxidative (P450) pathways (Abernathy and Casida 1973; Shono et al. 1979; Soderlund and Casida 1977). The previous discussed examples of the two resmethrin isomers and two permethrin isomers are good examples of this (Shono et al. 1979; Soderlund and Casida 1977). This general trend of pyrethroid metabolism and toxicity is further confirmed by the studies of Soderlund and Casida (1977) in which the metabolism of numerous pyrethroids and pyrethroid analogs were examined. For nearly every pyrethroid studied the cis-isomers were metabolized at slower rates by oxidative metabolism compared to the trans-isomers which were preferentially metabolized more rapidly by hydrolysis. The work on bioresmethrin and cismethrin, however, are the only studies that have attempted to correlate rates of metabolism with brain concentrations (Abernathy and Casida 1973; White et al. 1976). Comparison of other pyrethroid potencies in relation to metabolic rates has utilized acute oral LD_{50} (administered dose) values as the dose metric for comparison (see Table 1.1 as an example). Further examination of the relationship between metabolism and potency is therefore needed.

A potentially important aspect in species extrapolation during risk assessment is understanding how individuals in a population may differ. To understand how individuals in a population may differ in their ability to metabolize pyrethroids it would be helpful to understand individual enzymes that metabolize them. The expression and activity of individual P450s and esterase in the human population are available for some P450s and carboxylesterases (Hosokowa et al. 1995; Rodrigues 1999). If an individual enzyme is responsible for the metabolism of a pyrethroid there is greater potential for significant variability in clearance rates within the population. In contrast, if there are numerous enzymes capable of metabolizing a pyrethroid there is likely to be less variability in

clearance rates in the general population. While the pathways that metabolize pyrethroids are fairly well understood in laboratory animals, and appear similar in humans, much less is known about the individual enzymes responsible for the biotransformation of the pyrethroids.

Until recently there have been no reports on individual esterases or P450s, which metabolize the pyrethroids. A recent study by Anand et al. (2006) briefly reported on the metabolism of deltamethrin by rat P450's. In this study CYPs 1A1, 1A2 and 2C11 were identified as P450s capable of metabolizing deltamethrin. CYPs 2A1, 2A2, 2B1, 2C6, 2C12, 2C13, 2D1, 2D2, 2E1, 3A1, and 3A2 were incapable of metabolizing deltamethrin. These experiments were done at a single saturating concentration. CYP 1A1 and 1A2 metabolized deltamethrin at a rate of approximately 9 and 13ng/pmole P450/min. CYP 2C11 metabolize deltamethrin at a rate of approximately 2ng/pmole P450/min. The activity of these enzymes however does not appear to account for the oxidative metabolism of deltamethrin observed in rat liver microsomes reported by Anand et al. (2006). Rat CYPs 1A1 and 1A2 are not highly expressed in the rat (Guengerich et al. 1982) or human liver (Rodrigues 1999) under basal conditions. These P450s are therefore unlikely to be responsible for the metabolism of deltamethrin in the rat liver microsomes. The observed rate of metabolism by CYP 2C11 accounted for elimination of approximately 2% of the mass of deltamethrin in the described assay conditions by Anand et al. (2006). It is therefore unlikely that CYP2C11 alone is responsible for the metabolism of deltamethrin in rat liver microsomes. Based on these results it is likely that we still do not know which P450s are responsible for the metabolism of the pyrethroids in rats. There are currently no literature reports on the metabolism of pyrethroids by human P450s.

The hydrolytic metabolism of pyrethroids has been studied more extensively in recent years compared to the P450's. Esterases were previously identified as the enzymes which are responsible for the hydrolytic metabolism of pyrethroids (Abernathy and Casida 1973; Casida et al. 1975). More recentlycarboxylesterases have been identified as the family of esterase which hydrolyze the pyrethroids in animals and humans (Shan and Hammock 2001; Stock et al. 2004). These studies purified, cloned, and expressed two pyrethroid hydrolyzing carboxylesterases from mouse liver. Huang et al. (2005) explored the hydrolysis of pyrethroid-like substrates by human, murine, porcine, and rabbit carboxylesterases. These studies also further confirmed that the trans-isomer pyrethroid-like substrates were more rapidly metabolized as compared to their cis-isomer counterparts by purified esterases.

Ross et al. (2006) also explored the hydrolysis of a number of pyrethroids by a rabbit carboxylesterase and two rat carboxylesterases, which all share greater than 80% homology in their amino acid sequence. Each of these enzyme metabolized trans-permethrin more effectively than cis-permethrin. In addition two cyano containing pyrethroids, deltamethrin and cis-cypermethrin, were metabolized by the rabbit esterase significantly slower than cis-permethrin, which does not contain a cyano moiety (Ross et al. 2006). This appears to verify that the cyano group further inhibits the hydrolysis of pyrethroids as was previously believed.

Esterase hydrolysis of pyrethroids may also be important in the blood. The blood of rats contains carboxylesterases (McCracken et al. 1993). Anand et al. 2006 described the ability of the serum of rats to metabolize the pyrethroid deltamethrin. Human blood however does not contain carboxylesterases (Li et al. 2005). There is potentially a significant species differences in the metabolism of pyrethroids in the blood that could impact systemic concentrations of pyrethroids.

Limited work has focused on the human metabolism of pyrethroids. Nishi et al. (2006) identified two human liver carboxylesterases, hCE-1 and hCE-2, which hydrolyze pyrethroids. Ross et al (2006) also examined the metabolism of select pyrethroids by these human carboxylesteraes and found them capable of metabolizing pyrethroids.

Choi et al.'s (2002) examined the metabolism of permethrin by human liver microsomes. The extensive hydrolytic metabolism of trans-permethrin in rat and mouse liver microsomes (Shono et al. 1979; Soderlund and Casida 1977) is also evident in human liver microsomes (Choi et al. 2002). This corresponds well with the observed metabolism of trans-permethrin by the human liver carboxylesterase (Nishi et al. 2006; Ross et al. 2006). The cis-isomer of permethrin, which is metabolized predominantly by P450's in laboratory animals (Shono et al. 1979; Soderlund and Casida 1977), was not detectably metabolized in human liver microsomes in the presence or absence of an NADPH regenerating system (Choi et al. 2002). This raises questions regarding species differences in oxidative metabolism of pyrethroids. Is this due to species differences in the expression or catalytic ability of P450's that metbaolize cis-isomer pyrethroids, the more potent isomer in laboratory animal studies? This would be of significant concern to human health as decreased metabolism of pyrethroids in humans could lead to increased exposure at the target tissue and therefore increased potency. This lack of, or very slow, hepatic metabolism of cis-permethrin corresponds with blood concentration data obtained by Gotoh et al. (1998) from the permethrin emulsion intoxication case previously described. The ingested emulsion mixture was approximately 56% trans- and 44% cis-permethrin. Examination of serum concentrations after ingestion revealed trans- and cis-permethrin were at 96 and 118ng/ml respectively upon admission of the patient. These levels rose to 253 and 615ng/ml of trans- and cis-permethrin, respectively,

3 hours after admission. Trans-permethrin was then rapidly eliminated from the blood and was at undetectable levels after 25 hours, cis-permethrin however was still detectable after 10 days.

Because human pyrethoid toxicity appears to be mediated by the same mechanism of action as in laboratory animals, it is appropriate to hypothesize that there may be a relationship between metabolism and potency in humans as well. In addition, the example of cis-permethrin demonstrates that there may be species differences in the metabolism of pyrethroids. Such differences could impact target tissue dosimetry, and the potency of pyrethroids in humans as compared to laboratory animals. This demonstrates that there is a need to further understand how human metabolism of pyrethroids compares to laboratory animals in pathway, rate of metabolism, and enzymes which metabolize the pyrethroids. Further understanding of these differences would help to reduce the uncertainties surrounding extrapolation of relative potencies of individual pyrethroids between laboratory animals and humans.

ABSORPTION, DISTRIBUTION, ELIMINATION AND TOXICOKINETIC MODELING OF PYRETHROIDS

Literature descriptions of the absorption, distribution, and metabolism/elimination of the pyrethroids vary widely in methods, chemicals utilized and data gathered. The available data while incomplete for a single chemical may be taken as a whole to better understand the important determinants of pyrethroid tissue dosimetry.

Absorption

Oral absorption is an area of great uncertainty with the pyrethroids and likely the least understood. A number of different studies have identified large portions of oral doses unchanged in the feces (Cole et al. 1982; Ruzo et al 1978; Bosch 1990; Crawford et al. 1981a,b). This is possibly the result of limited solubility of the chemical in the gastrointestinal tract. The pyrethroids are highly lipohillic chemicals with Log K_{ow} values in the rage of 4-6. Crofton et al. (1995) found that vehicle plays a role in the potency of pyrethroids, and this is likely due to the different absorption characteristics of each vehicle, altering the rate and amount of chemical entering the animal. More recently Kim et al., (2007) conducted a set of bioavailability experiments with different vehicles in rats. IV and oral dosing studies were conducted with two vehicles. Deltamethrin was dissolved in glycerol formal or suspended in Alkamuls (formerly known as Emulphor®). The soluble preparation of deltamethrin had higher peak blood concentrations and greater bioavailability of an oral dose (15% compared to 1.7%) (Kim et al. 2007). These data underscore the importance of accurately describing absorption of pyrethroids as it can directly impact the toxic potency of the pyrethroid by affecting systemic concentrations.

Other routes of exposure have not been thoroughly examined. Dermal exposure has been found to lead to minimal systemic absorption of the pyrethroids (unpublished, Hughes et al.). No known studies of inhalation exposure have been conducted.

Distribution

Marei et al (1982) examined the persistence of a number of pyrethroids in the fat and brains of rats. Their findings indicate rapid disposition of the pyrethroids into both the brain

and fat with peak levels of pyrethroid within the first few hours after dosing. The concentration of pyrethroid in fat was significantly higher than in the brain and persisted longer. In addition pretreatment with metabolic enzyme inhibitors increased the disposition of trans-permethrin into the fat and brain; however this did not occur with cis-permethrin.

A series of studies was conducted on toxicokinetics of deltamethrin and cismethrin in rats (Gray et al. 1980; Gray and Rickard 1981; Gray and Rickard 1982b). Studies by Gray and Rickard (1981;1982) followed the disposition of deltamethrin after intravenous injection of a toxic dose. Deltamethrin was rapidly distributed into all body tissues with the highest concentrations in fat (Gray and Rickard 1981). An important finding of these studies was lower than expected brain concentrations of deltamethrin based on previous findings with cismethrin (Gray et al. 1980). Comparing equitoxic doses of cismethrin and deltamethrin all tissues examined except the brain had tissue concentration ratios equal to the dose ratio (Gray et al 1980; Gray and Rickard 1981). This raises questions surrounding the impact of metabolism on distribution of pyrethroids to the brain. If metabolic differences between deltamethrin and cismethrin were resulting in the differences in brain concentrations it would be expected that other tissue would be affected in a similar manner. It was postulated that the difference in brain distribution were due to decreased blood-brain-barrier (BBB) permeability based on greater polarity of the deltamethrin molecule as compared to cismethrin (Gray and Rickard 1981). The distribution of pyrethroids into the brain is not well understood and even though the pyrethroids are structurally similar compounds the differences in their metabolism clearly demonstrate that their interaction with biological molecules can be significantly different. Distribution of pyrethroids into the brain may be affected by biological processes other than simple descriptions of diffusion from the blood.

Elimination

Crawford et al. (1981a,b) and Gaughan et al. (1976) studied the *in vivo* fate of radiolabeled cypermethrin and permethrin respectively. Cypermethrin and permethrin differ only in the presence of the alpha-cyano group on the cypermethrin molecule, and both are mixtures of cis- and trans-isomers. Both chemicals had similar metabolic fates *in vivo* in which the trans-isomer was eliminated from the animals more rapidly than the cis-isomer. In addition greater quantities of hydroxylated metabolites were identified from the cis-isomers and greater quantities of hydrolysis products were identified from the trans-isomers in direct correlation with *in vitro* studies of their metabolism.

Cole et al. (1982) examined the *in vivo* fate of the pyrethroids tralomethrin, traolcythrin, deltamethrin, and cis-cypermethrin in rats orally dosed. Greater than 70% of the dose of these pyrethroids was eliminated in the urine and feces in the first 24 hours. Radiolabeled pyrethroid was detectably eliminated in both urine and feces up to 7 days post dose (Cole et al. 1982). Ruzo et al. (1978) conducted a similar study with deltamethrin. Rats were dosed with three similar doses (0.64, 0.9, and 1.60 mg/Kg) each of which was radiolabled in a different position of the pyrethroid molecule. In this study greater than 80% of the doses were eliminated from the rat within the first 24 hours with detectable levels of radiolabel also evident for up to 8 days in the urine and feces. Radiolabel detected in the urine was that of metabolites only, no parent chemical was identified in the urine. Parent deltamethrin and small amounts of hydroxylated deltamethrin metabolites were found in the feces. Crawford et al. (1981a) found approximately 1% of the total radioactivity of an oral dose of cypermethrin in the bile of rats. Crawford et al. (1981b) also identified small amounts of hydroxylated cypermethrin in the feces of rats. Biliary elimination of

hydroxylated metabolites prior to their hydrolytic cleavage likely accounts for the identification of hydroxylated metabolites of cypermethrin and deltamethrin in feces.

Bosch et al. (1990) also studied the urinary and fecal excretion of deltamethrin in rats. Two doses, 0.55 mg/kg and 5.5 mg/kg, were utilized. Between 17 and 35% of the dose was found unchanged with only trace levels of hydroxylated metabolites identified in the feces indicating incomplete absorption of deltamethrin. Dose dependency of absorption of deltamethrin is potentially apparent in this study as fecal excretion of the 0.55 mg/kg dose averaged 26% of the dose in male and female rats while excretion of the 5.5 mg/kg dose averaged 36% of the dose (Bosch 1990). Urinalysis indicated only hydrolyzed metabolites of deltamethrin were excreted in the urine.

Toxicokinetic Modeling

Anadon et al. (1991,1996) published limited toxicokinetic studies of permethrin and deltamethrin in rats. Deltamethrin and permethrin were rapidly absorbed with peak tissue concentrations occurring within hours similar to previous reports (Cole et al. 1982; Crawford et al. 1981). Anadon et al.'s (1991,1996) toxicokinetic modeling of deltamethrin and permethrin were fitted to two compartment models where maximal plasma concentrations were reached after 2-4hrs with elimination half lives of 38hr and 12hr respectively. Volume of distribution was reported by Anadon et al. (1991, 1996) for permethrin at 1.7 L while the value for deltamethrin was calculated as 5.33 L. Bioavailability of the parent chemical was significantly different for these two chemicals as well. Bioavailability of permethrin was reported to be 60% while it was only 15% for deltamethrin. These differences may be indicative of a number of differences in the pharmacokinetics of these two pyrethroids even

though they are structurally similar chemicals. Differences in absorption, distribution, and metabolism of these two chemicals may be responsible for the observed differences.

A PBPK model of deltamethrin has recently been developed in the rat by Mirfazaelian et al. (2006). This model revealed continued uncertainties in the modeling of deltamethrin which if addressed may increase confidence in species extrapolation. The model of Mirfazaelian et al. (2006) required a saturable efflux process to describe fecal excretion in which a greater percentage of an oral dose was absorbed at higher doses. This is contrary to published data regarding fecal excretion of pyrethroids. The work of Bosch 1990; Cole et al., 1982; Ruzo et al., 1978 show that varying amounts of deltamethrin (between 15-35%) are excreted unchanged in the feces of male rats with no clear dose dependency in the results.

The PBPK model of Mirfazaelian et al. (2006) however also make clear some of the major uncertainties involved with modeling of pyrethroids. As with previous studies distribution of deltamethrin into the brain was low with brain-blood ratios of less than one. This again raises questions about the influence of the BBB permeability, the possibility of export transporters along the BBB, and the impact of metabolic rate on brain concentrations. The model also does not describe the tissue time course of the liver. Understanding the distribution of the chemical in the liver may be important in determining the appropriate structure of the model. The importance of metabolic differences between chemicals would be greater in a model of a flow limited liver compartment as compared to a diffusion limited liver compartment. This could play a significant role in understanding how species differences in the metabolism of a pyrethroid may affect exposure-dose relationships between rats and humans.

Based on the available data it appears evident that the pyrethroids are rapidly, however incompletely, absorbed. They rapidly distribute throughout the body with the greatest disposition in the fat. There appear to be multiple factors that can play a role in determining exposure dose relationships in the brain in rats including, extent of absorption, rate of metabolic elimination and the ability of the chemical to penetrate the blood brain barrier.

Very little is known about the human kinetics for pyrethroids. The study of blood concentrations from an accidental exposure to a permethrin mixture by Gotoh et al. (1998) is the only published literature on the subject. This study reveled that trans-permethrin was rapidly eliminated from the blood while cis-permethrin was eliminated very slowly. How this might relate to the toxicokinetics of other cis-isomer pyrethroids in humans, typically the more toxic isomers, is unknown. It is clearly important to understand as much as possible about toxicokinetic similarities/differences between species to most accurately extrapolate dose response data in a risk assessment.

THE USE OF UNCERTATINTY/SAFETY FACTORS AND PBPK MODELING FOR SPECIES EXTRAPOLATION IN RISK ASSESSMENT

Risk assessment consists of four parts; hazard identification, dose-response assessment, exposure assessment, and risk characterization. With little to no human data typically available for environmental chemicals, animal data and default uncertainty/safety factors are routinely utilized in human health risk assessment to extrapolate dose response. The use of uncertainty or safety factors has been utilized in human health risk assessment for over 30 years (Renwick and Lazarus 1998). They have been utilized to convert no-observed-

adverse-effect levels (NOAEL) for noncancer end points into human intake levels that are believed to be "without appreciable health risk" (Renwick and Lazarus 1998; WHO, 1987). NOAELs are generally determined through appropriate animal studies and human reference doses are established by applying various uncertainty factors. The 1987 World Health Organization's (WHO) Environmental Health Criteria (EHC vol 70) recommends a safety factor of 100 for extrapolation of an animal NOAEL. The 100 factor for uncertainty in species extrapolation can be broken down into a factor of 10 for interspecies differences and 10 for inter-individual differences in the human population (WHO, 1987). Uncertainty factors are also added due to reliance on acute instead of chronic studies, database inadequacy, and for conversion of lowest-observed-adverse-effect level (LOAEL) to a NOAEL (Andersen 1995: Renwick and Lazarus 1998). Uncertainty factors however are used irrespective of the species used in the animal studies (Schneider et al. 2004). Further safety factors can also be added based on risk to susceptible populations such as children or the elderly.

More recently an appreciation for the use of pharmacokinetic and pharmacodynamic data which may augment or refine the understanding of species differences have been shown to be able to displace some of the uncertainty in risk assessment (Andersen 1995; Renwick and Lazarus 1998). The 10 fold factors each for inter-species extrapolation and inter-individual variability can be broken down into individual components for toxicodynamics and toxicokinetics to account for available data (Barnes and Dourson 1988; Renwick 1993). Thus, if data were available to better inform about species differences in a risk assessment a portion of the uncertainty factor may be removed. In WHOs 1994 EHC vol. 170 this paradigm was accepted and it was stated that "In situations where appropriate toxicokinetic

and/or toxicodynamic data exist for a particular compound, then the relevant uncertainty factor should be replaced by the data derived factor".

The need for better methods of extrapolating equivalent doses by predicting target tissue dose across species, including humans, has lead to the development and use of PBPK models in risk assessment. PBPK models utilize physiological and biochemical parameters from both the test species and humans to describe the differences in the disposition of a chemical at the target site. While changes in physiological parameters across species are independent of chemical a number of parameters are chemical dependent. It is necessary to determine differences in biochemical parameters such as absorption, metabolism, and excretion in both the test species and humans in order to more accurately extrapolate tissue dosimetry. PBPK models have been utilized to improve species extrapolation of equivalent dose in dose-response assessments for previous human health risk assessment. An example of which is the use of a PBPK model to assess the risk of exposure to methylene chloride (Andersen et al. 1987). The use of the PBPK model showed that the default methods of applying uncertainty factors overestimated the risk to humans from methylene chloride by 100-200-fold (Andersend et al. 1987). PBPK models have also been utilized in risk assessments for vinyl chloride, and 2-butoxyethanol (Clewell et al. 2002). Combined with animal studies these models were able to provide more accurate assessments of the risks to humans from exposure to these chemicals as compared to the use of default uncertainty factors.

PBPK modeling is also a tool that unlike uncertainty factors can be used to predict internal dose based on different exposure scenarios across species including humans (Andersen 2005). If an understanding of the appropriate biochemical and physiological

parameters is available in the test species and humans a PBPK model can be utilized. This allows an assessment of how species differences in pharmacokinetic parameters may effect the calculation of an equivalent dose based on a target tissue concentration. PBPK modeling also allows for a possible examination of how population variability in PK parameters could lead to susceptible populations. For example if there is considerable variability in the human population with respect to the expression or activity of a metabolizing enzyme (due to genetic variability, age, life style, other environmental exposures) which is responsible for the metabolism of a xenobiotic a portion of the population may be more or less susceptible to an exposure.

Important to understanding population susceptibility and the risks that are posed to the human population from an environmental contaminant is an understanding of human exposure. Understanding who is exposed to an environmental contaminant (general population, occupational groups, children, elderly) and by which route is important for not only identifying who is at the greatest risk from exposure, but what the appropriate method for modeling that exposure will be.

HUMAN EXPOSURE AND CURRENT REGUULATION OF PYRETHROIDS

Pyrethroids are used in the protection of a wide range of commercial crops, ornamentals, and trees. In addition, they are used in domestic insect control and are ectoparasiticides in both human and veterinary medicine (Roberts and Hutson 1999). Due to their numerous uses and increasing use world wide the potential for ubiquitous human exposure to pyrethroids exists. Evidence of this is seen in exposure studies of both occupational cohorts (Zhang et al. 1991) (Smith et al. 2002) and the general population. Heudorf and Angerer (2001), and Schettgen et al. (2002) found chemical residues of multiple

pyrethroid pesticides in human urine samples in urban populations of individuals with no known use exposure. Schettgen et al. (2002) propose that the majority of this exposure is due to oral intake of pyrethroid residues in the daily diet. The USDA's pesticide data program (PDP) demonstrates the presence of pyrethroids in a variety of foods at part per billion and part per million concentrations. In addition the CDC's Third National Report on Human Exposure to Environmental Chemicals details human exposure to pyrethroids utilizing data from the National Health and Nutritional Examination Survey (NHANES), which also reports urinary levels of pyrethroid metabolites. These studies indicate that humans are exposed to a variety of pyrethroid pesticides by both occupational, direct use, and dietary means. The major route of exposure to pyrethroids is expected to be orally through dietary and hand to mouth exposure and should be the focus of risk assessment efforts.

Previously the pyrethroids have been regulated on a single chemical basis utilizing default uncertainty factors to arrive at human equivalent doses or references doses. Deltamethrin for example has a rat no observable adverse effect level (NOAEL) of 1 mg/kg. This has been extrapolated to a safe human exposure level by the United States Environemental Protection Agency (USEPA) by dividing by 100 (a 10 fold factor for species difference and a 10 fold factor for human population variability). This yields a human reference dose (RfD) calculation of 0.01mg/kg/day for deltamethrin (Federal Register 2004).

The 1996 food quality protection act (FQPA) mandated that the USEPA conduct cumulative risk assessments of environmental chemicals that act by a common mechanism of toxicity. The EPA defines common mechanism of toxicity as "…substances that cause a common toxic effect to human health by the same, or essentially the same, sequences of major biochemical events. Hence, the underlying basis of the toxicity is the same, or

essentially the same, for each chemical." (EPA 2002). The neurotoxic actions of all pyrethroids are primarily the result of the interaction of the parent chemical with nerve axon sodium channels in mammals. There is limited evidence that other biochemical pathways may be involved in the neurobehavioral toxicity of pyrethroids, including interactions with calcium, chloride and GABA channels in the central nervous system (see review by Soderlund et al 2002). However because the preponderance of evidence points to the sodium channel as the primary biochemical pathway by which the pyrethroids induce their toxicity they are currently under evaluation to assess if they can be considered in a common mechanism group of chemicals.

Research is thus needed to aid a potential risk assessment. Work which includes understanding of mixture neurtoxicology, whether these chemicals act dose additively or not, as well as mixture pharmacokinetics will be useful. Understanding mixture pharmacokinetics however necessitates an understanding of individual pyrethroid pharmacokinetics.

CURRENT RESEARCH NEEDS

While there is a large volume of literature on the toxicity and metabolic fate of pyrethroids in animals, it is unknown how this translates to human risk. It would be beneficial to risk assessors to have a better understanding of pyrethroid pharmacokinetics between species to better extrapolate relative potencies. In the absence of human data the optimal way to conduct a species extrapolation is the use of PBPK models that can quantitatively describe differences in the pharmacokinetics and tissue dosimetry of pyrethroids across species.

As described above (Fig 1.1), the parallelogram approach to species extrapolation is the most desirable method for developing a PBPK model in the absences of human data. A PBPK model can be utilized in a risk assessment in the absences of actual human derived data to calculate equivalent dose. The steps to developing a PBPK model through the parallelogram approach include. (1) Developing a hypothesis based model that includes descriptions of the proposed PK determinants of disposition (tissue dosimetry). (2) Determining the influence of PK determinants (rodent *in vitro*) on a surrogate mammalian system in the proposed PBPK model. This can be done by examining the tissues, pathways, rates, and enzymes responsible for the metabolism of the pyrethroids. (3) Comparing important PK parameters in the human and a surrogate mammalian system. Conduct parallel experiments in available rat and human tissue fractions. (4) incorporate *in vitro* human parameters into a PBPK model with pertinent human anatomical/physiological data.

A PBPK model of pyrethroid exposure needs to be able to describe the absorption, distribution, metabolism/elimination of the toxicant (the parent chemical for pyrethroids). Based on the current knowledge of pyrethroids the following hypothesis driven model can be identified. Human exposure estimates suggest the oral exposure pathway appears to dominate as the primary route of exposure, thus the gastrointestinal tract, the rate of absorption, and the percent parent chemical absorbed (or fecal elimination) will be important model parameters. Liver and adipose tissue should be included in the model as organs involved in the metabolism and distribution of the pyrethroids, respectively. Metabolism must be understood in both the mammalian surrogate (the rat) and humans to properly extrapolate a PBPK model. The effects of pyrethroids on the CNS appear to mediate the neurotoxicity. There is however suggestive evidence that the PNS may also be involved in

the neurotoxicity. Based on the current understanding of the mode of action, the model should include a CNS tissue compartment (brain compartment), as a likely target tissue. Since the PNS is a rather diffuse system and the exact site of action of pyrethroids on this system is uncertain, blood concentrations may be an adequate surrogate for this tissue. Understanding both blood and brain concentrations will enable identification of an appropriate dose metric if the PBPK model is combined with a model of pyrethroid pharmacodynamics in a risk assessment. The inclusion of additional compartments such as rapidly and slowly perfused tissue may be necessary to further describe the disposition of the pyrethroids throughout the body. The description of model compartments as either flow or diffusion limited, particularly the brain and liver compartments, is important to understanding the influence of metabolism on brain concentrations and therefore toxic potency.

In addition to extrapolation of dose response, understanding human population variability is also an important variable in a population based risk assessment. There is currently little to no data regarding which individual enzymes metabolize the pyrethroids. A comparison of the enzymes that metabolize the pyrethroids between species may aid the understanding of how factors that influence the expression and activity of metabolizing enzymes may impact the toxic potency of the pyrethroids across a population. This may aid in elucidating potential variability in the human population and possibly identify susceptible populations. In addition knowledge of the enzymes that metabolize the pyrethroids will aid in the understanding of potential pharmacokinetic interactions at the level of metabolism in a cumulative risk assessment as well as in concomitant exposure with other environmental contaminants.

HYPOTHESIS, SPECIFIC AIMS, AND CONTENT OF DISSERTATION

While a general review of pyrethroids has been presented here, this dissertation focuses on the pharmacokinetics of two pyrethroids, deltamethrin and esfenvalerate. These two pyrethroids were chosen for the following reasons; (1) They are both single isomer pyrethroids, (2) and are two of the most potent pyrethroids based on oral LD_{50} in rats and mice. (3) They have consistently been identified in human exposure assessments, and (4) initial experiments showed that they displayed species differences in their metabolism between rats and humans that would allow valuable comparisons to be made.

The working hypothesis of this dissertation is that the clearance of pyrethroids from the liver will drive blood concentrations of pyrethroids and therefore impact brain concentrations and potency of pyrethroids. The specific aims of this research are to; (1) Explore the hepatic metabolism of deltamethrin and esfenvalerate to understand differences in rates and pathways of clearance in rats and humans, (2) examine rat and human extrahepatic tissues for their ability to metabolize these pyrethroids, (3) determine the enzymes responsible for the metabolism of deltamethrin and esfenvalerate in rats and humans, (4) develop a PBPK models of exposure to deltamethrin in the rat, and (5) extrapolate the rat PBPK model to a human PBPK model which can be used to derive human equivalent doses based on target tissue dosimetry.

Chapter II of this dissertation was based on the hypothesis that hepatic metabolism is similar between rats and humans. Results however identified species differences in the hepatic metabolism of deltamethrin and esfenvalerate. Also included in chapter II is a comparison of rat and human carboxylesterases. The ability of rat and human hepatic

carboxylesterase to metabolize deltamethrin and esfenvalerate were examined in an effort to elucidate the mechanisms behind the observed species differences.

Chapter III focuses on understanding the role of oxidative metabolism in the species differences in the hepatic metabolism of deltamethrin and esfenvalerate. The hypothesis was that different rat and human P450s metabolize these two pyrethroids based on the species difference observed in chapter II. Also included in chapter III is an examination of the metabolism of deltamethrin and esfenvalerate by rat and human serum and serum esterases to explore potential species differences.

Chapter IV explores the working hypothesis that the rate of hepatic metabolism will drive blood concentrations and therefore brain concentrations influencing the toxic potency of deltamethrin between rats and humans. A PBPK model was developed for rats and extrapolated to humans to examine this hypothesis.

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CHAPTER II

SPECIES DIFFERENCE IN THE *IN VITRO* METABOLISM OF DELTAMETHRIN AND ESFENVALERATE: DIFFERENTIAL OXIDATIVE AND HYDROLYTIC METABOLISM BY HUMANS AND RATS

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Species difference in the *in vitro* metabolism of deltamethrin

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Abbreviations
CL_{INT}, Intrinsic Hepatic Clearance
TEPP, tetraethylpyrophosphate
hCE-1, Human carboxylesterase 1
hCE-2, Human carboxylesterase 2
cis-per, cis-permethrin;
esfen, esfenvalerate;
delta, deltamethrin;
biores, bioresmethrin;
trans-per, trans-permethrin.

ABSTRACT

Pyrethroids are neurotoxic pesticides whose pharmacokinetic behavior plays a role in their potency. This study examined the elimination of esfenvalerate and deltamethrin from rat and human liver microsomes. A parent depletion approach in the presence and absence of NADPH was utilized to assess species differences in biotransformation pathways, rates of elimination, and intrinsic hepatic clearance. Esfenvalerate was eliminated primarily via NADPH-dependent oxidative metabolism in both rat and human liver microsomes. The intrinsic hepatic clearance (CL_{INT}) of esfenvalerate was estimated to be three- fold greater in rodents than in humans on a per kg body weight basis. Deltamethrin was also eliminated primarily via NADPH-dependent oxidative metabolism in rat liver microsomes; however, in human liver microsomes, deltamethrin was eliminated almost entirely via NADPH-independent hydrolytic metabolism. The CL_{INT} for deltamethrin was estimated to be two-fold more rapid in humans than in rats on a per kg body weight basis. Metabolism by purified rat and human carboxylesterases (CEs) were utilized to further examine the species in hydrolysis of deltamethrin and esfenvalerate. Results of CE metabolism revealed that human carboxylesterase 1 (hCE-1) was markedly more active towards deltamethrin than the class 1 rat CEs Hydrolase A and B and the class 2 human CE (hCE-2); however, Hydrolase A metabolized esfenvalerate 2-fold faster than hCE-1, while Hydrolase B and hCE-1 hydrolyzed esfenvalerate at equal rates. These studies demonstrate a significant species difference in the *in vitro* pathways of biotransformation of deltamethrin in rat and human liver microsomes, which is due in part to differences in the intrinsic activities of rat and human carboxylestersases.

INTRODUCTION

Pyrethroids are synthetic analogues of the natural pyrethrins, the insecticidal components of extracts from the pyrethrum flower (*Chrysanthemum cinerariaefolium)*. The pyrethroids modulate nerve axon sodium channels, resulting in neurotoxic effects (Narahashi 1982; Smith et al., 1997). The adverse effects produced by pyrethroids are due to the parent compounds in that no evidence currently exists that pyrethroid metabolites alter sodium channels and are neurotoxic. For the limited number of pyrethroids evaluated, the brain concentrations of pesticide appear to correlate with acute neurotoxicity (Rickard and Brodie, 1985; White et al., 1976). Pharmacokinetic parameters, particularly clearance of the parent chemical from the blood, will influence the effective concentration in the brain and therefore can have a significant influence on their toxic potency.

The metabolic pathway and rate of phase I biotransformation of pyrethroids is dependent upon their chemical structure and stereochemistry (Shono et al., 1979; Soderlund and Casida, 1977; Ueda et al., 1975). In laboratory animals, different metabolic pathways preferentially transform cis- and trans- isomers of pyrethroids; trans-isomers are typically transformed by the more rapid hydrolytic pathways, while cis-isomers are preferentially transformed by slower oxidative pathways (Shono et al., 1979; Soderlund and Casida, 1977). This difference correlates with the greater toxicity of several cis-isomers of individual pyrethroids (Soderlund et al., 2002). For example, cismethrin is about 50 times more acutely toxic in mice than its trans-isomer, bioresmethrin, when based on administered dose. This is due in part to the approximate 10-fold greater rate of metabolism of bioresmethrin than cismethrin (Abernathy and Casida, 1973).

Species differences in the contributions of hydrolytic and oxidative pathways to the metabolism of pyrethroids exist between mice and rats (Shono et al., 1979; Soderlund and Casida, 1977). For example, trans-permethrin is metabolized predominately by hydrolytic metabolism in rats. In contrast, both oxidative and hydrolytic pathways contribute to trans-permethrin metabolism in mice (Shono et al., 1979; Soderlund and Casida, 1977). For cis-permethrin, oxidative metabolism is 3- and 8-fold greater than hydrolysis in rats and mice, respectively (Shono et al., 1979). While there are numerous studies that have examined laboratory animal metabolism of pyrethroids, there are relatively few examples of human metabolism of pyrethroids (Choi et al., 2002; Nishi et al., 2006; Ross et al., 2006). In these studies, the extensive hydrolysis of trans-permethrin in rat and mouse liver microsomes (Shono et al., 1979; Soderlund and Casida, 1977) and by mouse carboxylesterases (Stok et al., 2004) was also evident in human liver microsomes (Choi et al., 2002; Ross et al., 2006). For cis-permethrin however, Choi et al. (2002) reported that there was no detectable oxidative or hydrolytic metabolism in human liver fractions (Choi et al, 2002). Ross et al. (2006) observed limited hydrolysis of cis-permethrin in human liver microsomes as compared to rat liver microsomes. Ross et al., (2006) and Nishi et al. (2006) also reported limited hydrolysis of cis-permethrin by purified human carboxylesterases. These results with cis-permethrin show that species differences in pyrethroid metabolism may exist between laboratory animals and humans. Differences in pathways or rates of metabolism of pyrethroids between species could lead to altered systemic bioavailability, exposure dose relationships, and toxic potency for a pyrethroid.

The present study examines the potential for species differences in the phase I biotransformation of two pyrethroids, deltamethrin and esfenvalerate (Fig 2.1). Rat and

human liver microsomes were used to compare the contribution of oxidative and hydrolytic pathways of metabolism between species. These two pathways are the primary known mechanisms of pyrethroid detoxification; no evidence exists that conjugative or reductive mechanisms of parent pyrethroid are involved. In laboratory animals there are numerous metabolites (Roberts and Hutson 1999) of deltamethrin and esfenvalerate likely produced by a number of enzymes including multiple cytochrome P450's (Anand et al. 2006) (Dayal et al. 2003) and esterases (Ross et al. 2006; Nishi et al. 2006). Therefore, liver microsomes represent a simple method of comparing the phase I detoxification step in pyrethroid metabolism between species. Since phase I biotransformation of a pyrethroid is the detoxification step, it allows the use of the parent depletion approach to examine hepatic elimination and estimate intrinsic clearance (Obach 1999). For the purposes of this manuscript, the term elimination is synonymous with metabolism or biotransformation. Based on results obtained with liver microsomes, purified enzyme preparations of rat and human carboxylesterases were also utilized in this research to better understand observed differences in rat and human metabolism of these two pyrethroids.

MATERIALS AND METHODS

Chemicals

Deltamethrin (98.9% purity) ((S)-alpha-cyano-3-phenoxybenzyl (1R)-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) (cyano-3-phenoxybenzyl (αS)-2-(4-chlorophenyl)-3-methylbutyrate) was a gift from Dupont (Johnston, IA USA). These chemicals were used in the microsomal elimination studies. Bifenthrin and tetraethylpyrophosphate (TEPP) were obtained from ChemService Inc (West Chester, PA). Deltamethrin (> 98%) and esfenvalerate (>98%) used for the *in vitro* carboxylesterase kinetics studies were obtained from ChemService Inc (West Chester, PA). LC/MS analysis of pyrethroids from the different 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, sucrose, EDTA, KCl, NaHepes, glycerol, dithiothreitol (DTT), Trizma-base, β-nicotinamide adenine dinucleotide phosphate reduced form (NADPH), and 3-phenoxybenzaldehyde were purchased from Sigma-Aldrich (St. Louis, MO USA). Unless otherwise specified chemicals were of the highest grade commercially available.

Animals

All animal procedures were approved by the USEPA National Health and Environmental Effects Research Laboratory Institutional Animal Care and Use Committee. Male Long Evans rats (70 days old) were obtained from Charles River Laboratory (Raleigh, NC USA). Animals were allowed to acclimate for a minimum of four days in an

Association for Assessment and Accreditation of Laboratory Animal Care approved animal facility prior to their use. Two animals were housed per plastic cage (45 cm x 24 cm x 20 cm) with heat-treated pine shavings bedding. Animals were maintained at 21-±2°C, 50 ± 10% humidity and a photoperiod of 12L:12D (0600-1800 hr). Feed (Purina Rodent Chow 5001, Barnes Supply Co., Durham, NC USA) and tap water were provided *ad libitum*.

Rat liver microsomal preparation

Animals were anesthetized with CO_2 and sacrificed via cardiac puncture. Livers were removed for microsomal preparation according to the method described by DeVito et al (1997). Three separate pools of rat liver microsomes were prepared. Each pool was prepared from the livers of two rats. From each pool of microsomes 1.0 mL aliquots were flash frozen in liquid nitrogen and stored at -80°C until use. Microsomal protein (MSP) content was quantified using the Bio-Rad Bradford protein assay (Bio-Rad, Hercules, CA USA) standardized with bovine serum albumin.

Human microsomes

Pooled human liver microsomes were purchased from CellzDirect (Phoenix, AZ USA) (Lot# HMMC-PL020), Cedra (Austin, TX USA) (Lot#821-1), and Xenotech (Lenexa, KS USA) (Lot#0310241). These microsomes were stored at -80°C until use.

Determination of K_m apparent (K_{mapp}) in rat and human liver microsomes

To ensure that the elimination assays (see below) were performed at concentrations $\ll K_{mapp}$, estimates of the K_{mapp} for deltamethrin and esfenvalerate elimination in rat and

human liver microsomes were determined using a single microsomal sample from each species. The reaction volume was 1.5 mL and consisted of 0.1 M Tris (pH 7.4), 1.0 mg MSP/mL and 1.0 mg NADPH /mL. Following preincubation for 10 min at 37 °C, the reaction was initiated by the addition of 60 μL of working stock solutions of deltamethrin or esfenvalerate dissolved in 50% acetonitrile solution to obtain final assay concentrations of 1, 2, 5, 7, 10, 20, and 50 μM pyrethroid. Pilot studies demonstrated that this percentage of acetonitrile did not to interfere with oxidative or hydrolytic metabolism in these assays (data not shown). Assays were carried out in duplicate in a shaking water bath and 250 μL aliquots of the reaction mixture were removed from the reaction vial at 0, 2.5, 5.0, 7.5 and 10.0 min. These time points were previously found to be in a linear range of elimination of deltamethrin and esfenvalerate (data not shown). Aliquots were immediately placed in 1.25-ml cold 50% ACN containing 0.1 mg/ml bifenthrin (internal surrogate of recovery) to stop the reaction. Samples were vortexed for 5 min and centrifuged at approximately 1,500 x g for 10 min. The supernatant was removed and placed in autosampler vials for LC/MS/MS analyses (see below). Non enzymatic controls were performed in the Tris buffer in the absence of microsomal protein to ensure all elimination of chemical was enzymatic. Concentration of substrate was monitored over the time course and converted to moles of substrate remaining. Substrate remaining was converted to product formed and plotted vs. time to produce a reaction velocity. K_{mapp} values in rat and human liver microsomes were estimated for deltamethrin with Lineweaver-Burk linear regression analysis from GraphPad Prism (v 4.0, GraphPad Software, San Diego California, USA). The 50 uM reactions appear to be approaching maximal elimination rates. However, because a clear maximum was not defined, a Lineweaver-Burk analysis was utilized for deltamethrin analysis. The K_{mapp} in rat

and human microsomes for esfenvalerate were estimated with Graph Pad Prism software using Michaelis-Menten nonlinear regression analysis.

Rat and human microsomal elimination assays

Microsomal elimination assays with deltamethrin and esfenvalerate were carried out using the parent depletion approach described above. Briefly, 1uM pyrethroid was found from kinetic studies to be below the K_{mapp} for both deltamethrin and esfenvalerate elimination from both rat and human liver microsomes. 1uM pyrethroid was incubated from 0-10min in 1.5mL 0.1M Tris containing 1.0mg MSP/mL, and 1.0 mg NADPH/mL. NADPH independent assays were carried out from 0-30min to insure sufficient elimination to calculate elimination rates. Assays were carried out in duplicate in a shaking water bath at 37°C and 250μl aliquots were removed at each time point for LC/MS/MS analysis. Assays were repeated in the presence of 200uM TEPP to inhibit esterase activity (Soderlund and Casida, 1977). A volume of 10μl of 30mM TEPP in methanol was added to the assay prior to incubating for 10min at 37°C before the addition of pyrethroid.

Identification, recovery, and quantification of deltamethrin and esfenvalerate.

An Agilent (Palo Alto, CA USA) 1100 series LC/MSD VL ion trap mass spectrometer and HP Chemstation software were used for identification and quantification of pyrethroid parent chemicals. Isocratic elution of chemicals was accomplished with an Agilent Zorbax Eclipse XDB – C18 column (4.6 x 100 mm, 3.5 μm pore size) and XDB C-18 guard column with a mobile phase of 90% methanol and 10% 5 mM ammonium formate at a flow rate of 0.5 ml/min. Deltamethrin identification and quantitation was accomplished

by fragmentation of the ammonium adducted ion $[M+NH_4]^+$ (m/z 523) to produce the parent ion $[M+H]^+$ (m/z 506). Esfenvalerate identification and quantitation was accomplished via the ammonium adducted ion $[M+NH_4]^+$ (m/z 437). Bifenthrin was identified and quantified using the ammonium adducted ion $[M+NH_4]^+$ (m/z 440). Recovery of deltamethrin and esfenvalerate from microsomal assays was assessed at all concentrations utilized in standard curves (0.1, 0.5, 1, 2, 5, 7, 10, 20, and 50μM) and compared to an equivalent concentration in the absence of microsomal protein. Recovery was found to be greater than 95% at all concentrations. Precision of points on standard curves were calculated to be within 5% of standards in buffer. Quantification was accomplished using the peak area ratios of the analyte and internal surrogate. Standard curves were developed from standards in the reaction buffer containing microsomal protein. Standard curves were linear over the range of concentrations used in the experiments with r^2 values of at least 0.99. Accuracy limits of 20% were utilized for inclusion in standard curves by the Agilent Chemstation software.

Calculation of elimination rates, elimination rate constants, and intrinsic hepatic clearance rates

Elimination rate, elimination rate constants and estimates of intrinsic hepatic clearance of deltamethrin and esfenvalerate were determined from the elimination of parent chemical from the microsomal assay at a single concentration (1 μM) over a time course of 0-10min. The concentration of the parent chemical was measured at each time point and converted to moles of substrate remaining. Calculation of elimination rates was accomplished by linear regression of substrate remaining vs. time plots. First order elimination rate constants (k) were determined from the plot of the ln of the % remaining vs.

time where the slope of the linear regression line = k (min^{-1}). The 1μM concentration was found to be below the K_{mapp} for each chemical in both rat and human liver microsomes (Table 2.1). At substrate concentrations significantly below K_m the clearance of a chemical is constant (Iwatsubo et al., 1997), and can be calculated using the first order elimination rate constant, k (Obach, 1999). Intrinsic clearance (CL_{INT}), on a per kg body weight basis was estimated based on the equation described below (Obach et al., 1997; Obach, 1999).

CL_{int} = *k*(min^{-1}) ** ml incubation/mg microsomes * mg microsomes/g liver * g liver/kg body weight.*

It was assumed that humans and rats have 40 and 25.7gm of liver per Kg body weight, respectively (Davies and Morris, 1993). Microsomal content of the livers were assumed to be 52.5 and 45 mg microsomal protein per gm of liver for humans (Iwatsubo et al., 1997) and rats (Houston, 1994), respectively.

Human and rat carboxylesterases

The recombinant carboxylesterases, human CE-1 (hCE-1) and CE-2 (hCE-2), were expressed in a *Spodoptera frugiperda*-derived cell line using baculovirus and purified to homogeneity as previously described (Morton and Potter, 2000). Rat CEs termed Hydrolases A and B (Morgan et al., 1994), were purified to homogeneity from male Sprague-Dawley rat liver by the procedure of Sanghani et al. (2002) with slight modification. This entailed removal of an 80-kDa impurity present in Hydrolase A by anion exchange chromatography. The purified rat CEs were digested with trypsin and analyzed by MALDI-TOF/TOF mass

spectrometery. The proteins were shown to be identical to rat Hydrolase A (also termed ES-10) and Hydrolase B (termed RL1) (Morgan et al., 1994).

Hydrolysis reactions catalyzed by CEs

Hydrolysis of deltamethrin and esfenvalerate by rat and human CEs were performed at a single saturating concentration of pyrethroid (50μM) in order to compare the hydrolysis rates of each enzyme (specific activity). Kinetic experiments with deltamethrin were also performed to obtain k_{cat}, K_m and k_{cat}/K_m parameters for each CE; these parameters were not obtained for esfenvalerate in this study. Hydrolysis of pyrethroids by rat and human CEs was performed in reaction volumes of 100 μL as described previously (Ross et al., 2006). In the kinetic experiments, varying amounts of deltamethrin (5–100 μM, final concentration) 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 CE enzyme (2.5 μg per 100μL reaction) and the reaction was allowed to proceed for 30 min at 37 °C. The reactions were quenched by the addition of an equal volume of ice-cold acetonitrile. The samples were centrifuged for 5 min at 13,200 x *g* (4 °C) and an aliquot was analyzed by HPLC to quantify the hydrolysis products. Non-enzymatic reactions were also performed (i.e., no CE enzyme was added to the buffer/pyrethroid mixture) and found to have negligible rates (data not shown). Specific activity reactions of each CE with deltamethrin and esfenvalerate were performed in the same manner as above except that a single pyrethroid concentration of 50 μM was used. Substrate concentration-velocity reactions and specific activity reaction were performed by repetitive assays (N=3 independent experiments). The pyretrhoids were added to reaction

mixtures from stock solution prepared with acetonitrile and the organic solvent content in the reactions were kept <1% v/v.

Quantitation of CE-catalyzed hydrolytic products by HPLC analysis

Authentic standards are available for the hydrolysis products of deltamethrin and esfenvalerate. Furthermore, in contrast to the extensive number of hydroxylated pyrethroid metabolites produced by cytochrome P450 metabolism, there are only two hydrolysis products, the acid and alcohol metabolites. Thus, we have detected and quantified the products of the hydrolysis reactions by HPLC analysis. HPLC-UV analysis of pyrethroid hydrolytic products was performed 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). The mobile phases used were solvent A (1:1 v/v, water: acetonitrile containing 0.1% v/v acetic acid) and solvent B (100% acetonitrile containing 0.1% v/v acetic acid). The analytes were eluted with the following linear gradient program: 0 min (100% A, 0% B), 6 min (100% A, 0% B), 20 min (50% A, 50% B), 25 min (50% A, 50% B), and 30 min (100% A, 0% B), at a flow rate of 0.2 ml/min. Products were detected at 230 nm. Calibration standards of the hydrolysis products were routinely run along with the samples. For esfenvalerate and deltamethrin, hydrolysis rates were based on the production of 3-phenoxybenzaldehyde, which is spontaneously formed from the cyanohydrin (Wheelock et al., 2003).

Kinetic analysis and statistics

Non-linear regression of substrate concentration versus reaction velocity curves were analyzed using SigmaPlot v. 8.02 software (Systat Software, Inc., Point Richmond, CA) by

fitting experimental data to the Michaelis-Menten equation. The kinetic parameters reported for the human and rat CE's are the mean (± SD) of three independent kinetic assays. The specific activity data are reported as the mean (± SD) of three replicates.

RESULTS

Elimination of deltamethrin and esfenvalerate from rat liver microsomes

Both oxidative and hydrolytic pathways mediate the biotransformation of pyrethroids by hepatic microsomes. Initial studies indicate that the K_{mapp} for deltamethrin and esfenvalerate in rat and human liver microsomal incubations ranged between 21 and 75 μM (Table 2.1). Thus, substrate concentrations of 1μM were used in the elimination assays in order to estimate first order rate constants (Obach et al., 1999). Elimination of deltamethrin and esfenvalerate from rat liver microsomes occurred predominantly via NADPH-dependent metabolic pathways (Fig. 2.2). The rate of NADPH-independent elimination of deltamethrin was only 20% of the total elimination rate in the presence of NADPH (Table 2.2). The addition of the esterase inhibitor TEPP completely inhibited the NADPH-independent elimination of deltamethrin (Table 2.2). TEPP also inhibited elimination of deltamethrin in the presence of NADPH by 20%, which is consistent with the role of esterases in deltamethrin elimination. The rate of NADPH-independent elimination of esfenvalerate was 11% the total elimination (Table 2.2), however, esfenvalerate elimination in the presence of NADPH was decreased by nearly 50% following addition of TEPP. These results are inconsistent with TEPP inhibiting only esterase metabolism. TEPP is an organophosphate pesticide that is also metabolized by cytochrome P450's (Kulkarmi and Hodgson 1984). Thus, competitive inhibition of cytochrome P450 metabolism of esfenvalerate by TEPP may be occurring in rat liver microsomes. In addition, this did not occur with deltamethrin, indicating potential differences in the cytochrome P450's metabolizing deltamethrin and esfenvalerate in rat liver microsomes. Deltamethrin metabolism was completely inhibited by TEPP in the absence of NADPH (Table 2.2).

Elimination of deltamethrin and esfenvalerate from human liver microsomes

The elimination of deltamethrin from human liver microsomes occurs almost entirely by NADPH-independent metabolic pathways (Fig. 2.3). The rate of elimination of deltamethrin from human liver microsomes was similar in the presence and absence of NADPH. The addition of TEPP into the reaction mixture decreased the total elimination rate by nearly 90% (Table 2.2). The elimination of esfenvalerate from human liver microsomes occurred predominantly via NADPH-dependent metabolic pathways (Fig. 2.3). The NADPH-independent elimination rate of esfenvalerate was 12% of the elimination rate in the presence of NADPH (Table 2.2). In contrast to rat microsomes, TEPP decreased elimination of esfenvalerate in the presence of NADPH by only 10%, consistent with the role of esterases in the elimination of esfenvalerate (Table 2.2). This may indicate that there are differences in the enzymes metabolizing esfenvalerate or TEPP between species. There was no detectable elimination of esfenvalerate following the addition of TEPP to the reaction mixture in the absence of NADPH.

Rat and human intrinsic hepatic clearance estimates for deltamethrin and esfenvalerate

Intrinsic hepatic clearance (Cl_{int}) values of deltamethrin and esfenvalerate for rats and humans were scaled to a per kg body weight basis for purposes of comparison. The scaled estimate for deltamethrin Cl_{int} in humans was approximately 2-fold more rapid than in rats (Table 2.1). In contrast, the scaled Cl_{int} for esfenvalerate was approximately 3-fold more rapid in rats than in humans.

Hydrolysis of deltamethrin and esfenvalerate by purified human and rat carboxylesterases

The rates of hydrolysis of deltamethrin and esfenvalerate were examined by two purified human CEs (hCE-1 and hCE-2) and two purified rat CEs (Hydrolases A and B). The most effective CE for deltamethrin hydrolysis was hCE-1, which was 25-, 4-, and 16-fold more active than hCE-2, Hydrolase A, and Hydrolase B, respectively (Table 2.3). Consistent with the differential rates of hydrolysis of deltamethrin and esfenvalerate by human liver microsomes described above, the rates of deltamethrin hydrolysis by hCE-1 and hCE-2 were markedly faster than those for esfenvalerate (4- and 5-fold, respectively; Table 2.3). Rat Hydrolase A was the most effective CE catalyst of esfenvalerate hydrolysis, with 2-fold more activity than either rat Hydrolase B or hCE-1, and 73-fold more activity than hCE-2 (Table 2.3).

The kinetic parameters for deltamethrin hydrolysis were also estimated for each human and rat CE (Table 2.4). Among the four esterases examined, hCE-1 had the highest K_m and k_{cat}, and the highest catalytic efficiency (k_{cat}/K_m). Figure 2.4 demonstrates results from a representative substrate-velocity experiment comparing hCE-1 and rat Hydrolase A. The rates of deltamethrin hydrolysis catalyzed by hCE-1 were more rapid at all concentrations utilized as compared to rat Hydrolase A. It should also be noted that the kinetic parameters shown in Table 2.4 were estimated by non-linear regression methods using all concentrations of substrate (5–100 μM) (Fig. 2.4) and that similar kinetic parameters were also obtained when the kinetic data was analyzed instead using the Lineweaver-Burk plot (data not shown). A reliable CE kinetic parameter for comparison with respect to deltamethrin hydrolysis is the turnover numbers (k_{cat}). The k_{cat} values

obtained are in good agreement with the measured specific activity values that were determined at a substrate concentration of 50 μM (Table 2.3). While this is a relatively high concentration of substrate, it is necessary in order to detect the hydrolysis product 3-phenoxybenzaldehyde by HPLC analysis. Figure 2.5 compares the k_{cat} values of rat Hydrolase A and hCE-1 for deltamethrin and esfenvalerate hydrolysis with those of other pyrethroids (Ross et al. 2006). This result clearly demonstrates the species difference in the activities of rat Hydrolase A and hCE-1 that is unique for deltamethrin when compared to the general similarity in hydrolytic rates observed with the other pyrethroids.

DISCUSSION

The biochemical pathways that contribute to the metabolism of pyrethroid pesticides in laboratory animals are understood relatively well (Soderlund and Casida 1977; Casida and Ruzo, 1980). Fewer studies have examined the metabolism of pyrethroids by humans (Choi et al 2002; Ross et al 2006; Nishi et al 2006). Recent studies by Ross et al. (2006) suggest that there may be quantitative and qualitative differences in the metabolism of pyrethroids by rat and human esterases. The present study compares the elimination of deltamethrin and esfenvalerate from rat and human liver microsomes. Consistent with previous work (Soderlund and Casida 1977; Shono et al. 1979; Anand et al., 2006) the elimination of deltamethrin and esfenvalerate in rat liver microsomes occurred primarily through an NADPH-dependent oxidative pathway. Esfenvalerate elimination from human liver microsomes also occurs primarily by the NADPH-dependent oxidative pathways. In contrast, NADPH-independent hydrolytic pathways predominately eliminate deltamethrin in human liver microsomes.

The observed species difference in the metabolism of deltamethrin led to the examination of the metabolism of deltamethrin and esfenvalerate by purified rat and human CE enzymes. Human carboxylesterase 1 (hCE-1) had a specific activity that was 4- to 15-fold greater than rat Hydrolases A and B when rates of deltamethrin hydrolysis were compared (Table 2.3). The specific activity obtained for Hydrolase A in our current study is in agreement with the deltamethrin hydrolysis activity obtained using a rabbit carboxylesterase (Ross et al., 2006), which is 99% identical to Hydrolase A in terms of amino acid sequence homology. The differential hydrolysis rates by human and rat CEs suggests that differences in esterase activity are likely responsible, at least in part, for the

differences in the metabolism of deltamethrin observed between rat and human liver microsomes. Indeed, the k_{cat} values for the human and rat CEs (which is a measure of the rate of enzymatic reaction at saturating concentrations of substrate) did correlate with the species difference in deltamethrin metabolism using the liver microsomes; however, differences in k_{cat}/K_m values were not remarkably different between hCE-1 and Hydrolase A (Table 2.4). Caution needs to be exercised when correlating kinetic parameters of pure enzymes with the species difference in deltamethrin hydrolysis rates found using hepatic microsomes. This is because of k_{cat}/K_m and k_{cat} values are a metric of an enzyme's intrinsic catalytic efficiency and turnover in a pure preparation, not in a crude mixture of proteins found in tissue fractions where protein-protein interaction may modulate enzyme activity (Saghatelian et al., 2004). Also, differences in hydrolysis rates in hepatic microsomes between species may reflect the spectrum of esterase isoforms that are present in each species and their relative expression levels in liver. For example, if hCE-1 and Hydrolase A have similar catalytic efficiencies and thus metabolize deltamethrin equally well at low concentrations of substrate (which does not saturate the enzyme), then the observed differences in hydrolytic rates using hepatic microsomes may reflect differences in the relative expression levels of hCE-1 versus Hydrolase A. Alternatively, it cannot be excluded that an unidentified esterase is present in human liver microsomes that is much more efficient (much larger k_{cat}/K_m) than the rat liver esterases, thus accounting for the observed differential hydrolysis rates.

In contrast to hCE-1, human carboxylesterase 2 (hCE-2)was shown to have significantly less activity towards deltamethrin. hCE-1 is expressed abundantly in human liver, while hCE-2 is expressed at relatively lower levels in this tissue (Satoh et al, 2002).

Thus, based on our findings, it is likely that hCE-1 plays a greater role in the metabolism of deltamethrin in human liver than does hCE-2. Recently Nishi et al. (2006) reported on the activity of hCE-1 and hCE-2 towards a number of pyrethroids including deltamethrin and esfenvalerate. The specific activities in Nishi et al. (2006) are similar to those reported here except in one case. In their studies, hCE-2 had greater activity towards deltamethrin than hCE-1. While this is inconsistent with the data presented in this study, this may be explained by differences in the intrinsic activity of the preparations used. The intrinsic activity of hCE-2 in Nishi et al., (2006) was more than two fold greater than the hCE-2 used in our studies (reported in Ross et al., 2006) towards the standard substrate *p*-nitrophenyl acetate.

Carboxylesterases (EC 3.1.1.1) are members of the esterase family of serine hydrolase enzymes (Satoh and Hosokawa, 1998). The catalytic mechanism of CEs requires a triad of amino acid residues (Ser, His, and Glu or Asp) that are essential for activity. The rat CE isozymes named Hydrolase A and Hydrolase B are the two most abundant carboxylesterases present in rat liver, accounting for 80% of the total hepatic carboxylesterase protein under basal conditions (Morgan et al., 1994; Sanghani et al., 2002). These rat enzymes share ~70% amino acid sequence identity with each other and have overlapping substrate specificities. Hydrolases A and B have amino acid sequences that are approximately 70-80% identical with the major human hepatic carboxylesterase (hCE-1) (Redinbo and Potter, 2005). Thus, these CE isozymes are classified as being within the class 1 family of CE enzymes. In contrast, hCE-2 is quite distinct from the rat CEs and hCE-1 in terms of sequence identity and substrate specificity (Satoh and Hosokawa, 1998). hCE-2 shares less than 50% sequence identity with hCE-1 and Hydrolases A and B and is classified as a class 2 carboxylesterase.

The amino acid sequences that flank the catalytic triad of Ser, His, and Glu residues in hCE-1 and Hydrolase A are nearly identical to each other, thus they are highly conserved. The differences in catalytic specificity observed for hCE-1 and Hydrolase A toward deltamethrin is potentially related to differences in the types of amino acids that line the substrate-binding gorge and/or active site of the enzymes. Future studies that create site-specific mutants of hCE-1 and/or Hydrolase A may yield insight into the underlying specificity differences observed between rat and human CEs. Furthermore, computational approaches that involve molecular modeling of hCE-1 and Hydrolase A will be of potential value.

The present study indicates that deltamethrin (a cis-isomer) is metabolized primarily by an NADPH-independent hydrolytic pathway in human liver microsomes. This finding is not consistent with structure-metabolism relationships found in laboratory animals. A number of studies demonstrate that in laboratory animals oxidative pathways generally metabolize cis-isomers of pyrethroids more rapidly compared to their trans-isomers, which are generally more rapidly metabolized by hydrolytic pathways (Soderlund and Casida, 1977; Shono et al., 1979). Recently, Anand et al. (2006) described the ability of a number of rat hepatic CYP450s to metabolize deltamethrin. Their results indicate that CYP1A1, CYP1A2, and CYP2C11 may be active in the metabolism of deltamethrin in rat liver microsomes, with CYP1A2 being the most active. Based on these results we examined the metabolism of deltamethrin by human CYP1A2, but did not see significant evidence of metabolism (unpublished data, Godin et al.). This suggests that there may be marked differences in CYP450 activities between rats and humans toward deltamethrin. It is also possible that the difference in oxidative metabolism is related to differences in expression of these enzymes

between rat and human liver. For example CYP2C11 is highly expressed in the rat liver (Guengerich et al., 1982), while one of its human homologues 2C18 has very low expression in the liver (Rodrigues 1999). A comparison of the full complement of rat and human CYP450 isozymes would be needed to confirm these initial findings and to understand the lack of P450 mediated metabolism in human liver microsomes. The lack of CYP450 mediated metabolism of deltamethrin in human liver microsomes may be as important in the species difference as is the differences in the activities of rat and human carboxylesterases.

In addition to differences in the pathways of biotransformation of the parent chemical there were also differences in the rates of elimination and calculated intrinsic hepatic clearance for deltamethrin and esfenvalerate. Since the parent chemical is the primary toxicant for pyrethroids, elimination of the parent chemical can be considered the detoxification step in their metabolism. The CL_{int} of esfenvalerate, which was eliminated by similar pathways in both species, was estimated to be nearly three-fold greater in rats than in humans. In contrast, the CL_{int} of deltamethrin was estimated to be twice as rapid in humans that in rats. In addition, deltamethrin was eliminated by different pathways in rat and human liver microsomes.

These results indicate that laboratory rodents may not be a good model for understanding and extrapolating the results of metabolism studies of all pyrethroids in a human health risk assessment. It is therefore necessary to more completely understand the human metabolism of individual pyrethroids to reduce uncertainties in a risk assessment.

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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.

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Table 2.1. K_{mapp} and scaled intrinsic hepatic clearance (CL_{INT}) values for deltamethrin and esfenvalerate in rats and humans.

Deltamethrin	**K_{mapp}** (μM)	**Clearance (mL/min/kg b.w)**
Rat Microsomes	39	89.0 ± 23.7
Human Microsomes	75	162.1± 32.7
Esfenvalerate		
Rat Microsomes	22	108.2 ± 2.2
Human Microsomes	21	37.3 ± 10.3

Data is mean ± SD (N=3)

Table 2.2. Elimination rates for deltamethrin and esfenvalerate from rat and human liver microsomes.

	Elimination rate (pmoles/min/mg microsomal protein)	
Deltamethrin	**+ NADPH**	**- NADPH**
Rat Microsomes	30.4 ± 7.3	8.8 ± 0.23
Rat Microsomes + TEPP	24.6 ± 3.7	ND
Human Microsomes	52.8 ± 6.2	58.0 ± 3.0
Human Microsomes + TEPP	5.8 ± 5.4	ND
Esfenvalerate		
Rat Microsomes	45.3 ± 2.4	4.8 ± 1.4
Rat Microsomes + TEPP	23.4 ± 9.0	ND
Human Microsomes	20.9 ± 4.4	2.6 ± 1.6
Human Microsomes + TEPP	18.9 ± 2.7	ND

+ NADPH, total clearance (oxidative and hydrolytic) of parent chemical from microsomal assay.
– NADPH, NADPH-independent hydrolytic clearance of parent chemical from microsomal incubation.
+ TEPP, tetraethylpyrophosphate (200µM) used to inhibit hydrolytic metabolism.
ND, no detectable elimination.
Data is mean ± SD. (N=3)

Table 2.3. Specific activity of human and rat carboxylesterase hydrolysis of deltamethrin and esfenvalerate

Enzyme	Deltamethrin specific activity[a] (nmol/min/mg)	Deltamethrin fold difference[b]	Esfenvalerate specific activity[a] (nmol/min/mg)	Esfenvalerate fold difference[b]
human CE-1[a]	12.2 ± 0.6	1.00	3.1 ± 0.2	0.25
human CE-2[a]	0.5 ± 0.0	0.04	0.1 ± 0.0	0.01
rat Hydrolase A	3.0 ± 0.2	0.25	6.6 ± 0.3	0.54
rat Hydrolase B	0.8 ± 0.1	0.07	3.0 ± 0.3	0.25

[a] 50 μM deltamethrin or esfenvalerate in assay (*n*=3). Incubation time of 30 min. Rates were based on the formation of 3-phenoxybenzaldeyde.
[b] Relative to the human CE-1 specific aactivity for deltamethrin hydrolysis.

Table 2.4. Hydrolysis of deltamethrin: Kinetic parameters of human and rat carboxylesterases.

Enzyme	Km (μM)	Vmax (nmol/min/mg)	kcat (min^{-1})	kcat/Km (min^{-1} mM^{-1})
human CE-1[a]	22.6 ± 3.7	21.4 ± 7.2	1.3 ± 0.4	56.3
human CE-2[a]	1.6 ± 1.6	0.6 ± 0.1	0.035 ± 0.003	21.1
rat Hydrolase A	6.3 ± 0.4	4.5 ± 0.8	0.27 ± 0.04	42.7
rat Hydrolase B	2.0 ± 4.9	1.1 ± 0.3	0.07 ± 0.02	35.2

[a] Eight different concentration of deltamethrin were assayed (5-100μM) in each kinetic experiment—rates are based on the formation of 3-phenoxynezaldehyde. Parameters are the means ± SD obtained from three independent kinetic experiments.

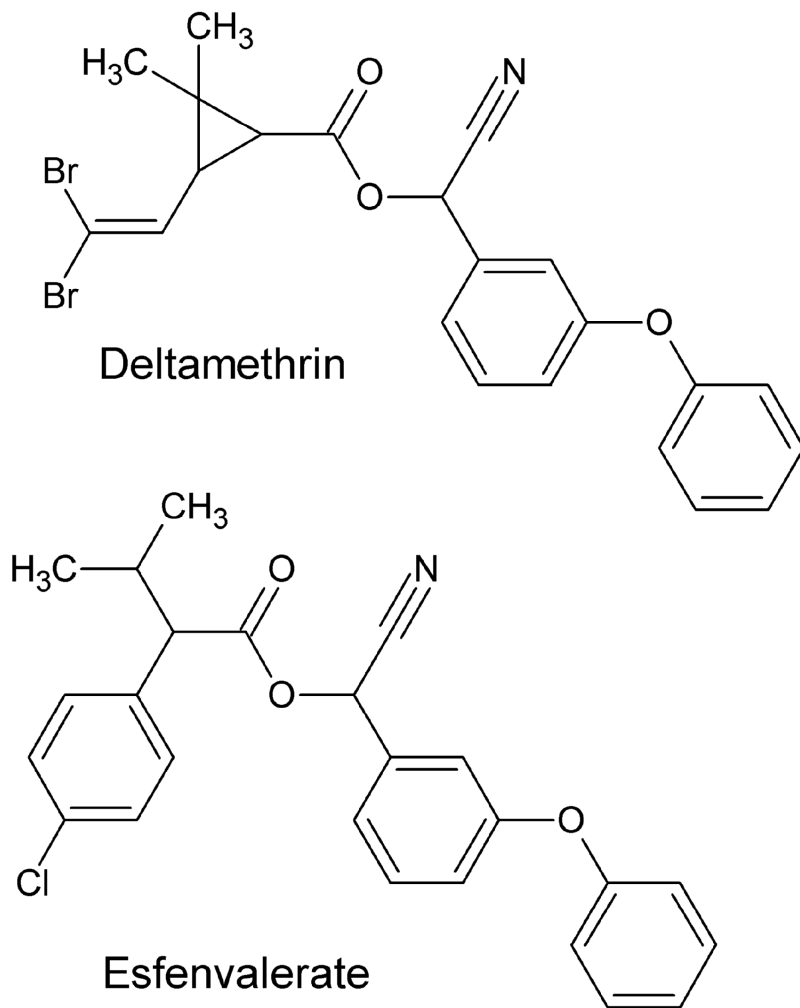

Figure 2.1. Structures of the pyrethroid pesticides deltamethrin and esfenvalerate.

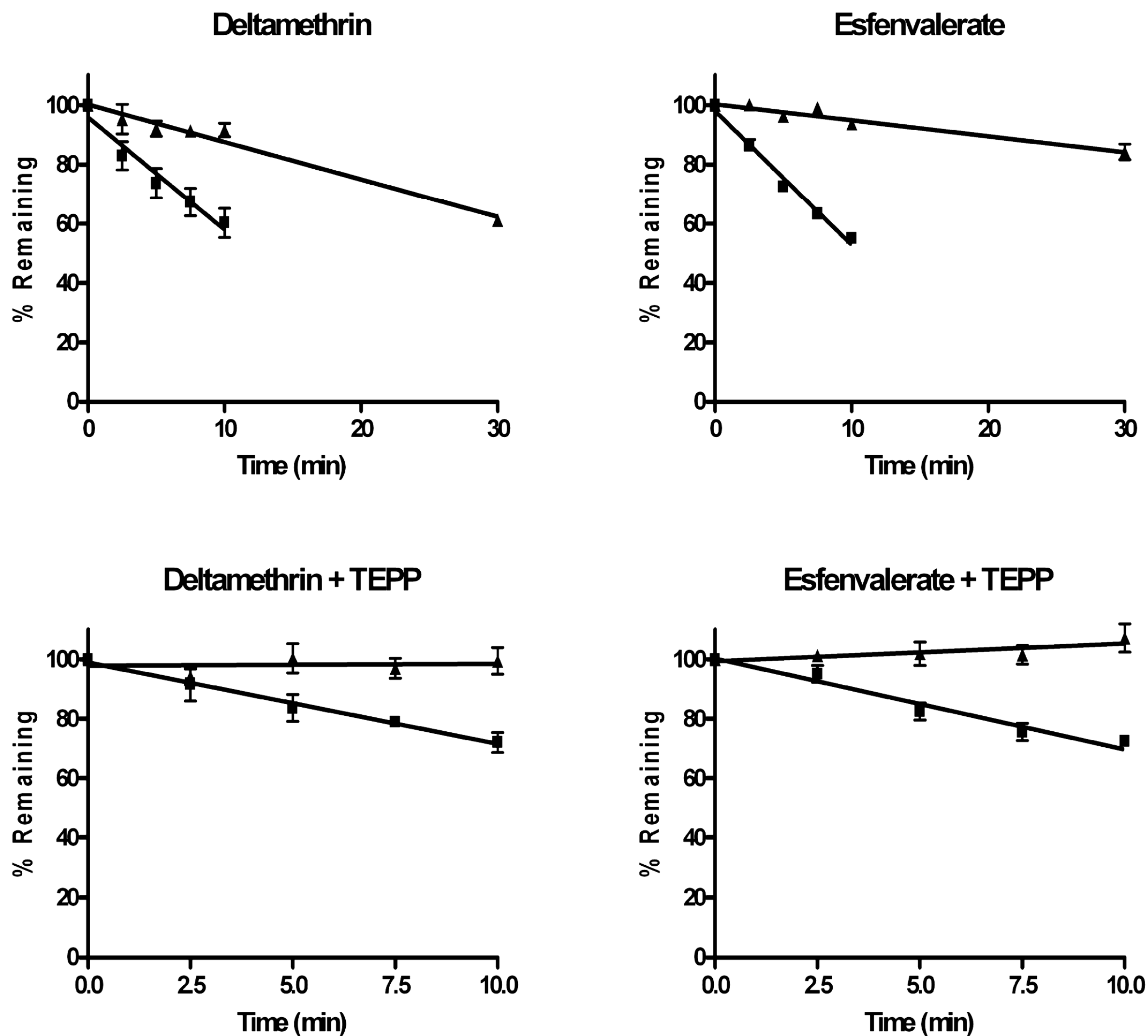

Figure 2.2. Elimination of deltamethrin and esfenvalerate from rat liver microsomes. Elimination examined at 1µM deltamethrin or esfenvalerate in the presence of (■ solid line) or absence of (▲ dashed line) NADPH, with and without TEPP. Line represents linear regression of data points. Data represented as the mean ± SD (N=3).

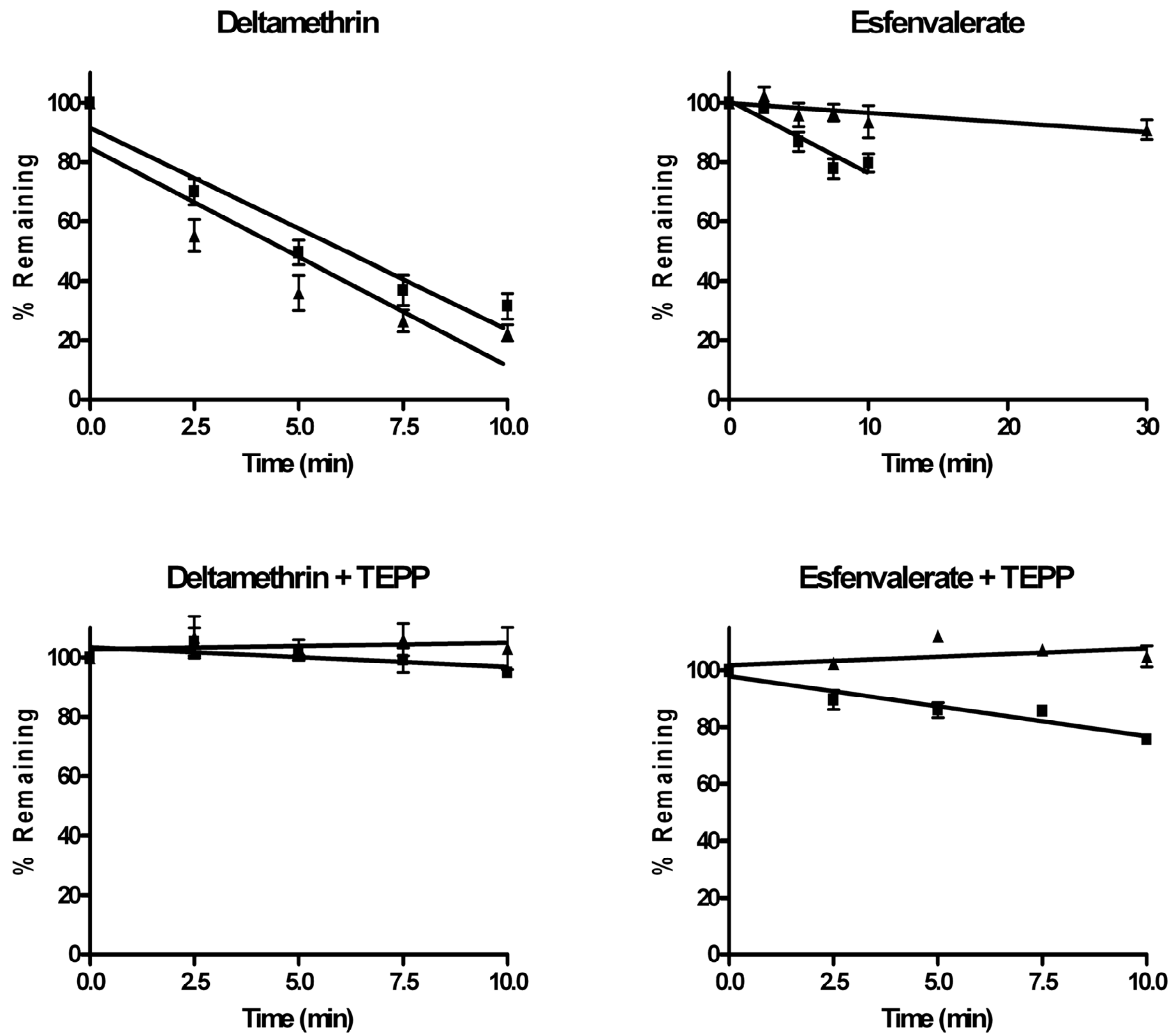

Figure 2.3. Elimination of deltamethrin and esfenvalerate from human liver microsomes. Elimination examined at 1μM deltamethrin or esfenvalerate in the presence of (■ solid line) or absence of (▲ dashed line) NADPH, with and without TEPP. Line represents linear regression of data points. Data represented as the mean ± SD (N=3).

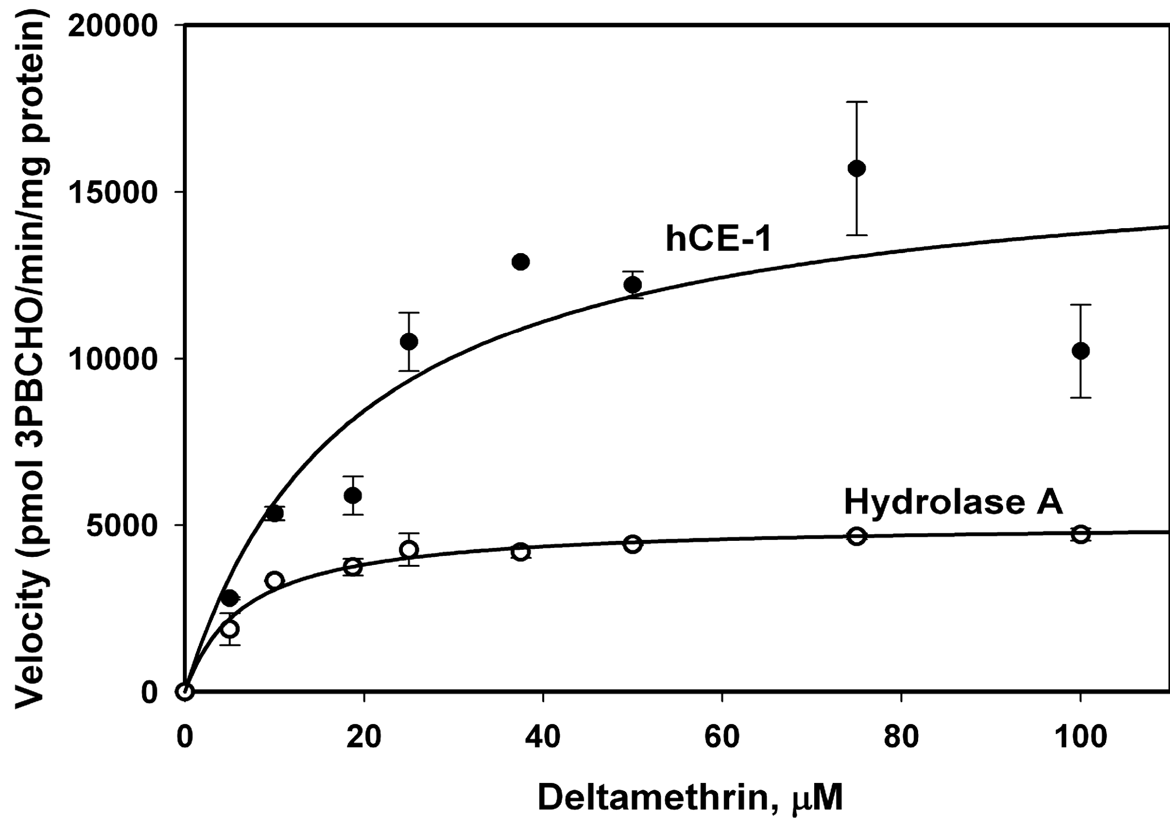

Figure 2.4. Kinetics of deltamethrin hydrolysis by hCE-1 and Hydrolase A. Velocity was measured by the amount of 3-phenoxybenzyladehyde (3PBCHO) released during the reaction. Data (symbols) were fit to the Michaelis-Menten equation and the non-linear regression results are plotted (lines). Each point represents the mean ± SD (N=3).

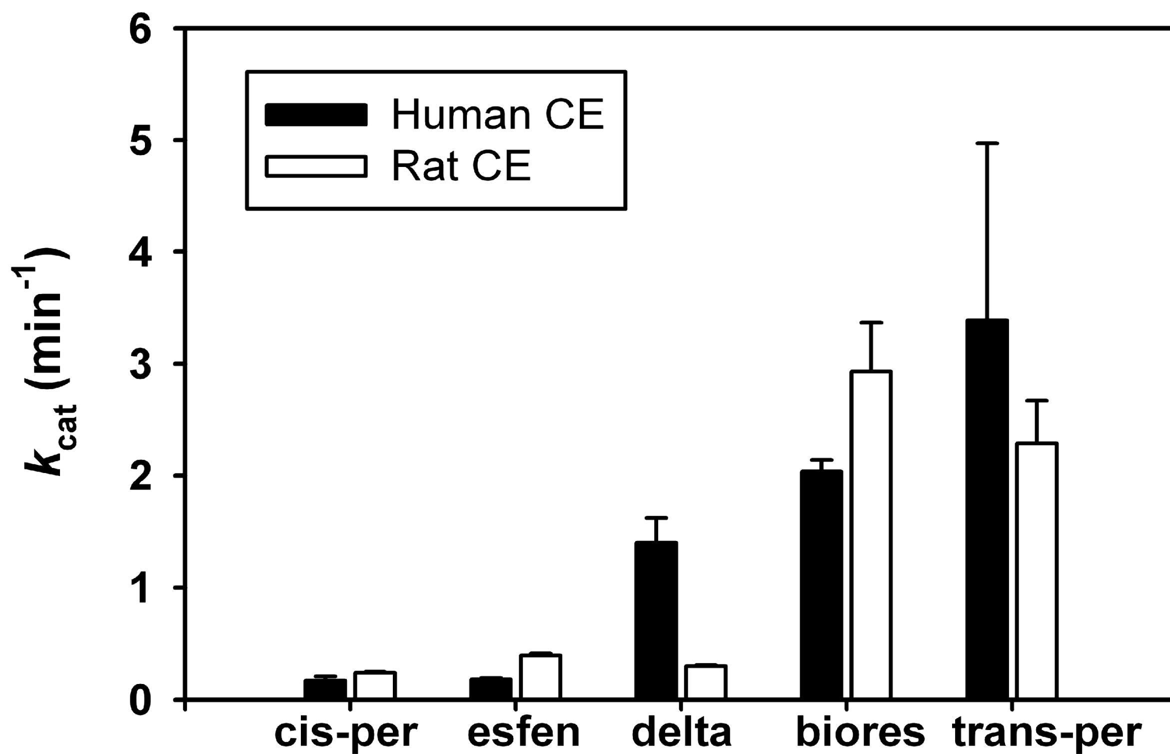

Figure 2.5. Comparison of turnover numbers (k_{cat}) for human CE (hCE-1) and rat CE (Hydrolase A). Hydrolysis of five different pyrethroids are compared. Data for cis-per, trans-per, and biores are from Ross et al. (2006). Abbreviations: cis-per, cis-permethrin; esfen, esfenvalerate; delta, deltamethrin; biores, bioresmethrin; trans-per, trans-permethrin.

CHAPTER III

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 a esterase which metabolize delta and esfen

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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 *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.

MATERIALS AND METHODS

Chemicals

Deltamethrin (98.9% purity) ((αS)--cyano-3-phenoxybenzyl (1R, 3R)-*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) ((αS)-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, β-nicotinamide adenine dinucleotide phosphate reduced form (NADPH), 3-phenoxybenzyl alcohol (3PBAlc), *cis/trans*-3-(2′,2′-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid (a 1:1 mixture of *cis* and *trans* isomers) [also called *cis/trans*-dichlorochrysanthemic acid (Cl_2CA)], 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. Vmax and Km 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 (Vmax/Km) 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 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 N_2. 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 butyrlcholinesterases (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 polyvinyldifluoride 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. 3.1). However, rat CYPs 1A2, 2A1, 2B1, 2C12, and 2C13 did not metabolize either compound (Fig. 3.1). Rat CYP2C6 eliminated the greatest percentage of deltamethrin followed by 1A1>2C11> 3A2 (Fig. 3.1). Rat CYP2C6 also eliminated the greatest percentage of esfenvalerate followed by 2C11>3A2> 1A1 (Fig. 3.1). 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. 3.1). 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. 3.2). Esfenvalerate was metabolized by human CYPs 2C8, 2C9*1, 2C9*2, 2C19, 3A4 and 3A5 (Fig. 3.2). However,

human CYPs 1A2, 2A6, 2B6, 2E1, and 2D6*1 did not metabolize either deltamethrin or esfenvalerate (Fig. 3.2). CYP2C19 eliminated the greatest percentage of deltamethrin followed by 2C8 > 3A5 > 3A4 (Fig. 3.2). 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. 3.2), while CYPs 2C9*1 and 2C9*2 metabolized esfenvalerate but not deltamethrin (Fig. 3.2). These CYP isozymes 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 3.1). Rat CYP3A2 eliminated deltamethrin at a significantly slower rate than CYPs 2C6 and 2C11; however, it also exhibited a lower K_m value (Table 3.1).

Human CYP2C8 and 2C19 have similar K_m and V_{max} values for deltamethrin (Table 3.1). 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 3.1). The catalytic efficiency of CYP 3A5 was found to be greater than both 2C8 and 2C19 for deltamethrin.

K_m, $V_{ma,x}$ and Vmax/Km parameters for deltamethrin were similar for rat and human CYPs. The large standard error assoicatated with the estimates of K_m and V_{max} for some enzymes is likely due to the inability to accurately determine Vmax 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_{max} values, and were similar to the values for deltamethrin (Table 3.1). Rat CYP3A2 had lower K_m and V_{max} values then 2C6 and 2C11 (Table 3.1). Of the human CYPs examined each had similar V_{max} values (Table 3.1). However, the K_m for CYP2C19 was ~5-6-fold lower than the K_m for 2C8 and 2C9*1. As with deltamethrin, Km and Vmax 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 3.1). As previously noted, kinetic paramaters 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 μM 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 (3PBAlc) and *trans*-dichlorochyrsanthemic acid (Cl_2CA). Both metabolites can be analyzed by HPLC (Fig. 3.3). Hydrolysis of the type II pyrethroid, deltamethrin, by purified CEs liberates *cis*-dibromochrysanthemic acid (Br_2CA), which is a stable metabolite, and a cyanohydrin that spontaneously converts to 3-phenoxybenzaldehyde (3PBCHO) at pH>7 (see scheme in Fig. 3.4). Br_2CA and 3PBCHO are also conveniently quantified by HPLC analysis (Fig. 3.3) and thus product formation rates can be determined. The hydrolysis of esfenvalerate also liberates the same cyanohydrin that spontaneously yields 3PBCHO. When deltamethrin is incubated with hepatic microsomes, 3PBCHO can undergo redox reactions to produce 3PBAlc and 3-phenoxybenozoic acid (3PBCOOH) (Fig. 3.4), 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. 3.5A and B). The specific activity of deltamethrin hydrolysis by the purified CE was nearly 2-fold greater than the activity for esfenvalerate (Fig. 3.5B). This compares well with the greater specific activity of the pooled rat serum with deltamethrin than with esfenvalerate (Fig. 3.5A). 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. 3.5C). In contrast, deltamethrin displayed hyperbolic kinetics with the purified CE (Fig. 3.5D). The estimated k_{cat} and K_m values for the rat serum CE-catalyzed deltamethrin hydrolysis (Fig. 3.5D) were 0.48 min^{-1} and 12.6 μM, respectively, and the calculated k_{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. 3.6). 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. 3.6). 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 3.1). 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 3.2). An important exception was their metabolism by the human CYP2C9 isozymes (Fig 3.2) (Table 3.1). While esfenvalerate was metabolized effectively by the 2C9 isozymes, deltamethrin was not. Although 2C19 eliminated the greatest percentage of both deltamethrin and esfenvalerate, (Fig 3.2), 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_{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 pmoles/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. 3.6). 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. 3.5A). 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. 3.5B). Similar results were observed in rat serum (Fig. 3.5A). Concentration-velocity plots for deltamethrin in rat serum revealed non-

hyperbolic kinetics (Fig. 3.5C), 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. 3.5D). 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 pararmeters 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 (ie. 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.

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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 and NIEHS Research Grant T32-ES07126. Research support to M.K.R and J.A.C. was provided by NIH grant P20 RR017661.

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Table 3.1. Kinetic parameters for deltamethrin and esfenvalerate metabolism by rat and human CYPs.

	Km (μM)	V_{max} (pmoles/min/pmoleP450)	V_{max}/K_m [a]	V_{max}/K_m [b]
Deltamethrin				
Rat CYPs				
2C6	21.6 ± 9.4	150.0 ± 36.6	6.9	3.4 ± 0.3
2C11	31.9 ± 25.7	205.8 ± 107.6	6.5	5.2 ± 0.7
3A2	6.4 ± 3.8	25.9 ± 5.8	4.0	2.1 ± 0.2
Human CYPs				
2C8	10.2 ± 9.5	42.7 ± 16.6	4.2	1.6 ± 0.1
2C19	9.0 ± 5.6	61.6 ± 17.7	6.8	2.2 ± 0.5
3A5	-	-	-	4.8 ± 0.6
Esfenvalerate				
Rat CYPs				
2C6	38.2 ± 26.2	158.2 ± 72.8	4.1	3.2 ± 0.3
2C11	33.5 ± 21.8	219.4 ± 92.8	6.5	4.4 ± 0.4
3A2	4.3 ± 1.8	39.5 ± 6.1	9.2	2.4 ± 0.5
Human CYPs				
2C8	19.1 ± 11.6	59.2 ± 21.6	3.1	1.6 ± 0.3
2C9*1	24.3 ± 5.6	79.8 ± 6.3	3.3	2.9 ± 0.2
2C19	4.1 ± 2.2	64.4 ± 12.3	15.7	4.4 ± 0.6
3A5	-	-	-	4.7 ± 0.7

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

[a]Calculated value using kinetic parameters obtained from non-linear regression analysis

[b]Catalytic efficiency estimated from the slope of the linear regression analysis of concentration versus velocity plots.

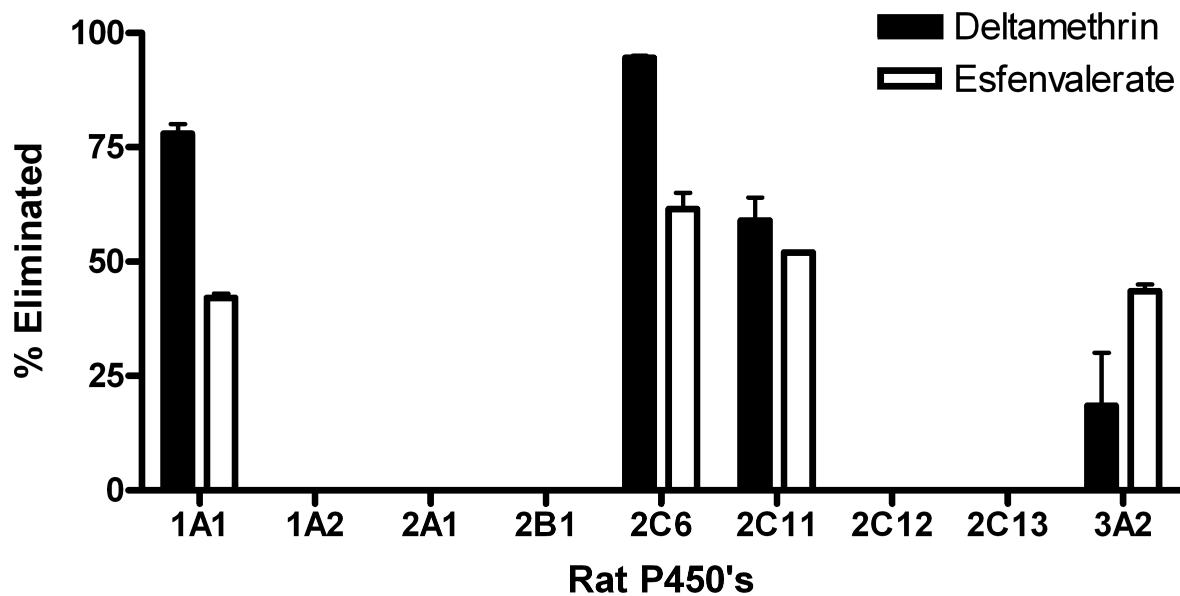

Figure 3.1. Elimination of deltamethrin and esfenvalerate by rat CYPs.
Elimination of 1 uM deltamethrin (black bars) or esfenvalerate (white bars) by rat CYP isoforms. Assays were run with 10 pmoles P450 isozyme/ml. Results are expressed as the average of the % eliminated over 10 min of duplicate samples.

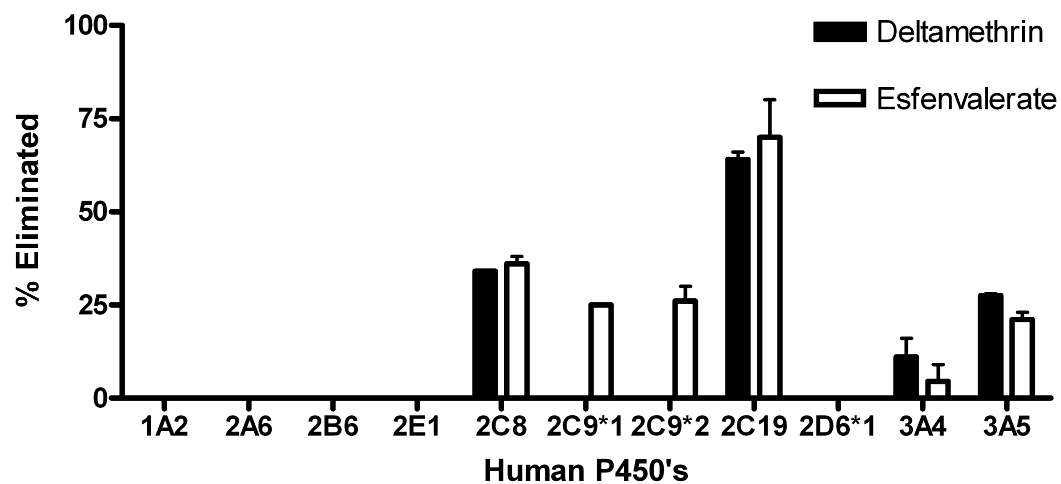

Figure 3.2. Elimination of deltamethrin and esfenvalerate by human CYPs. Elimination of 1 uM deltamethrin (black bars) or esfenvalerate (white bars) by human CYP isoforms. Assays were run with 10 pmoles P450 isozyme/ml. Results are the average of the % eliminated over 10 min of duplicate samples.

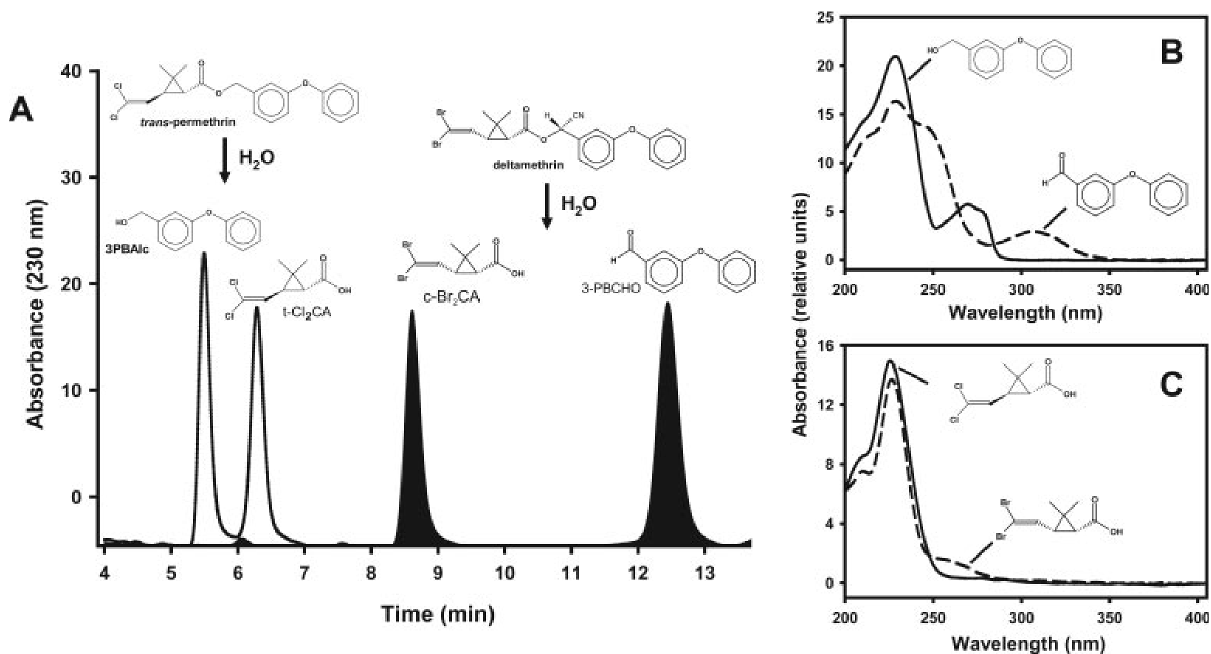

Figure 3.3. Comparison of hydrolysis products of trans-permethrin and deltamethrin by esterases. A, Overlay of HPLC chromatograms of hydrolysis products derived from each pyrethroid catalyzed by human carboxylesterase 1. B and C, UV spectra of the hydrolysis products of trans-permethrin (type I pyrethroid) and deltamethrin (type II pyrethroid).

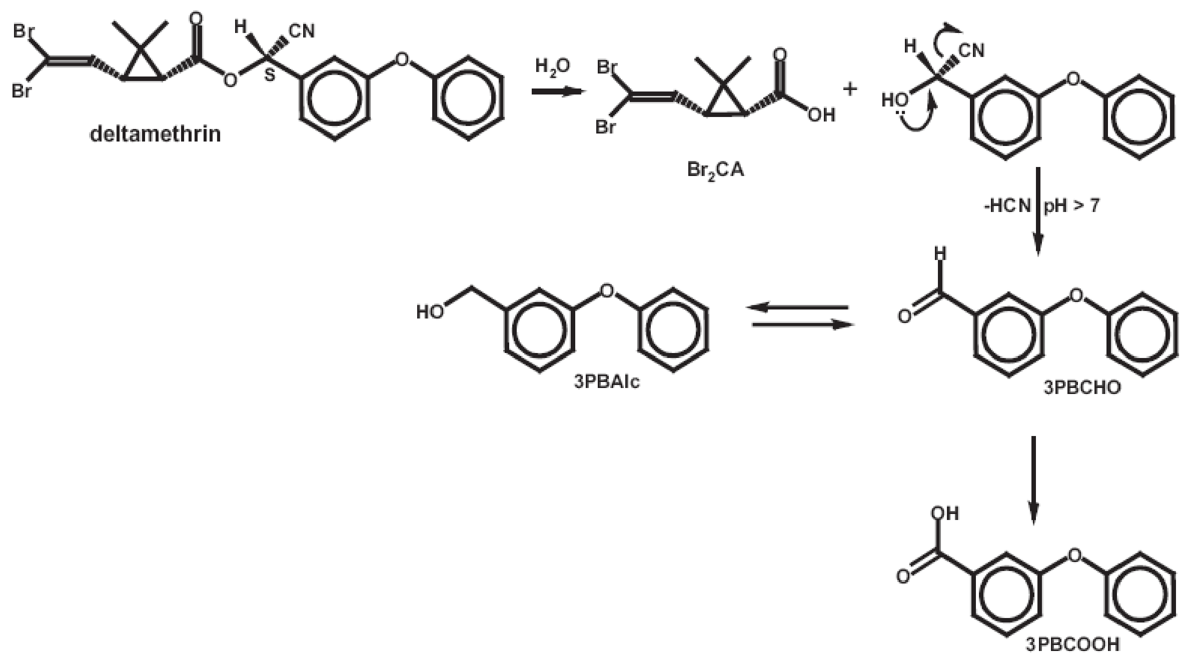

Figure 3.4. Hydrolytic metabolism of deltamethrin.

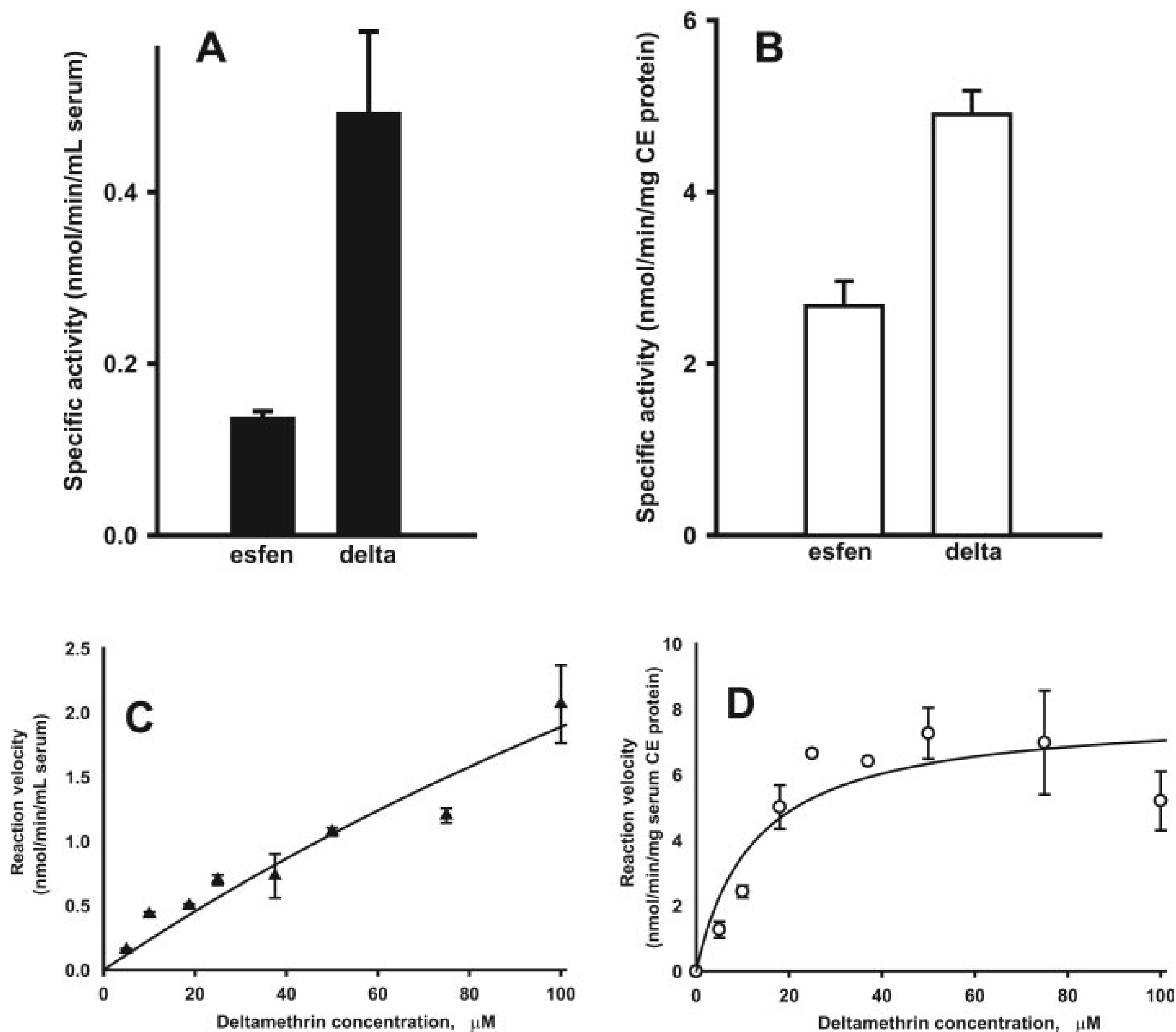

Figure 3.5. Comparison of hydrolysis rates of esfenvalerate and deltamethrin by whole rat serum and purified rat serum CE.

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.

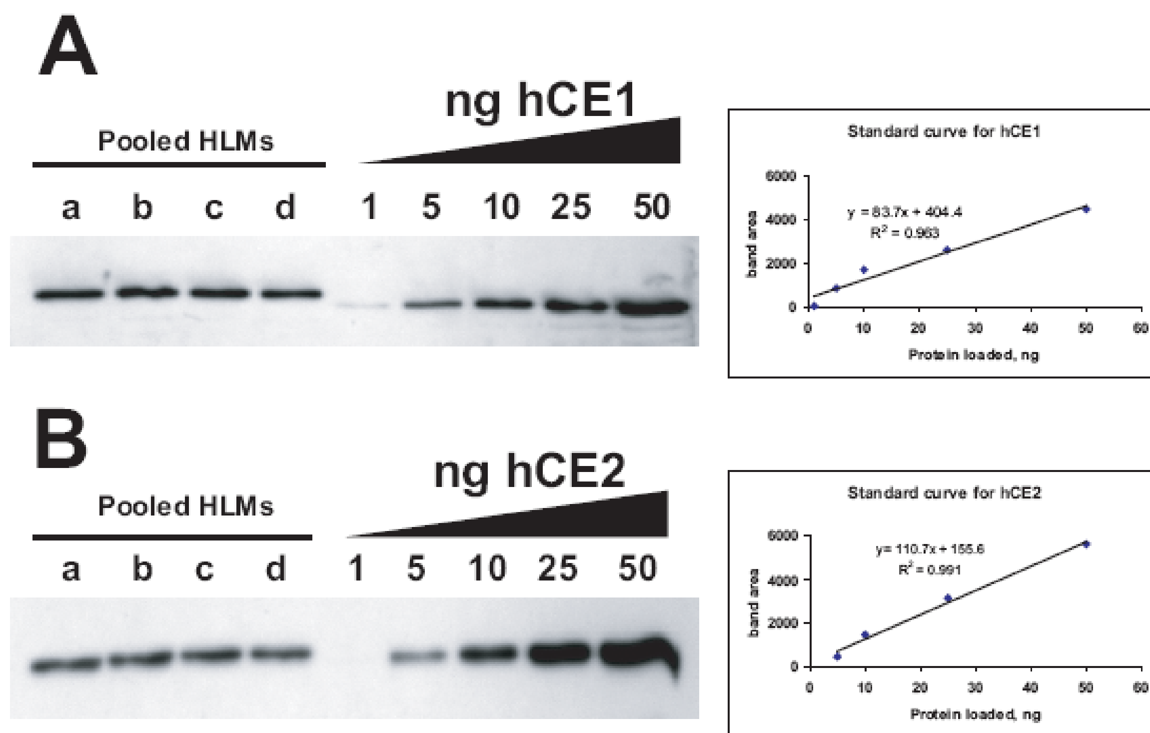

Figure 3.6. Quantitative immunoblotting of hCE1 protein (A) and hCE2 protein (B) in pooled human liver microsomes from four sources.

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).

CHAPTER IV

Physiologically Based Pharmacokinetic Modeling of Deltamethrin

PBPK Modeling of Deltamethrin

# of Text Pages – 31
# of Tables – 1
# of Figures – 11
# of References – 38

Abstract # of words – 258
Introduction # of words – 850
Discussion # of words – 1601

Abbreviations
PBPK – Physiologically based pharmacokinetic modeling

ABSTRACT

The pyrethroid pesticide deltamethrin is cleared nearly twice as rapidly in human liver microsomes compared to rat liver microsomes. A species difference such as this could influence the relative toxic potency of deltamethrin in rats and humans. A PBPK model was utilized to examine the impact of this species difference on exposure-dose relationships. A previously-developed PBPK model for deltamethrin in the rat by Mirfazaelian et al. (2006) suggests that absorption is inversely dose-dependent, with little absorption of this pesticide at environmentally relevant exposures. In addition, the Mirfazaelian et al. model employs a combination of flow- and diffusion-limited compartments and divides the blood compartment into plasma and red blood cells. Oral bioavailability studies were conducted to examine the dose-dependency in absorption of deltamethrin in the rat. There was no significant difference in the fraction absorbed of oral doses of 0.3 and 3.0 mg deltamethrin/kg. In contrast to the previous model, the current model described all tissue compartments with diffusion-limited kinetics and the blood as a single compartment. These changes resulted in an improved ability of the current deltamethrin PBPK model to describe the shape of the deltamethrin tissue concentration-time curves for both literature data and data from the present oral bioavailability studies. The description of the liver using diffusion- limited kinetics reduced–the impact of the species difference in metabolism since diffusion is the rate-limiting step in the metabolic elimination of deltamethrin. A proposed human PBPK model was also developed to explore differences in target tissue concentration of deltamethrin at the current reference dose (RfD) for human safety assessment.

INTRODUCTION

Pyrethroids are synthetic pesticides used in the protection of a wide range of commercial crops, ornamentals, and trees. They are also used in domestic insect control and as ectoparasiticides in both human and veterinary medicine (Roberts and Hutson 1999). Due to restriction and removal of other pesticides from the market, the use of pyrethroids continues to increase. Pyrethroid use has risen from 2.8% of the market share of lawn and garden pesticide use in 1993 to an expected 30% in 2008 (Freedonia Market Research, 2004). Numerous studies describe human exposure to pyrethroids (Zhang et al. 1991; Smith et al. 2002; Heudorf and Angerer 2001; Schettgen et al. 2002). Not only are humans exposed to pyrethroids, reported cases of acute pyrethroid poisoning reveal a similar set of symptoms (He et al. 1989) to those seen in laboratory animals, suggesting similar modes of action across species. A better understanding of the human health risks associated with pyrethroid exposure is therefore increasingly important.

Understanding human health risks associated with environmental contaminants such as the pyrethroids necessitates extrapolation from laboratory animal data. In the absence of human data, physiologically based pharmacokinetic (PBPK) models can improve the scientific basis of extrapolating the relationships between external exposures to internal measures of dose between laboratory animals and humans (Andersen, 2003). PBPK models allow the incorporation of species-specific physiological and biochemical data to aid species extrapolation of exposure dose relationships.

Recently, species differences in the *in vitro* hepatic and serum metabolism of the pyrethroid deltamethrin were identified between rats and humans (Godin et al 2006, 2007). Intrinsic hepatic clearance of deltamethrin clearance is nearly twice as rapid in human

hepatic microsomes as compared to rat hepatic microsomes (Godin et al., 2006). In addition, rat serum contains carboxylesterases which metabolize deltamethrin (Annand et al. 2006; Godin et al., 2007), while deltamethrin remains intact in human serum because of its lack of carboxylesterases (Godin et al., 2007; Crow et al. 2007). Pharmacokinetic (PK) differences such as these have the potential to influence exposure-dose relationships at target tissues and thus alter the relative potency of a chemical between a test species and humans. Application of PBPK models allow for the quantitative examination of how species differences, such as those for deltamethrin metabolism, affect the extrapolation of exposure-dose relationships.

Mirfazaelian et al (2006) developed a PBPK model for deltamethrin in the adult male Sprague-Dawley rat, and applied a saturable efflux process in the gastrointestinal (GI) compartment that predicts very low absorption of deltamethrin at low, environmentally relevant exposures. However, the available data in the literature does not demonstrate an inverse dose-dependent absorption of deltamethrin as the model indicates (see Fig 1). Fecal excretion of deltamethrin is approximately 13- 37% of an administered oral dose and weakly linearly dose dependent (Bosch 1990; Ruzo et al., 1978).

The Mirfazaelian et al. model describes the fat and slowly perfused tissues as diffusion-limited while using flow-limited descriptions for the brain, liver and richly perfused compartments. Lacking in the assessment of this model is data from the liver, which may be important to understand whether deltamethrin displays diffusion-limited or flow-limited pharmacokinetics. The liver is the primary organ for metabolic clearance of deltamethrin. Literature regarding liver concentration time courses for deltamethrin is limited to two IV studies (Gray and Rickard 1981 and 1982). These two studies reported only total radioactivity in the liver. The use of total radioactivity as the measure of tissue dose limits

the utility of these data for model development or evaluation because a major portion of the radioactivity could be deltamethrin metabolites. The choice of describing the liver as either flow- or diffusion-limited has implications for the contribution of species differences in hepatic clearance of deltamethrin to species differences in the toxicity of deltamethrin. In the flow-limited description hepatic clearance will have a larger impact on blood concentrations than in the diffusion-limited description. This difference arises because in the diffusion-limited description diffusion limitation dampens the influence of the intrinsic hepatic clearance rate on the blood levels of the chemical. Given the pivotal role of the liver in the disposition of deltamethrin, it is important to include data for this compartment in the assessment of the model.

Several of the assumptions outlined above in the Mirfazaelian et al. (2006) model were because of inconsistencies in the literature or from a lack of data. Based on these identified uncertainties and data gaps, a series of experiments were performed in this work. An oral bioavailability experiment was conducted to explore possible dose-dependent absorption of deltamethrin. In addition, a time-course study examining tissue disposition of deltamethrin after an oral dose was conducted to aid model development and assessment. Using these new data and the Mirfazaelian et al (2006) model as a framework, a refined PBPK model for deltamethrin in rats was developed. This refined model was then used as the basis for the development of a human PBPK model for deltamethrin. Predictions obtained with the human and rat model suggested that, for the same oral exposure, humans would have a marginally higher peak concentration in the target tissue, despite having the intrinsic ability to metabolize deltamethrin more rapidly.

MATERIALS AND METHODS

Chemicals

Deltamethrin (98.9% purity) ((αS)--cyano-3-phenoxybenzyl (1R, 3R)-*cis*-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropane carboxylate) was a gift from Bayer Crop Sciences (Research Triangle Park, NC, USA). Labeled cis- and trans-permethrin (phenoxy-$^{13}C_6$) were purchased from Cambridge Isotope Laboratories (Andover, MA). Chromasolv® acetonitrile and methanol for LC/MS applications were from Riedel-de Haën (Seelze, Germany). Solvents, including acetone, hexanes (Fisher Scientific, Pittsburgh, PA) and methanol (VWR, West Chester, PA), were pesticide grade. Glycerol formal, heparin, dextrose, and saline were purchased from Fisher Scientific (Pittsburgh, PA). Unless otherwise specified all chemicals were of the highest grade commercially available.

Animals

The USEPA National Health and Environmental Effects Research Laboratory Institutional Animal Care and Use Committee approved all animal procedures. Male Long Evans rats (approximately 70 days old) were obtained from Charles River Laboratory (Raleigh, NC USA) with or without jugular vein catheters. Animals were allowed to acclimate for a minimum of four days in an Association for Assessment and Accreditation of Laboratory Animal Care approved animal facility prior to their use. Two animals were housed per plastic cage (45 cm x 24 cm x 20 cm) with heat-treated pine shavings bedding for disposition stuidies. Cannulated animals for bioavailability studies were housed one animal to a cage. Animals were maintained at 21 ± 2°C, 50 ± 10% humidity and a photoperiod of 12L:12D (0600-1800 hr). Feed (Purina Rodent Chow 5001, Barnes Supply Co., Durham,

NC USA) and tap water were provided *ad libitum*. Animals were exposed to deltamethrin in either glycerol formal (iv) or corn oil (po) at 1 mg/ml dosing volume. Doses included a 1 mg/kg iv dose and 0.3 and 3.0 mg/kg oral doses via gavage. Oral doses were determined from the work of Wolansk~~i~~y et al. (2006), which examined the dose response relationship of deltamethrin for decreased motor activity. The 3.0 mg/kg dose was approximately an ED_{30} for decreased motor activity and the 0.3 mg/kg dose was used to compare absorption differences at a lower and more environmentally relevant dose.

Blood Collection

Cannulated rats were dosed orally via gavage or iv through the indwelling jugular vein catheter. There were four animals for each dose by the oral route and four animals for the iv route. Serial blood samples were taken at time points ranging from 5 min – 36 hr post-dosing. 300 uL aliquots of blood were removed via the catheter and immediately flash frozen in liquid nitrogen. Between each sampling time, catheters were rinsed with 300 uL saline and filled with a void volume of heparanized dextrose as a lock solution to maintain catheter patency.

Liver, Brain, and Fat Collection

A second group of rats were exposed via gavage with 0.3 or 3.0 mg/kg deltamethrin in corn oil at a volume of 1 ml/kg. At time points ranging from 1-48 hrs, animals were sacrificed via CO_2-induced anesthesia and blood was collected via cardiac puncture. Brain, liver and fat were immediately removed, weighed, and flash frozen in liquid nitrogen. Tissue

samples were stored at -80 °C until residue analysis was performed. For each dose, there were 4 rats per time point.

Tissue Extraction

Brain, liver, and fat were homogenized in a Spex CertiPrep 6850 freezer/mill (Metuchen, NJ) to form a fine homogeneous tissue powder. Aliquots of thawed blood (0.3 ml) and tissue homogenate (300-500 mg) were vortex extracted with 20:80 acetone:hexane. 25 μL of 6 μM labeled trans-permethrin (phenoxy-$^{13}C_6$) was added prior to extraction as a surrogate of recovery. Samples were vortexed for 10 min in 16 x 100 mm culture tubes with 5 ml solvent and centrifuged at 4000 x G for 10 min. The organic layer was collected. The process was repeated twice more with 3 ml solvent, combining organic fractions of each extraction. Pyrethroid-containing organic fractions were dried under nitrogen and reconstituted in 1 ml hexane. Brain samples were then loaded onto a hexane rinsed Sep-pak 500 mg silica solid phase extraction (SPE) columns (Waters, Inc., Milford, MA). SPE columns were washed with 5 ml hexane and the pyrethroids were eluted with 5 ml 94:6 hexane:ethyl acetate. Column eluants were dried under a stream of nitrogen, and reconstituted in 1 ml of 90:10 methanol:water with 25 μL of 6 μM cis-permethrin added as an internal standard of instrument efficiency for LC/MS analysis. The solid phase cleanup was automated using a RapidTrace SPE Workstation (Hopkinton, MA). Liver and fat extracts were loaded onto a silica containing analytical GPC preparatory column and run with a 70% ethyl acetate, 30% cyclopentane mobile phase at 5 mL/min. A 25 mL fraction containing the pyrethroids was collected with a fraction collector and then dried under a stream of nitrogen. Samples were reconstituted in 3 ml hexane. Liquid-liquid extraction

was conducted with equal volumes of the hexane extracts and hexane saturated acetonitrile. The liquid-liquid extraction was repeated 3 times and the acetonitrile fractions were combined, dried under a stream of nitrogen and reconstituted in 1 ml of 90:10 methanol:water with 25 µL of 6 µM Labeled cis-permethrin (phenoxy-$^{13}C_6$) added as an internal standard of instrument efficiency for LC/MS analysis.

Residue Determination

An Agilent (Palo Alto, CA USA) 1100 series LC/MSD VL ion trap mass spectrometer and HP Chemstation software were used for identification and quantification of pyrethroid parent chemicals as previously described by Godin et al (2006) with minor modifications. Briefly, isocratic elution of chemicals was accomplished with an Agilent Zorbax Eclipse XDB – C18 column (4.6 x 100 mm, 3.5 µm pore size) and XDB C-18 guard column with a mobile phase of 90% methanol and 10% 5 mM ammonium formate at a flow rate of 0.5 ml/min. Deltamethrin identification and quantification was accomplished by fragmentation of the ammonium adducted ion $[M+NH_4]^+$ (m/z 523) to produce the parent ion $[M+H]^+$ (m/z 506). Pyrethroids were quantified using at least a five-point calibration curve, prepared in tissue appropriate matrix, containing $^{13}C_6$-cis-permethrin internal standard. Residue concentrations were determined by the ratio of internal standard response to the analyte response. Calibration standards ranged from 1 to 500 ng/ml. Method limits of deltamethrin quantification were approximately 5 ng/ml or g for blood and brain, and 10 ng/g for liver and fat. Extraction integrity was ensured by adding trans-permethrin (phenoxy-$^{13}C_6$) surrogate standard to each sample prior to extraction. Surrogate recovery values of 80-120%

of expected were deemed acceptable and required no correction. Samples with surrogate recoveries above or below the acceptable range were reanalyzed.

Bioavailability Analysis

Bioavailability was calculated for 0.3 mg/kg and 3.0 mg/kg po doses using equation (1) and a 1mg/kg iv dose.

$$\text{Bioavailability} = (AUC_{po}/AUC_{iv})*(Dose_{iv}/Dose_{po}) \qquad (1)$$

Where AUC_{po} represents the area under the curve to infinity for the blood after an oral dose ($Dose_{po}$) and AUC_{iv} represents the area under the curve for the blood to infinity after an intravenous dose ($Dose_{iv}$).

PBPK Modeling

All PBPK modeling employed AcslXtreme software version 2.3.0.12 (The AEgis Technologies Group Inc., Huntsville, AL). An initial PBPK model for deltamethrin was developed based on the model of Mirfazaelian et al (2006); this initial model is further referred to as the mixed kinetics model. Tissue compartments included brain (target tissue), liver (metabolic compartment), fat, slowly perfused, and richly perfused tissues. Tissue compartments were connected via a single blood compartment. The model includes a two compartment GI tract for description of uptake and fecal excretion of an oral dose similar to that of Timchalk et al (2002). This model varied from that of Mirfazaelian et al (2006) by removal of the GI Juice compartment and the rate constant for 'stomach' (Ks) absorption.

These parameters were removed as they were found by preliminary modeling to have no influence on model predictions of peak and time to peak blood and tissue concentrations. The present model describes the blood as a single compartment since there was no difference in deltamethrin blood and plasma concentrations (Mirfazaelian et al., 2006). Fecal excretion was also modified as the saturable absorption process described by Mirfazaelian et al. (2006) is inconsistent with reported data on fecal excretion of deltamethrin (Fig. 1) (Bosch 1990; Cole et al., 1982; Crawford et al., 1981a, b; Ruzo et al., 1978) and the current bioavailability study (see Results below). Fecal excretion was therefore described by a first order rate constant (see Modeling Equations). The fecal excretion rate constant was therefore set so that excretion equaled 25% of the oral dose since this is approximately the median of the range of reported values in the literature (Bosch 1990; Cole et al., 1982; Crawford et al., 1981a, b; Ruzo et al., 1978). Figure 2 depicts the general model structure. Fat and slowly perfused tissues were modeled as diffusion-limited compartments and the brain, liver, and richly perfused compartments were modeled as flow-limited. This model is therefore referred to as the mixed kinetics model. All chemical parameters (partition coefficients) were from Mirfazaelian et al (2006) with the exception of hepatic clearance, which was taken from Godin et al. (2006). In order to achieve comparable simulations to Mirfazaelian et al. (2006), hepatic clearance required attenuation by a factor of 4 (from 5.3 – 1.325 L/hr/Kg). The initial mixed model, effectively equivalent to Mirfazaelian model, was unable to adequately simulate the data from the current study or from the iv dose study of Gray and Rickard 1982. Consistent with this finding, we note that Mirfazaelian et al. 2006 were also unable to adequately simulate the date of Gray and Rickard 1982.

Diffusion-Limited Model Development and Computational Approaches

Further model development focused on diffusion-limited descriptions in all tissue compartments (Fig 2 and Modeling Equations). This model is referred to as the diffusion-limited computational (comp) model. As in the previous model physiological parameters were determined from the available literature (see Table 1). Partition coefficients (P_T) for each tissue compartment were calculated by the computational approach of Poulin and Theil (2000). This method is based on tissue composition and describes the expected solubility of the chemical in each tissue compartment based on water and lipid content of different tissues. Description of oral absorption was based on the model of Timchalk et al (2002) with the exception of the rate constant for stomach absorption (K_s) which was removed. Initial results and optimization of K_s resulted in values close to zero, which did not influence peak and time to peak blood concentrations. Metabolism parameters for liver and blood clearance were based on the data of Godin et al. (2006, 2007). Permeability area coefficients (PA_TC) (diffusion limitation) were first fit to the 3 mg/kg po data from this study via visual fitting and subsequently optimized with AcslXtreme OpStat parameter estimation software.

AcslXtreme OpStat software utilizes maximum likelihood estimation to fit parameter values. The likelihood function assumes that the data are statistically independent and normally distributed. When the heteroscedascity parameter (γ) is set equal to 0, error is calculated as absolute error (normal) and when γ is equal to 2 error is calculated as relative error (log-normal). Model parameters were estimated with γ equal to 2 as data error was proportional to concentration (constant coefficient of variation).

Diffusion-Limited Model Optimization

The diffusion limited computational model severely overestimated brain tissue concentrations at all time points. Because of possible bias in other partition coefficients and the interdependence with permeability area coefficients, both sets of parameters were optimized for all compartments using Acsl OpStat. Furthermore, a diffusion-limited structure was maintained for all compartments. The optimized model is referred to as the optimized diffusion-limited model. Parameter optimization was done in the following stepwise manner. P_T and PA_TC for richly and slowly perfused compartments were optimized to the 1 mg/kg iv blood concentration data in order remove oral absorption as a confounding factor. Blood, liver, brain, and fat P_T and PA_TC were then optimized to fit the blood, liver, brain, and fat tissue concentration data from the 3.0 mg/kg po dose. All tissue distribution parameters were estimated with the fraction of the oral dose absorbed set to 25% as previously described. Oral absorption parameters were then estimated to further optimize the fit of model predictions to the blood, liver, fat and brain data from the 3 mg/kg oral dose. Final parameterization resulted in approximately 27% of the oral dose excreted in the feces. While estimated partition and permeability coefficients for the fat accurately predicted data from this study final parameters required adjustment post optimization via visual fit. This was due to underestimation of later time points from two literature studies (Ruzo et al. 1978; Mirfazaelian et al. 2006). Literature data included time points at 8, 14, and 21 days post exposure whereas the present study only included time points out to 48 hr. The discrepancy was determined to be due to the weighting of the numerous time points in the first 48 hours of this study by Acsl OpStat during the error minimization routine influencing final parameter estimates. This change did not influence model predictions in other tissue

compartments including target tissue concentrations as it did not significantly affect fat concentration predictions out through 48hrs (see results).

Sensitivity analysis

Sensitivity analysis was conducted utilizing the sensitivity analysis software in AcslXtreme OpStat. Analyses were conducted for the optimized diffusion-limited model structure. Model parameters were increased by 0.1% to assess their impact on model prediction of peak blood and brain concentrations. Sensitivity coefficients were normalized to both the response variable (model output value) and the parameters of interest (parameter being adjusted) (Evans and Anderson 2000; Mirfazaelian et al 2006).

Extrapolation from rat to human PBPK model

The optimized diffusion-limited (op) rat PBPK model was extrapolated to develop a human PBPK model for deltamethrin. The model was extrapolated by the use of human physiological parameters for cardiac output, cardiac output to individual tissue, volume of individual tissues, and blood volume fractions from the literature (Table 1). All other model parameters were held constant from the rat models except for metabolic parameters that were obtained from Godin et al. (2006; 2007).

RESULTS

Bioavailability analysis

Blood concentration data after iv dosing of 1 mg/kg deltamethrin in glycerol formal and 0.3 and 3.0 mg/kg oral doses in corn oil were used to assess the oral bioavailability of deltamethrin. The blood concentrations vs time graphs and the AUCs for the 1 mg/kg iv dose and 0.3 and 3.0 mg/kg oral doses are presented in Figures 3a-c and Table 2. The oral bioavailability of deltamethrin was 25% ± 5% (mean ± SD?) at the 0.3 mg/kg dose and 28% ± 7% at the 3.0 mg/kg dose (Table 2). Based on the dose range utilized and the tissue concentrations of deltamethrin observed (below K_m for deltamethrin metabolism, Godin et al 2006), hepatic clearance of deltamethrin is expected to be in the linear range. This result indicates that there is no significant difference in the extent of absorption of deltamethrin at these two doses. Bioavailability of deltamethrin was higher in this work as compared to previous reports by Kim et al (2007) and Anadon et al. (1996), which reported approximately 15% bioavailability at oral doses of 10 and 26 mg/kg, respectively. This difference is likely due to experimental conditions including dosing vehicle and volume (Crofton et al. 1995; Kim et al 2007).

Simulation of Blood Concentrations

IV exposure to deltamethrin leads to its rapid distribution to all tissues. A biphasic decline in deltamethrin concentrations in the blood (Fig 3a inset) was observed in the first 4 hr after injection of a bolus dose, a result similar to the data of Gray and Rickard (1982) (Fig 4.4). The optimized diffusion limited model accurately predicted the shape of this blood concentration time course data (Fig 4.3 and Fig 4.4).

Blood concentrations of deltamethrin after single 0.3 and 3.0 mg/kg oral doses were compared to model predictions (Figs 3b and c). Deltamethrin is rapidly absorbed after oral exposure with peak blood concentrations 1-2 hr post-exposure. Model predictions from the optimized diffusion limited model resulted in peak blood concentrations after 1.5 hrs. The optimized diffusion-limited model accurately predicted the rapid rise in blood concentration, peak concentration and the rapid elimination of deltamethrin from the blood at both doses. These results further confirm the bioavailability results indicating that there does not appear to be any dose-dependent absorption of deltamethrin at the doses examined. Metabolism of deltamethrin in the serum did not affect model predictions (data not shown) and only accounted for a fraction (less than 1%) of the metabolism of the absorbed dose in the model.

Simulation of Brain Concentrations

Brain concentrations of deltamethrin were at or below limits of detection in the 0.3 mg/kg exposure group at all time points examined. Brain concentration data peaked at 3 hrs in animals exposed to 3 mg deltamethrin/kg. The use of a computationally derived brain partition coefficient resulted in drastic over estimation of brain concentrations of deltamethrin by the model. Accurate prediction of peak brain concentrations could be obtained by adjusting the permeability area coefficient alone however doing so could not describe the shape of the concentration time curve in the brain. Lowering the brain partition coefficient by nearly 300-fold resulted in more accurate model predictions of the brain concentration-time course data (Table 1). The optimized diffusion-limited model predicted peak brain concentrations at 2.6 hrs and accurately fit declining concentrations of deltamethrin (Fig. 5). The delay in peak brain concentration relative to peak blood

concentrations is a good indication that the diffusion-limited description of the brain compartment is appropriate. Initial modeling attempts with the mixed model structure of Mirfazaelian et al (2006) resulted in more rapid peak brain concentrations (results not shown).

Simulation of Liver Concentrations after oral exposure

The concentration of deltamethrin in the liver rose rapidly after the oral dose of 3.0 mg/kg, peaking 1-2 hrs after exposure and decreasing to undetectable levels within 8-10 hrs of exposure (Fig 6). Liver concentrations were below detection limits in the 0.3 mg/~~k~~g dose group. The optimized diffusion-limited model accurately predicted peak tissue concentrations of deltamethrin as well as the rapid decline in tissue concentrations (Fig 6). The liver is the main tissue for metabolic elimination of pyrethroids and rapidly metabolizes deltamethrin (Godin et al 2006; Mirfazaelian et al 2006). The rapid decline of deltamethrin concentrations in the liver is consistent with its rapid metabolism in this tissue.

Simulation of Fat Concentrations after oral exposure

Fat concentrations of deltamethrin were determined out to 48 hr after oral exposures of 0.3 and 3.0 mg/kg. Fat concentrations of deltamethrin peaked within 8 hrs of dosing (Fig 7). The optimized diffusion limited model accurately predicted the concentration time curves at both exposures (Fig 7). Model predictions using the computationally derived fat partition coefficient were similar to model predictions using the final optimized partition coefficient (Fig 7). Despite a five-fold difference in the partition coefficients, the use of either value resulted in fits consistent with the data up to 48 hr ~~hours~~ post exposure.

Simulation of literature data

PBPK model prediction were compared to available literature data on deltamethrin tissue dosimetry obtained by Gray and Rickard (1982) (Fig 4.4, and Fig 4.8), Mirfazaelian et al. (2006) (Fig 4.9), and Ruzo et al. (1978) (Fig 4.10). Gray and Rickard (1982) exposed animals to 1.75 mg deltamethrin/kg iv and followed blood concentrations over 4 hrs post dosing. As previously described, the blood concentration data of Gray and Rickard (1982) displays a biphasic distribution over the first 4 hrs. This biphasic clearance is similar to that seen in the current study, which is accurately described by the optimized diffusion-limited model (Fig 4.3 and Fig 4.4). Gray and Rickard (1982) also reported concentrations of deltamethrin in three different regions of the brain with very little variability between regions. Fig 4.8 depicts model prediction compared to the average brain concentration of deltamethrin from Gray and Rickard (1982). The optimized diffusion-limited model predictions compared very well with this data. The brain data, similar to the blood data, has a biphasic time course which is also described well by the optimized diffusion-limited model.

There were some inconsistencies in predictions of the optimized diffusion-limited model of the Mirfazaelian et al (2006) data. Some of the inconsistencies are related to study design and data. For example, the fat concentration data from both studies are similar up to the last time point (48 hrs) examined in the present study. However, the Mirfazaelian et al data includes time points to 336 and 504 hr. The diffusion-limited model using the computationally derived partition coefficient for fat overpredicts the fat concentration data of Mirfazaelian et al (2006) at the later time points (not shown). Optimization towards the fat data from this study resulted in underprediction of the late time points. Visual fitting of the fat partition coefficient and permeability area coefficient resulted in good agreement with the

data for the 2 and 10 mg/kg oral doses from Mirfazaelian et al. (2006) (Fig 9 B and C). Simulation of blood concentrations from Mirfazaelian et al (2006) with the optimized diffusion-limited model also resulted in good agreement with the published data (Fig 9 B and C). In contrast, the optimized diffusion-limited model was unable to accurately describe the time course for the brain deltamethrin concentration of Mirfazaelian et al. (2006). The brain concentration time course from Mirfazaelian et al (2006) has a significantly different shape to the tissue concentration time curves than the data from this study (Fig 5 compared to Fig 9 B and C). Brain concentrations remain elevated for a greater period of time in the study of Mirfazaelian et al. (2006).

Ruzo et al. (1989) reported tissue residues of deltamethrin in the fat at 8 days (192~~)~~ hr) ~~hours~~ post oral dosing at three different dose levels. The final optimized diffusion limited model resulted in good agreement with these data (Fig 10). This further increases confidence in the visually optimized fat partition and permeability area coefficients determined with the data of Mirfazaelian et al. (2006).

Sensitivity Analysis

Sensitivity analysis was conducted on the optimized diffusion-limited model structure. Model parameters were increased by 0.1% to evaluate their influence on peak blood and brain concentrations of deltamethrin. Figure 11 presents the normalized sensitivity coefficients for each parameter in the model. The parameter that had the greatest impact on both blood and brain concentrations was the volume of the slowly perfused tissue compartment (vsc). This is the largest compartment in the model. The muscle accounts for most of its mass. Parameters that influence the liver, liver blood flow (qlc) and the diffusion

limitation (palc), were also highly sensitive model parameters. Increasing any of these parameters leads to increased diffusion of the chemical from blood into the liver, resulting in increased hepatic metabolism and decreased blood and brain concentrations of deltamethrin. Hepatic clearance is diffusion limited, so the metabolic clearance parameter is not itself a sensitive parameter.

Absorption parameters were also relatively sensitive. Fecal excretion (kfe), when increased, decreased peak blood and brain concentrations by reducing the amount of deltamethrin absorbed into the systemic circulation. Conversely, increases in Ki and Ksi, which describe the transfer and uptake of deltamethrin through the GI compartment, resulted in increases in peak blood concentrations due to more rapid and greater deltamethrin absorption. Partitioning (pb) and the diffusion limitation (pbrc) in the brain impact peak brain concentration but have no significant effect on blood concentrations.

Human Modeling

Utilizing the diffusion-limited (op) model structure, the rat model was extrapolated to a human model using human physiological and metabolic parameters. The human physiological parameters are from Brown et al. (1995) and are presented in Table 1. Human metabolic parameters for hepatic and serum clearance are from Godin et al (2006; ~~and~~ 2007).

Data from controlled human exposure to deltamethrin is limited to an oral absorption study in three healthy volunteers conducted by Roussel-Uclaf in 1984. Volunteers were given a single 3 mg oral dose of deltamethrin in a suspension of 10 mL of poly (oxyethlene glycol)-300 and diluted with water to a final volume of 150 mL. This corresponds to approximately a 0.43 mg/kg dose of deltamethrin in these subjects, all of whom were

approximately 70 kg. Data was reported as total radioactivity in the blood, urine, and feces and does not differentiate between parent chemical and metabolites. This makes comparisons with the current model difficult as it was based on actual measured deltamethrin concentrations. Cumulative urinary excretion of radioactivity averaged 48 ± 3% of the oral dose after 96 hrs. Cumulative fecal excretion averaged 17 ± 8%, with a range of 10-25%, of the administered dose after 72 hrs. This range is within literature values for fecal excretion in rats (Bosch 1990; Cole et al., 1982; Crawford et al., 1981a, b; Ruzo et al., 1978). Based on the limited number of subjects, the use of a large volume of an aqueous vehicle, and the use of total radioactivity as a measure of dose, it is difficult to compare the fecal excretion in the human study to those of rodent studies. Therefore, we decided to use the rat fecal excretion rate in the human model

The cumulative urinary and excretion in the human study accounts for approximately 48% of the total radioactivity of the administered dose (Roussel-Uclaf 1984). Based on literature reports, only hydrolyzed metabolites of pyrethroids are present in the urine and the parent chemical, with minor amounts of hydroxylated metabolites, are excreted in the feces (Bosch 1990; Cole et al., 1982; Crawford et al., 1981a, b; Ruzo et al., 1978). In vitro studies suggest that in humans, deltamethrin is primarily metabolized by hepatic esterases to hydrolyzed products (Godin et al 2006). Thus, it would be expected that the radioactivity in the urine from the human study was primarily hydrolyzed deltamethrin metabolites. Therefore, the percent of the administered dose metabolized in the human PBPK model may be able to be used as a surrogate to compare to the human urinary excretion data. The diffusion-limited (op) model, however, predicted 66% percent of an oral dose was

metabolized over 96 hr. Based on these data it is unclear whether there is a correlation between urinary excretion of radioactivity and the total predicted metabolized dose.

Simulation of brain tissue concentrations of a 1 mg/kg oral dose of deltamethrin (rat NOAEL), based on the current optimized model, revealed that the diffusion-limited model structure leads to brain concentrations that are 1.8-fold greater in the human model than in the rat model (Fig 12). Concentrations of deltamethrin in the brain also remain elevated for a significantly longer time in the human than in the rat model. These differences are the result of physiological differences in the brain of rats and humans, which are reflected in the model. Cardiac output to the brain and brain volume (% BW) in humans is five and four times greater, respectively, than in the rat, resulting in a greater predicted CNS exposure of deltamethrin in humans.

DISCUSSION

An initial PBPK model for deltamethrin by Mirfazaelian et al (2006) makes several assumptions necessitated by either inconsistent or limited data available from the literature. One of the inconsistencies involves oral absorption. Mirfazaelian et al (2006) assumed that oral absorption of deltamethrin was dose-dependent (inversely). Our analysis of the available literature indicated that there was a potentially linear relationship (Fig 1). In order to resolve ambiguities in the data, the present study examined the bioavailability of deltamethrin at two doses, one at the approximate ED_{30} (3.0 mg/kg) for motor activity (Wolansky et al. 2006) impairment and a second ten-fold lower (0.3 mg/kg). Results indicate that there is no significant difference in the oral bioavailability of deltamethrin at the dose levels examined. In addition the present model, incorporating a first order rate constant for hepatic clearance, was able to accurately predict blood concentrations across a range of doses (0.3 – 10.0 mg/kg) from these studies and literature studies (Gray and Rickard 1982; Mirfazaelian et al 2006; Ruzo et al 1978). These results indicate that there is no significant difference in the oral absorption of deltamethrin and that oral absorption of deltamethrin is not dose-dependent within the range of available data sets and dose range in the literature. Changes in oral bioavailability are most likely to be vehicle dependent (Kim et al 2007)

Due to the lack of dose-dependent absorption of deltamethrin, we developed a simpler model for oral absorption than was used by Mirfazaelian et al (2006). Our description of oral absorption was based on the PBPK model of Timchalk et al (2002) for chlorpyrifros, an organophosphate pesticide. The oral absorption consists of a two compartment model (Fig 2). Of note is the similarity in the K_{SI} parameter, as compared to the model of Timchalk et al (2002), which describes the passage of the chemical from

compartment 1 to compartment 2 in the GI tract and was necessary to describe time to peak blood concentration. This similarity may have some physiological relevance as a parameter describing gastric emptying and may be useful in future modeling of other pyrethroids. Parameter estimations were initially completed with K_{SI}, K_I and K_{FE} parameters set and held constant so that fecal excretion equaled 25% of the oral dose. Final optimization of absorption and excretion parameters to optimize fits in all other tissue compartments resulted in fecal excretion of 27% of the oral dose. Both values are within the range of fecal excretion found in the literature for deltamethrin and other pyrethroids (Bosch 1990; Cole et al., 1982; Crawford et al., 1981a, b; Ruzo et al., 1978). As stated previous, fecal excretion and therefore oral absorption is likely a vehicle dependent parameter (Kim et al 2007).

Initial modeling of deltamethrin based on the mixed model structure does not accurately describe tissue time course data for deltamethrin, particularly after an iv dose (data not shown) The mixture model required attenuation of the hepatic clearance constant from Godin et al (2006) by 4-fold in order to approximate tissue concentrations but still could not accurately describe the shape of the tissue concentration time curves. Because there is no evidence of significant protein binding of pyrethroids in hepatic microsomes (Abu-Qare and Abou-Donia, 2002; Hoellinger et al 1985) which could affect their free concentration in the liver, there does not appear to be a physiologically relevant reason to attenuate metabolism. Based on this result and the inability of the initial mixed kinetic model to describe the shape of tissue concentration time curves, the model was modified to describe all tissues as diffusion-limited. Diffusion limitation in the liver reduces the rate at which a chemical enters and exits a tissue compartment, decreasing the amount of chemical available for metabolism. Comparison of liver tissue concentrations to model predictions showed the diffusion-limited

model accurately described the rapid decrease in liver tissue concentrations of deltamethrin (Fig 6). The use of a diffusion-limited description of the liver allows for the incorporation of the hepatic clearance values of Godin et al (2006) without attenuation.

The clearest evidence supporting a diffusion-limited description of deltamethrin was found in the ability of the model to describe the shape of the blood concentration time curves after iv dosing. In this study and the study of Gray and Rickard (1982) iv administration of a bolus dose of deltamethrin was followed by a biphasic distribution of deltamethrin from the blood within a few hours of exposure (Figs 3a inset and 4). This was accurately described by the diffusion limited model in contrast to the initial mixed kinetic model. The diffusion-limited model was also able to describe the shape of the tissue concentration time curve in the brain after the same 1.75 mg/kg dose (Gray and Rickard 1982) (Fig 8). The brain, like the blood displayed a biphasic distribution that was accurately depicted by the diffusion-limited model, but not by the mixed model with flow limitations. While other model structures may be able to provide adequate descriptions of some deltamethrin tissue concentration time curves (see Mirfazaelian et al 2006), they do not appear to be able to accurately describe the rapid changes in tissue concentrations after an iv dose, while the diffusion limited model can.

The target tissue for pyrethroid neurotoxicity is the central nervous system (Rickard and Brodie 1985). Accurate prediction of brain concentrations is therefore central to the ability to scale rat toxicity data to humans. The diffusion-limited model accurately predicted brain tissue time course data from a 3 mg/kg dose in this study and after a 1.75 mg/kg iv dose (Gray and Rickard 1982). However, this model did not accurately predict the brain concentrations time curves from Mirfazaelian et al (2006) or Anadon et al (1996). There is

inconsistency in the data for brain deltamethrin concentrations between these studies. The data from Mirfazaelian et al (2006) (Fig 9) and Anandon et al (1996) indicate that concentrations in the brain remain elevated for extended periods. It is difficult to assess why differences exist from each of these studies. However, the work of Mirfazaelian et al (2006) and Anandon et al (1996) used HPLC/UV quantification. Gray and Rickard (1982) conducted their study utilizing radiolabeled deltamethrin with TLC. Our study utilized mass spectrometry to identify and quantify deltamethrin. Modeling indicates that our data correlates well with the data of Gray and Rickard (1982). Mass spectrometry and radiolabel analysis are more specific identification methods than HPLC/UV and this could be a potential reason for differences in the results across these studies.

Our modeling efforts utilized computational methods to estimate partition coefficients for the different tissues of the body. The computationally derived parameters were very near optimized values in tissue compartments except for the fat and brain (Table 1). The discrepancies between computational and optimized values in the brain and adipose tissue suggest that there are unidentified biological processes that regulate the distribution of deltamethrin to these tissues. It is also possible that there are limitations to the ability of the computational methods to accurately predict lipid rich tissues. In the brain this may be due to thermodynamic limitations of the compound crossing the blood brain barrier (BBB) or potential interactions with transporters at the BBB reducing the ability of deltamethrin to distribute into the brain. It is difficult to determine why discrepancies between the computationally derived fat partition coefficient and the optimized value necessary to fit the available fat data exist. The computational value appears to fit peak fat concentrations of deltamethrin (Fig 7) and allow accurate estimation of other tissue concentration profiles (Fig

3-9) because there is little discrepancy within the first 48 hours (Fig 7). The computational value however predicts that the level of deltamethrin in the fat remains elevated for considerably longer than literature data suggests. This could be due to minor metabolic processes in the fat or blood within the fat tissue, or due to non-enzymatic degradation of deltamethrin in the fat over the length of the study. It is currently unknown however, why the fat partition coefficient had to be lowered four-fold for such a highly lipophilic chemical such as deltamethrin. Modeling of another highly lipophillic compound, TCDD, also reveals that lower than expected partition coefficients, based on these computational methods, are needed to accurately model fat concentrations (Emond et al 2004). This may be indicative of deficiencies in the computational methods for predicting partition coefficients in lipohillic tissues for highly lipophillic compounds.

The rat model was used as the framework for developing a human deltamethrin PBPK model. Comparing predictions of the rat and human diffusion limited models at the rat NOAEL (1mg/Kg) (USEPA, 1998) for deltamethrin indicates that humans would have slightly greater brain deltamethrin concentrations (Fig. 12) despite the fact that humans metabolize deltamethrin more rapidly.

Model structure can have a significant impact on risk assessment of pyrethroids. A diffusion-limited model such as the one presented here minimizes the effect of the species difference in metabolism of deltamethrin (Godin et al 2006). In a diffusion-limited description of the liver, the rate limiting step in the clearance of deltamethrin is not the clearance rate but rather the rate of diffusion of compound from blood into tissue. Diffusion-limited kinetics would also have the effect of reducing the impact of inter-individual differences in the capacity for metabolism of deltamethrin in the human population. One of

the limitations of applying this model for risk assessments would be the description of the distribution of deltamethrin to the brain. Further research in this area is needed to better identify mechanisms for the lower than expected brain concentrations in rats. Understanding this process would enhance our confidence that the model predictions of target tissue concentrations are more accurate.

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Table 4.1. PBPK Model parameters

Parameter Description	Rat Model	Proposed Human Model
Body Weight (BW)	0.4	70
Cardiac Output (QCC) (L/hr/Kg^.75)	14.1[1]	14.1[1]
Cardiac Output to Liver (QLC) (% QCC)	0.183[1]	0.214[1]
Cardiac Output to Fat (QFC) (% QCC)	0.07[1]	0.052[1]
Cardiac Output to Brain (QBrC) (% QCC)	0.02[1]	0.114[1]
Cardiac Output to Slowly Perfused Tissues (QSC) (% QCC)	0.236[1]	0.282[1]
Cardiac Output to Richly Perfused Tissues (QRC) (% QCC)	QRC = 1-QLC-QFC-QBrC-QSC	
Blood Volume (VBlC) (%BW)	0.07[1]	0.07[1]
Liver Tissue Volume (VLC) (%BW)	0.03[1]	0.03[1]
Fat Tissue Volume (VFC) (%BW)	0.07[1]	0.22[1]
Brain Tissue Volume (VBrC) (%BW)	0.005[1]	0.02[1]
Slowly Perfused Tissue Volume (VSC) (%BW)	0.78[1]	0.62[1]
Richly Perfused Tissue Volume (VRC) (%BW)	VRC=1-VBlC-VLC-VFC-VBrC- VSC	
Blood Volume Fraction in Liver (%VLC)	0.21[1]	0.21[1]
Blood Volume Fraction in Fat (%VFC)	0.025[2]	0.025[2]
Blood Volume Fraction in Brain (%VBrC)	0.03[1]	0.03[1]
Blood Volume Fraction in Slowly Perfused Tissues (%VSC)	0.04[a]	0.04[a]
Blood Volume Fractoin in Richly Perfused Tissues (%VRC)	0.21[b]	0.21[b]
Liver Partition Coeffcient (P_L)	(9) 19[c]	19
Fat Partition Coeffcient (P_F)	(412) 75[2]	75
Brain Partition Coeffcient (P_{Br})	(30) 0.14[c]	0.14
Slowly Perfused Tissue Partition Coeffcient (P_S)	(7) 5.64[c]	5.64
Richly Perfused Tissue Partition Coeffcient (P_R)	(12) 8.10[c]	8.10
Liver Permeability Area Coefficient (PA_LC)	(0.28) 0.288[c]	0.288
Fat Permeability Area Coefficient (PA_FC)	(0.02) 0.025[c]	0.025
Brain Permeability Area Coefficient ($PA_{Br}C$)	(0.1) 0.002[c]	0.002
Slowly Perfused Tissue Permeability Area Coefficient (PA_SC)	(0.045) 0.043[c]	0.043
Richly Perfused Tissue Permeability Area Coeffcient (PA_RC)	(0.14) 0.093[c]	0.093
Stomach-Intestine Transfer Rate Constant (K_{SI}) (hr^{-1})	0.42[c]	0.42
Intestinal Uptake Rate Constant (K_I) (hr^{-1})	1.51[c]	1.48
Fecal Excretion Rate Constant (K_{FE}) (hr^{-1})	0.59[c]	0.6
Hepatic Clearance Rate Constant (K_{CL}) (L/hr/Kg BW)	5.3[3]	9.7[3]
Plasma Clerance Rate Constant (K_{BlC}) (L/hr//mL serum)	0.0012[4]	0[4]

Values in parentheses are computationally derived partition coefficients and corresponding permeability area coefficients. 1-Brown et al., 1997; 2- Mirfazaelian et al., 2006; 3-Godin et al., 2006; 4-Godin et al., 2007. a - set to Liver. b - set to muscle from Brown et al., 1997. c - estimated by AcslXtreme OpStat parameter estimation software.

Table 4.2. Deltamethrin Oral Bioavailability

	1mg/kg iv	0.3 mg/kg po	3.0 mg/kg po
Average $AUC_{0-\infty}$	2.22	0.167	1.86
STD	0.63	0.034	0.46
F%		25 ± 5	28 ± 7

Oral Bioavailability of Deltamethrin after oral doses of 0.3 and 3.0 mg/kg in corn oil.
N=4, mean ± SD

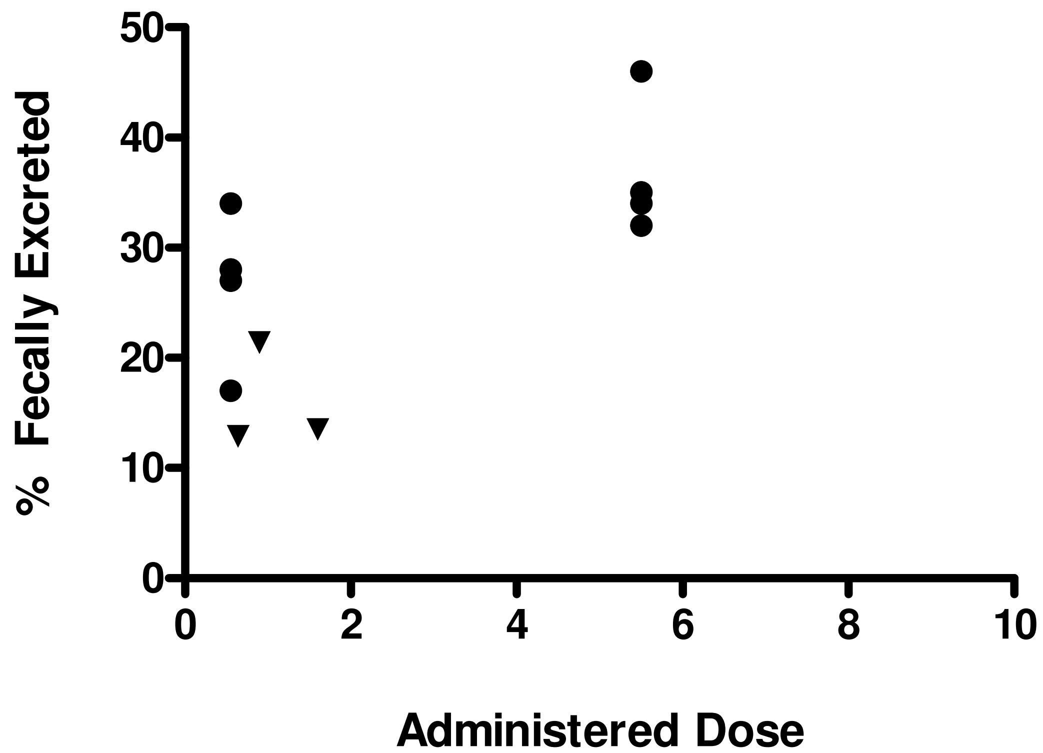


Figure 4.1. Fecal excretion of deltamethrin (literature values). Individual data points from ● - Bosch 1990 and▼ - Ruzo et al., 1978.

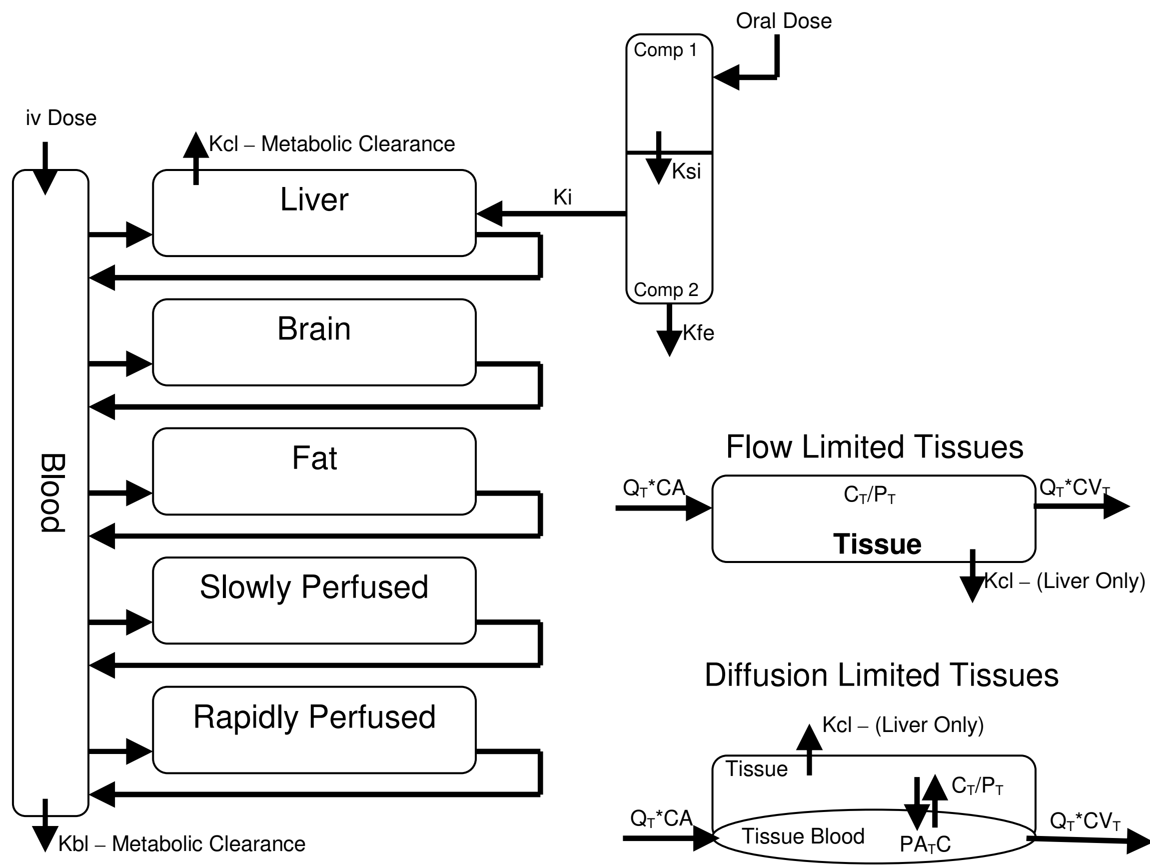
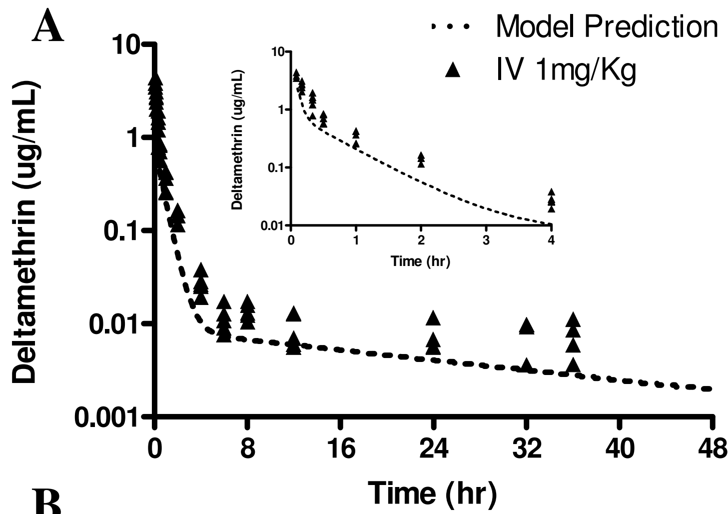
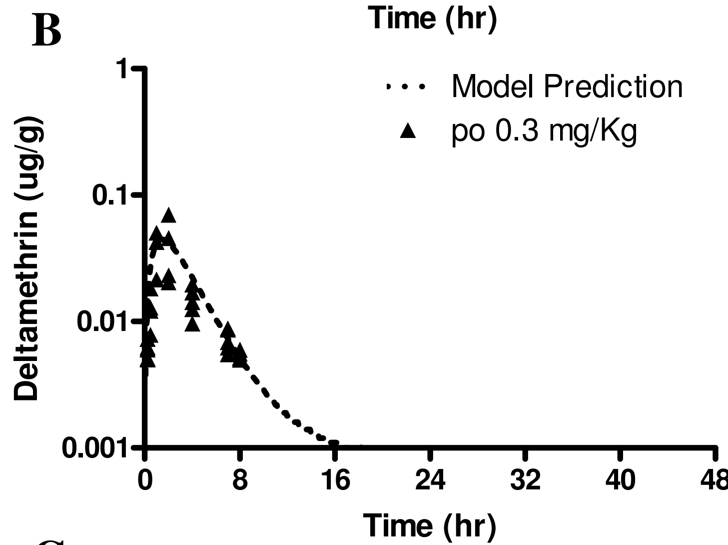


Figure 4.2. Basic PBPK Model Structure Representation





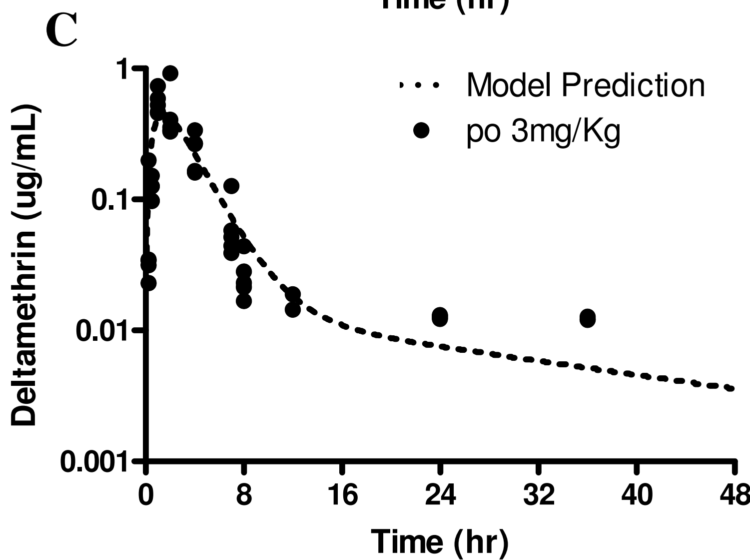


Figure 4.3. Model predictions of deltamethrin blood concentrations after iv and oral dosing.

A. Blood concentrations after an iv dose. Inset = 1-4 hours. Individual data points (▲, 1 mg/kg) (N=4). Dashed line represents predictions of the optimized diffusion-limited model.

B. Blood Concentratoins after a 0.3 mg/kg po dose individual data points(▲, 0.3 mg/kg) (N=4). Dashed line represents predictions of the optimized diffusion-limited model.

C. Blood Concentratoins after a 3.0 mg/kg po dose compared to individual data points(●, 3.0 mg/kg) (N=4). Dashed line represents predictions of the optimized diffusion-limited model.

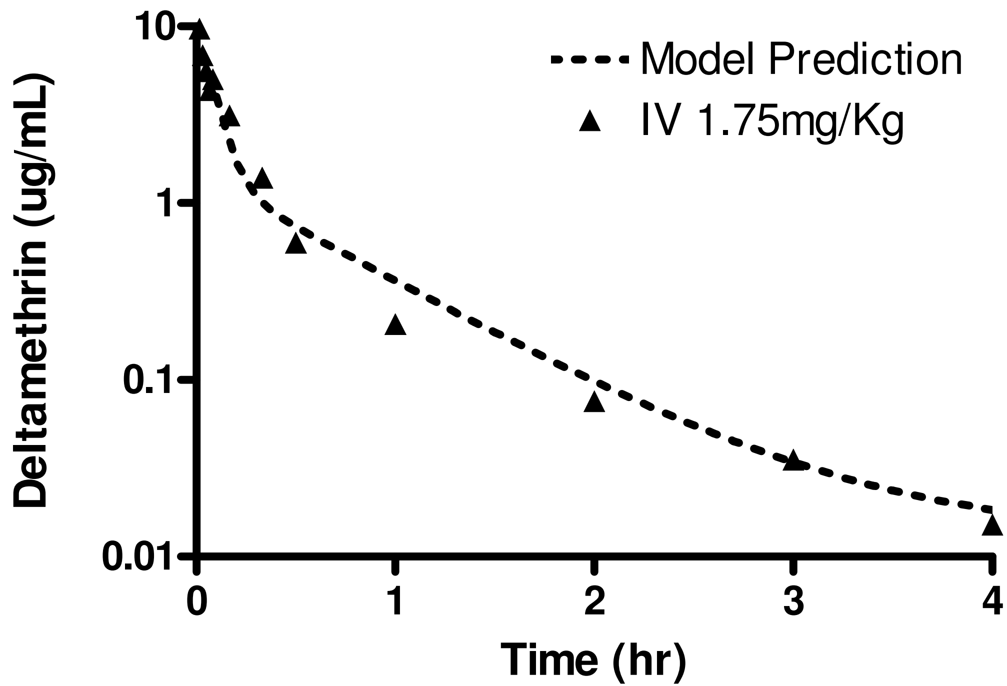


Figure 4.4. Model predictions of deltamethrin in blood after a 1.75 mg/kg iv dose (▲,data of Gray and Rickard 1982). Dashed line represents predictions of the optimized diffusion-limited model compared to data points.

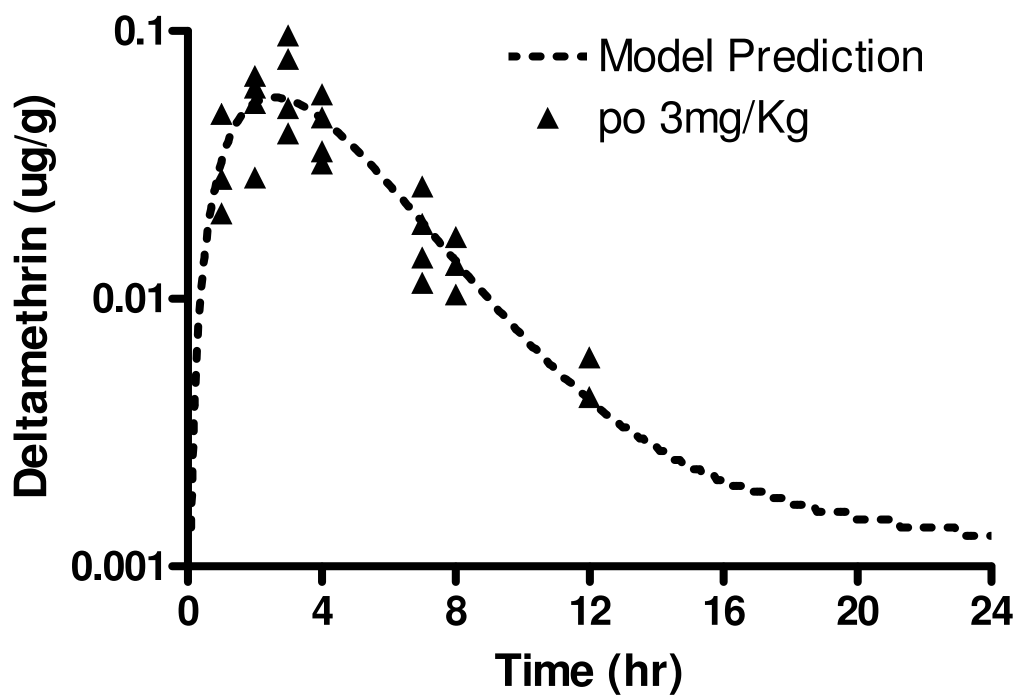


Figure 4.5. Model predictions compared to brain concentration of deltamethrin after oral dosing. Dashed line represents predictions of the optimized diffusion-limited model compared to data points (▲, 3mg/kg) (N=4).

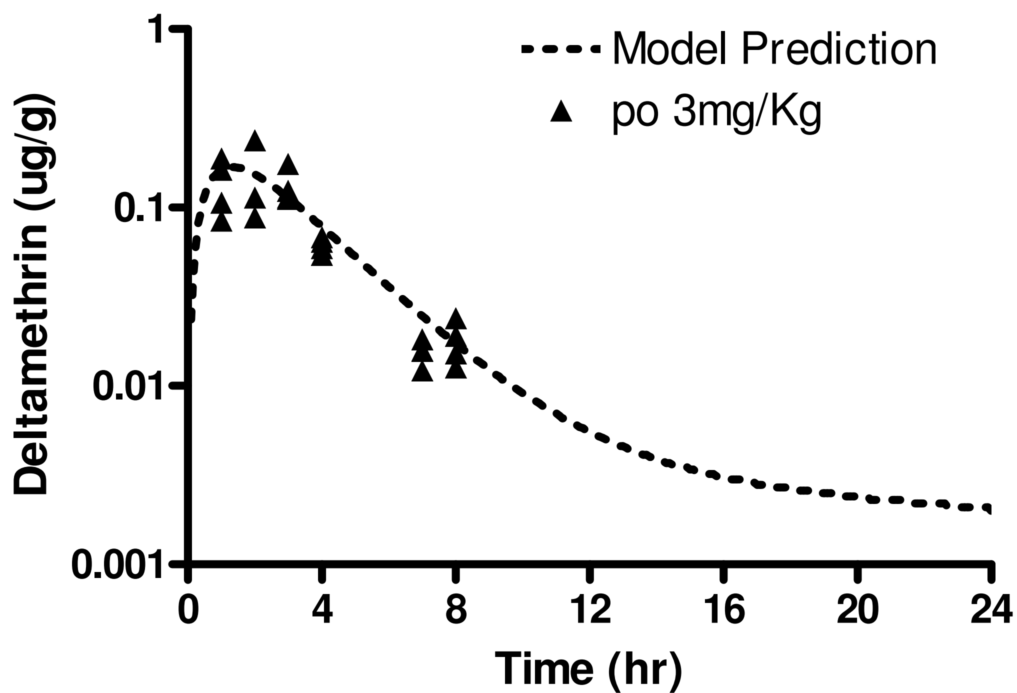


Figure 4.6. Model prediction compared to liver tissue concentration of deltamethrin after oral dosing. Dashed line represents predictions of the optimized diffusion-limited model compared to individual data points (▲, 3 mg/kg) (N=4).

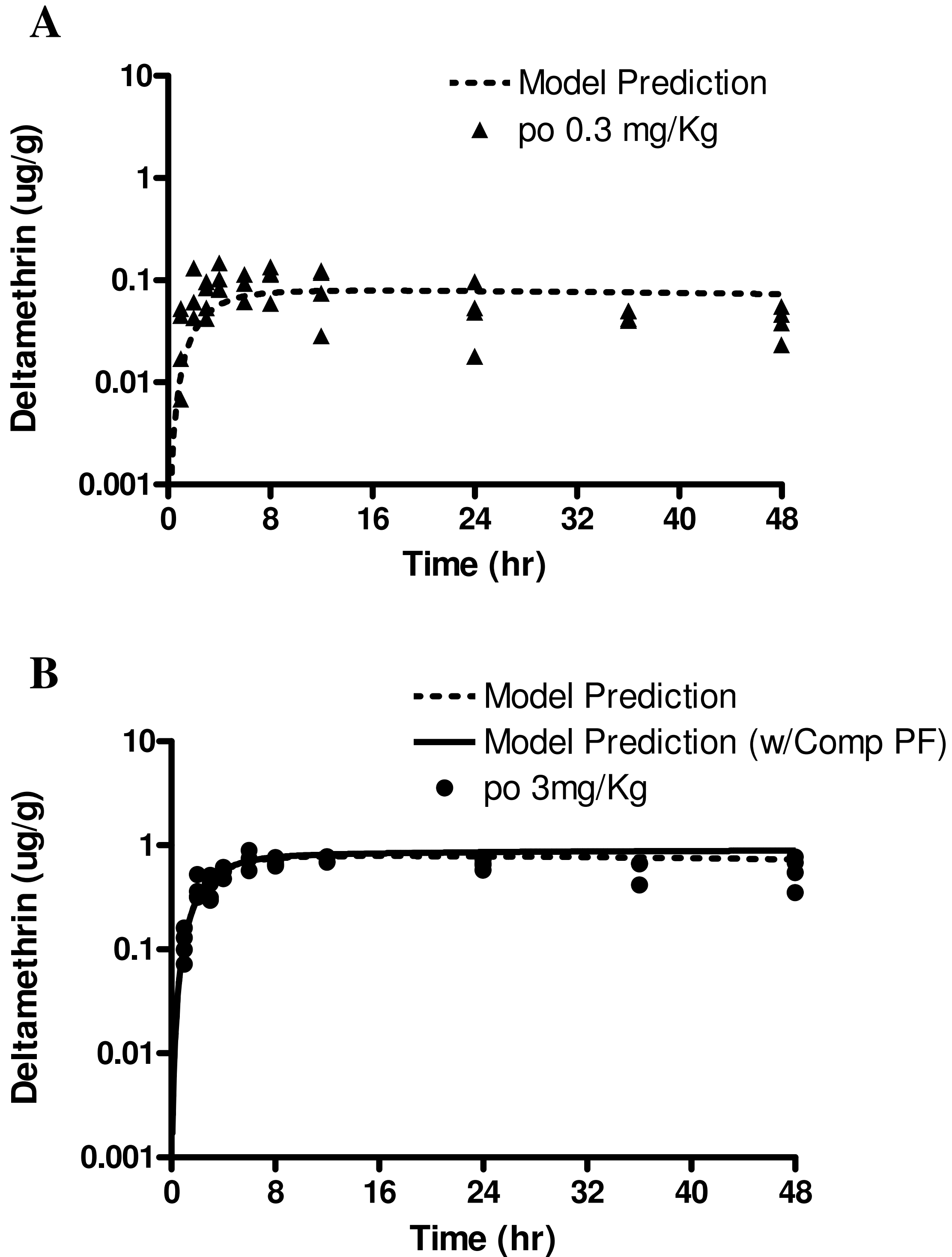


Figure 4.7. Model simulation of deltamethrin fat concentrations after oral dosing Dashed line represents predictions of the optimized diffusion-limited model compared to individual data points (A-▲, 0.3 mg/kg and B-●, 3.0 mg/kg) (N=4). Solid line represents prediction of the diffusion limited model with a computationally derived partition coefficient for the fat compartment.

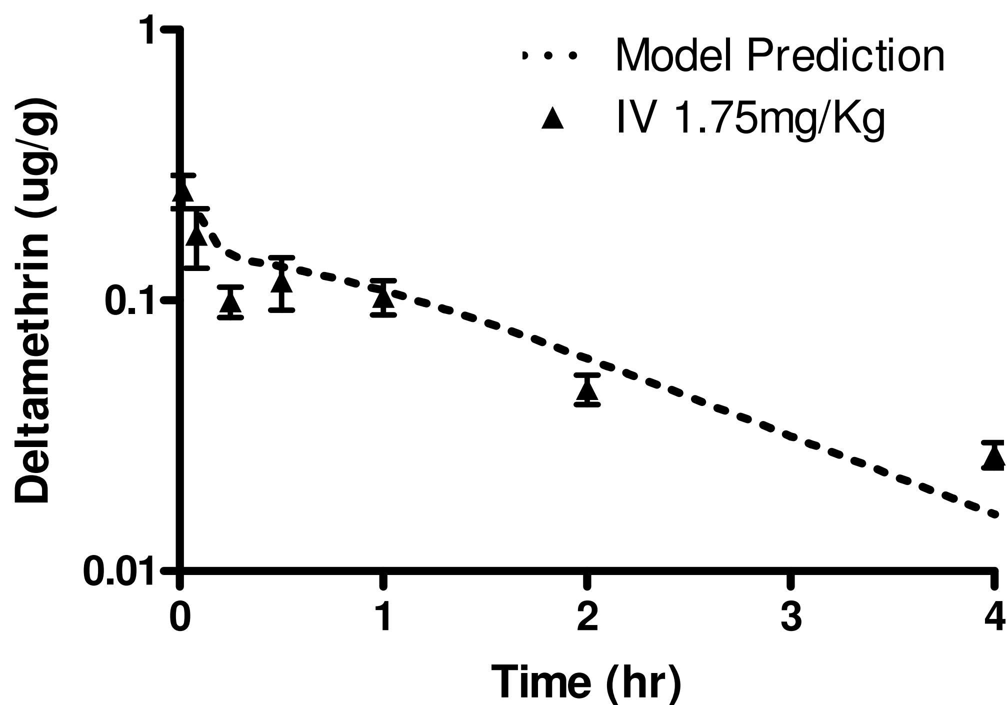


Figure 4.8. Model predictions of averaged brain concentrations of deltamethrin from the cerebrum, cerebellum and spinal cord after a 1.75 mg/kg iv dose (▲, data from Gray and Rickard 1982). Error bars equal standard deviation between average concentrations in each brain region. Dashed line represents predictions of the optimized diffusion-limited model.

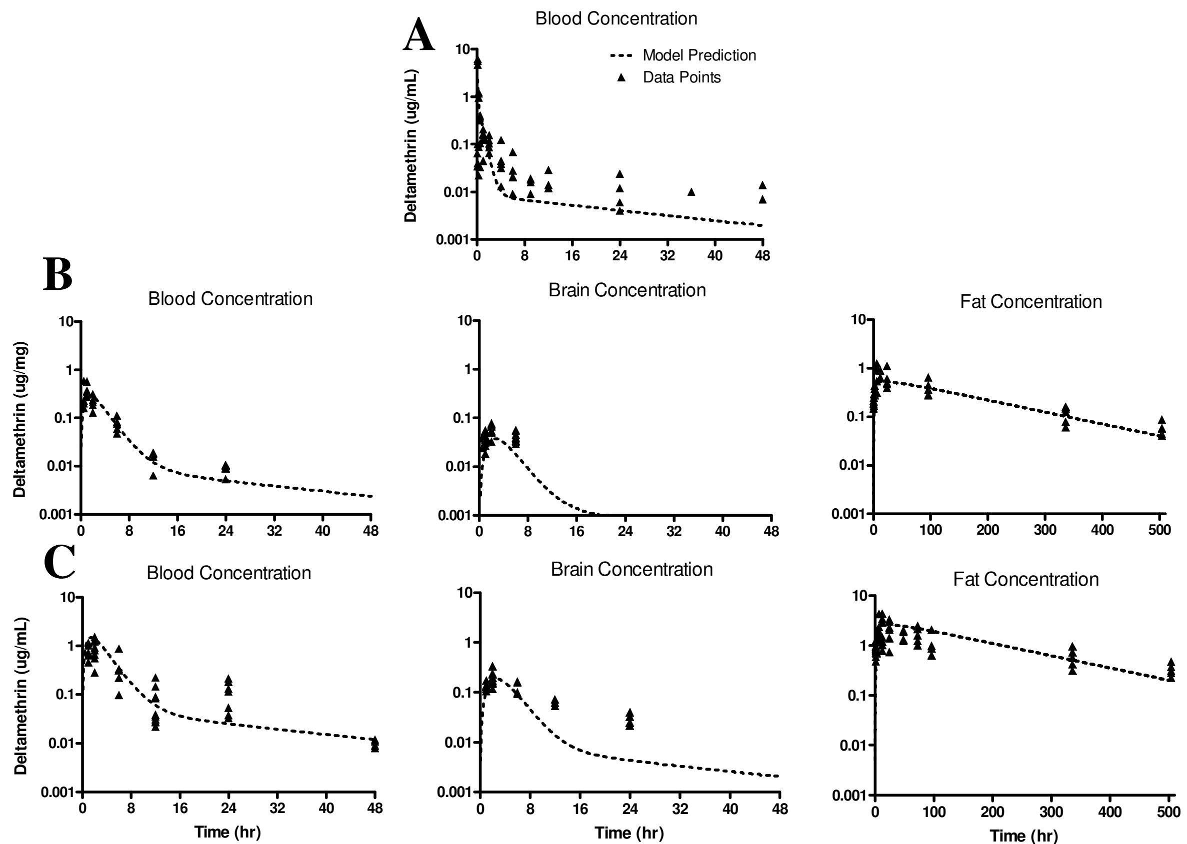


Figure 4.9. Model predictions of the deltamethrin tissue time course data of Mirfazaelian et al (2006) (▲). A) 1 mg/kg iv. B) 2 mg/kg po. C) 10 mg/kg po. Dashed line represents predictions of the optimized diffusion- limited model.

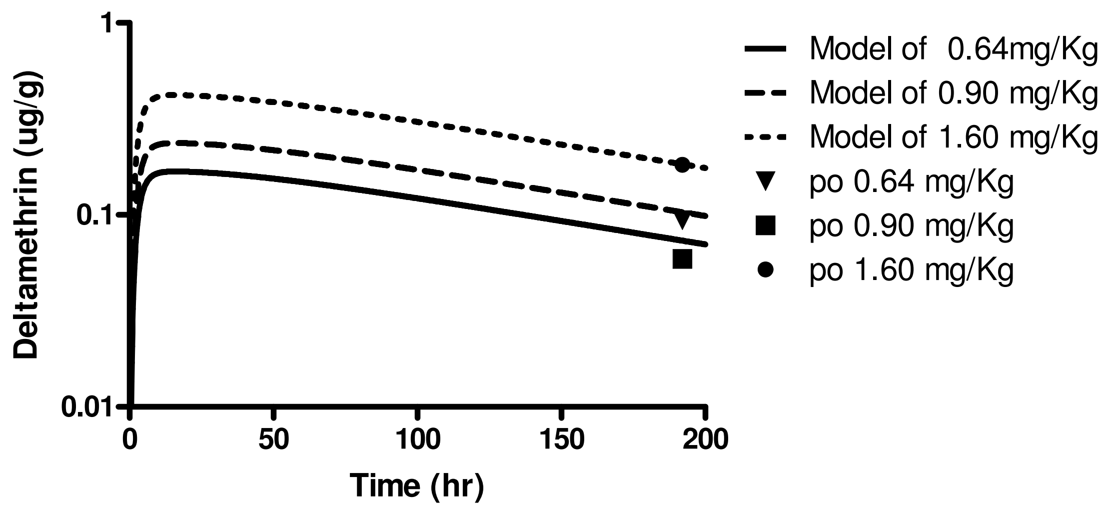


Figure 4.10. Model predictions vs fat concentration of deltamethrin from Ruzo et al. (1978). Lines represent predictions of the optimized diffusion limited model. ▼, 0.64 mg/kg oral dose; ●, 0.90 mg/kg oral dose; ■, 1.60 mg/kg oral dose.

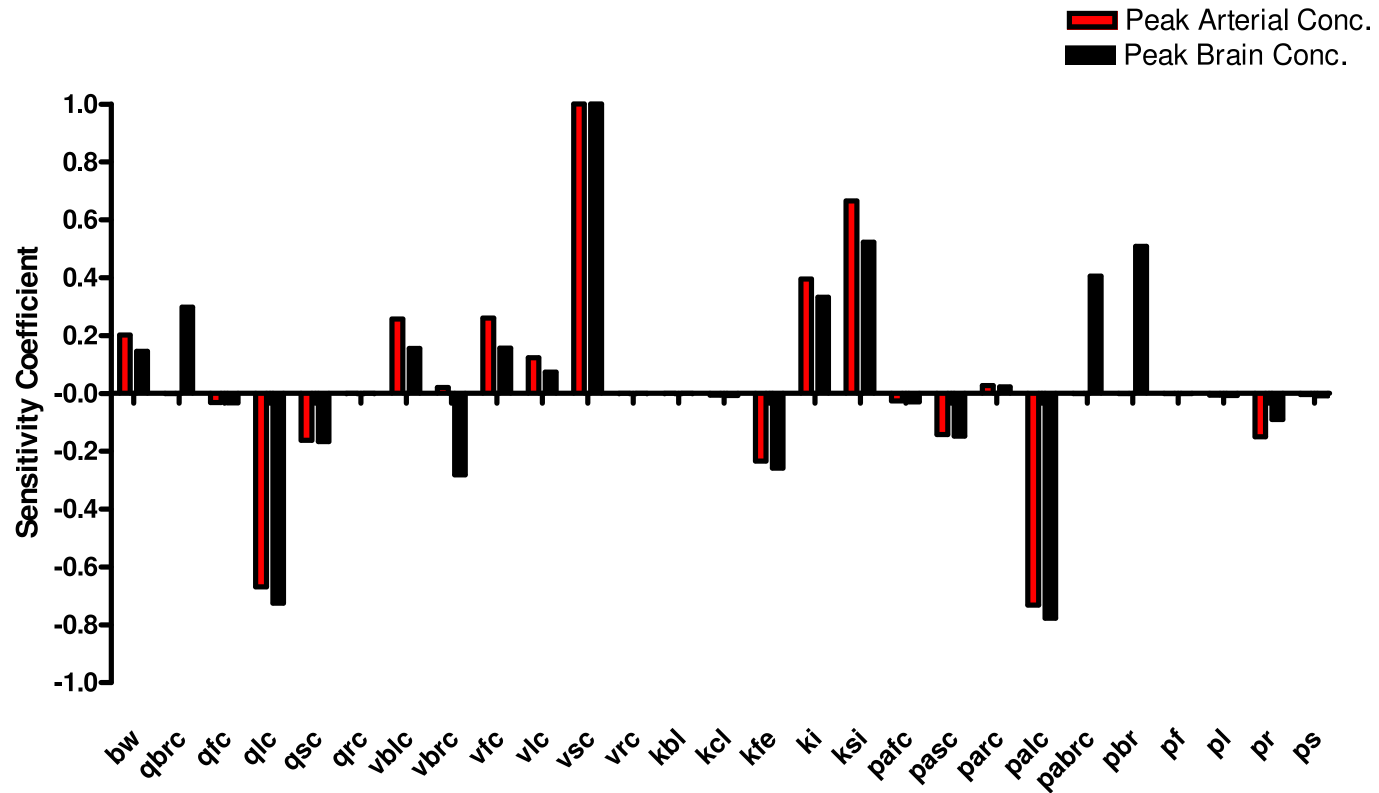


Figure 4.11. Sensitivity analysis of the optimized diffusion-limited model for peak arterial and brain concentrations of deltamethrin. See Table 1 for parameter descriptions.

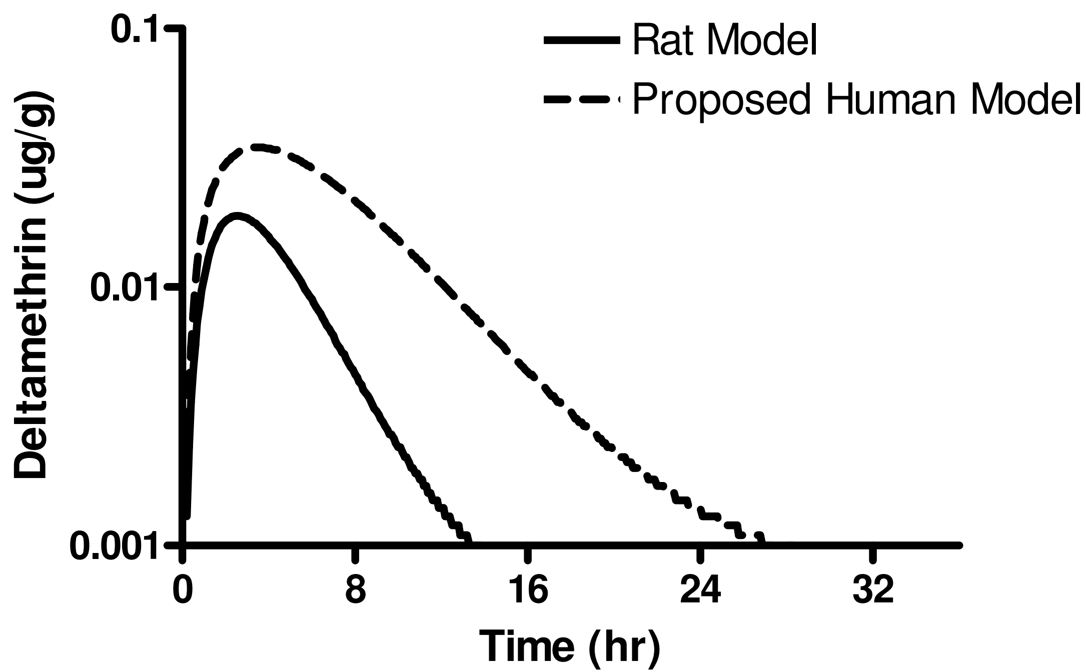


Figure 4.12. Model predictions of brain concentrations of deltamethrin from optimized diffusion limited rat model and proposed human model after a 1 mg/kg oral dose.

Modeling Equations

Oral Absorption and Fecal Excretion

Rate of mass input from intestines (mg/hr)
K_I*AI
AI = mass in intestines (mg)
Rate of fecal excretion (mg/hr)
K_{FE}*AI

iv Infusion

Rate of iv infusion (mg/hr)
RIC = ivDOSE*BW/TINF
ivDOSE = Mass dosed (mg/Kg)
TINF = Time of infusion (hr^{-1})

Blood Compartment

Rate of change in blood (mg/hr)
QL*CVL + QBr*CVBr + QF*CVF + QS*CVS + QR*CVR - QC*CA - RMBL + iv
Concentration in blood (mg/L)
CA = ABL/VBL
Rate of clearance from blood (mg/hr)
RMBL = (Kbl*CA)
Serum esterase clearance rate constant (L/hr)
Kbl = KblC*(VBL/2)
iv = Riv*Ivr
Ivr = iv dosing timing switch

Flow-Limited Compartments

Rate of blood:tissue exchange (mg/hr)
$Q_T*(CA-CV_T)$
Concentration in venous blood leaving tissue (mg/L)
$CV_T = C_T/P_T$
Concentration in tissue
$C_T = A_T/V_T$

Diffusion-limited tissues

Rate of change is extracellular tissue blood (mg/hr)
$Q_T*(CA-CV_T) + PA_T*(CI_T/P_T-CV_T)$
Concentration in venous blood leaving tissue
$CV_T = AE_T/(V_T*BV_T)$ (mg/L)
Rate of blood:tissue exchange (mg/hr)
$PA_T*(CV_T-CI_T/P_T)$
Concentration in intracellular tissue (mg/L)
$CI_T = AI_T/(V_T*(1-BV_T))$

Liver Compartment

Rate of Change in Liver blood

Q_L*(CA-CV_L) + PA_L*(CI_L/P_L-CV_L) + RAO

Rate of blood:liver exchange (mg/hr)

PA_L*(CV_L-CI_L/P_L)- RAM

Scaled intrinsic hepatic clearance

Cl_{int} = KCL*BW

Rate of metabolism in diffusion-limited liver

RAM = CL_{int}*CI_L

CHAPTER V

GENERAL DISCUSSION

Pyrethroid pesticides are currently undergoing evaluation for a potential cumulative risk assessment by the EPA Office of Pesticide Programs under the auspices of the Food Quality Protection Act of 1996. Risk assessment of environmental toxicants is a process that consists of numerous uncertainties. Typically, there is a lack of human data available for environmental contaminants; the case of the pyrethroids is no exception. This necessitates extrapolation of data from non-human species to humans. The extrapolation of toxicological data from laboratory animals to humans often simply relies on default safety assumptions in the absences of appropriate data. This dissertation has focused on developing appropriate data sets and models to address uncertainties in the description of pyrethroid pharmacokinetics.

SUMMARY OF RESEARCH RESULTS

The working hypothesis for this dissertation was that hepatic clearance of pyrethroids drives blood and brain concentrations of the pyrethroids influencing pyrethroid potency.

Metabolism of Deltamethrin and Esfenvalerate

The metabolic detoxification of pyrethroids has long been understood to take place through two general pathways, esterase-mediated hydrolysis and cytochrome P450 oxidation in the liver of laboratory animals (Abernathy and Casida 1973; Casida et al., 1975; Soderlund and Casida 1977). Identification of common metabolites between rats and humans (Cole et al. 1982; Heudorf and Angerer 2001) indicates that there are common pathways of metabolism for pyrethroids between species. However, till now there has been no data comparing the pathways and relative rates of metabolism of individual pyrethroids in laboratory species and humans.

Preliminary work for this dissertation revealed that hepatic metabolism of deltamethrin and esfenvalerate by rat and human microsomes differed in rate and/or pathway. Utilizing a parent depletion approach, deltamethrin and esfenvalerate were both metabolized by NADPH dependent oxidative metabolism in rat liver microsomes (RLM) at similar rates. Only minor amounts of NADPH independent hydrolysis of deltamethrin and esfenvalerate occurred in RLM (Table 2.2 and Figure 2.2). In contrast, in human liver microsomes (HLM) deltamethrin and esfenvalerate were metabolized primarily via different pathways. Esfenvalerate was metabolized via NADPH dependent oxidation in HLM, as in RLM; however, the rate of metabolic clearance was nearly three fold slower than in RLM (Table 2.1). Unexpectedly, deltamethrin was metabolized by NADPH independent hydrolysis in HLM nearly twice as rapidly as it is in RLM (Fig 2.3).

Deltamethrin is a cis-isomer pyrethroid. Cis-isomer pyrethroids are metabolized in rats and mice primarily by oxidative mechanisms (Shono et al. 1979; Soderlund and Casida 1977). Cis-isomer pyrethroids are hydrolyzed significantly slower by purified rat, mouse, and rabbit carboxylesterases compared to trans-isomer pyrethroids (Ross et al. 2006; Stok et al. 2004). In contrast, human carboxylesterase – 1 (hCE-1) rapidly metabolized deltamethrin (Table 2.3). The rate of deltamethrin hydrolysis by a rat carboxylesterase is similar to cis-permethrin and esfenvalerate while in humans its hydrolysis is similar to the trans-isomer pyrethroids trans-permethrin and bioresmethrin by hCE-1 (Figure 2.5).

The species difference in hydrolysis of deltamethrin as compared to esfenvalerate is in part the result of increased metabolism of deltamethrin by hCE-1 as compared to esfenvalerate (Fig 2.3). In addition comparison of hydrolysis of deltamethrin by hCE-1 and rat hydrolase A indicates that the human esterase has greater capacity to metabolize

deltamethrin (Table 2.4 and Fig 2.4). The differences in oxidative metabolism of esfenvalerate and deltamethrin between rats and humans however, appear to be simply due to differential expression of P450s, in rat and human liver microsomes, which metabolize the pyrethroids. Table 3.1 and Figures 3.1 and 3.2 shows that both deltamethrin and esfenvalerate are metabolized with similar K_m values and at similar rates by both rat and human P450s. The exception is that of human CYP2C9 which efficiently metabolizes esfenvalerate, but not deltamethrin. This likely explains the greater oxidative metabolism of esfenvalerate in human liver microsomes compared to deltamethrin. CYP2C9 is one of the most highly expressed CYPs in the human liver. Estimates of average individual CYP isozyme expression in human liver microsomes range from 1-100 pmoles P450/mg microsomal protein (Rodrigues, 1999). CYPs 3A4 and 2C9 are the most abundant of the human hepatic P450s expressed near 100 pmoles/mg microsomal protein (Rodrigues, 1999). The expression of pyrethroid metabolizing P450s in hepatic microsomes of rats however reveals that the expression of rat pyrethroid metabolizing P450s (CYPs 2C6, 2C11, and 3A2) ranges from 300 to >1000 pmoles P450/mg of microsomal protein (Guengerich 1982). This likely explains the differences in oxidative clearance of these pyrethroids between rats and humans. In addition comparison of the expression of hCE-1 to human P450s reveals that the expression of hCE-1 is approximately 12–1200-fold greater than the levels of individual CYPs in human liver microsomes (Godin et al 2007), describing why deltamethrin is so rapidly metabolized in HLM.

Extrahepatic metabolism of pyrethroids occurs in the blood, brain, lung and intestines of rats (Anand et al. 2006, Godin et al. 2007; Crow et al. 2007; Bruckner et al. 2007). Metabolism in the brain and lungs has been found to be minimal, requiring large quantities of

subcellular tissue in order to detect metabolism (Bruckner et al. 2007). Metabolism in these tissues would likely have no influence on local tissue concentrations and would have no impact on systemic clearance of the pyrethroids. Intestinal metabolism of the pyrethroids in rats appears to be chemical specific and dependent on the expression of intestinal esterases. Trans-permethrin is metabolized in rat intestinal microsomes however, no intestinal metabolism of deltamethrin occurred (Crow et al. 2007). This corresponds with the greater activity of rat esterases with trans-permethrin compared to deltamethrin (Godin et al. 2006; Ross et al. 2006). Based on similarities in deltamethrin and esfenvalerate hydrolysis by rat esterases this also indicates that esfenvalerate metabolism is unlikely in the intestines of rats as well (Godin et al. 2006; Ross et al. 2006). Human intestinal metabolism of pyrethroids is unlikely to influence disposition either. The major esterase in the human intestines is hCE-2 (Imai et al. 2005) which does not efficiently metabolize deltamethrin or esfenvalerate (Table 2.3) and corresponds to the lack of deltamethrin metabolism in human intestinal microsomes (Crow et al. 2007).

The blood of rats contains carboxylesterase that are capable of metabolizing pyrethroids (McCraken et al. 1993; Anand et al 2006). Deltamethrin is metabolized by rat serum and a purified rat serum carboxylesterase (Fig 3.5). The purified rat serum carboxylesterase metabolized deltamethrin with similar efficiency to the rat hepatic carboxylesterase, rat hydrolase A (Godin et al. 2006 and 2007). Hepatic concentrations of carboxylesterases are expected to be greater than serum carboxylesterase. Thus, serum metabolism was thought to be unlikely to influence systemic clearance. In contrast to rats, human serum does not contain carboxylesterases (Li et al. 2005) and does not metabolize deltamethrin (Godin et al. 2007).

The current findings regarding the metabolism of deltamethrin reveal that the vast majority of metabolism of deltamethrin is occurring in the liver of rats and humans. There are species difference in the pathways and rates of hepatic clearance in the liver of rats and humans that may influence systemic concentrations of deltamethrin. This could influence peak brain concentrations and therefore the relative potency of deltamethrin between rats and humans. Experimentally determined hepatic clearance rates were utilized in developing PBPK models in rats and humans to examine the impact of the species difference on exposure-dose relationships.

PBPK Modeling of Deltamethrin

To explore the impact of the species differences in the hepatic metabolism of deltamethrin on exposure-dose relationships, PBPK models were developed in the rat and extrapolated to humans. These models utilized species-specific physiological and metabolic parameters. To examine uncertainties in model structure, including absorption and model structure, oral bioavailability and time course studies were conducted.

A published PBPK model of deltamethrin utilized both flow- and diffusion-limited kinetics to describe the distribution of deltamethrin (Mirfazaelian et al. 2007). Initial modeling efforts utilized the model structure of Mirfazaelian et al. (2007), however, this model was unable to accurately describe the tissue concentration time course of deltamethrin from published data of Gray and Rickard (1982) (data not shown) or the data generated in chapter IV (Fig 4.3 - 4.10). A new model structure was therefore developed in which deltamethrin tissue kinetics were described by diffusion limitations in all tissue compartments. The model consists of tissue compartments for the brain (target tissue), liver

(metabolism), and fat, richly perfused and slowly perfused tissues (distribution). A single blood compartment connected each tissue compartment. These were the tissue compartments from the initial hypothesis driven model described in the general introduction.

Computationally derived tissue:blood partition coefficients were utilized to parameterize the model. Doing so allowed a hypothesis driven examination of deltamethrin tissue distribution based on expected tissue solubility. With the exception of the brain and the fat, the use of the computationally derived partition coefficients adequately described the distribution of deltamethrin. Computational determination of partition coefficients resulted in drastic overestimation of brain concentration. The brain partition coefficient had to be reduced by nearly 300-fold to accurately describe the data from the current study presented in chapter IV and that of Gray and Rickard (1982). This suggests that the BBB is having a significant influence on the distribution of deltamethrin into the brain. This may be due to simple diffusional limitations of the BBB or the interaction of deltamethrin with transporters on the endothelial lining of the BBB.

It is difficult to determine why discrepancies between the computationally derived fat partition coefficient and the optimized value necessary to fit the available fat data exist. The computational value appears to fit peak fat concentrations of deltamethrin out through 48 hrs (Fig 4.7) and allows accurate estimation of other tissue concentration profiles (Fig 4.3 - 4.9) because this occurs within the first 48hrs after exposure. The computational value, however, predicts that the level of deltamethrin in the fat remains elevated for considerably longer than literature data suggests. This may be due to minor amounts of metabolism in the fat, or possibly over the extended fat time course in the studies of Mirfazaelian et al (2006) and Ruzo et al. (1978) non-enzymatic degradation of deltamethrin occurs in the fat. Stability of

the pyrethroids in fat at physiological temperatures over the course of days or weeks is unknown. It is possible that the computational predictions are correct and elimination from the fat is occurring by a yet to be described processes. Further data sets on the stability of deltamethrin in the fat may result in a better understanding of this discrepancy between the computational and optimized fat partition coefficients.

The diffusion-limited model was able to more accurately describe the tissue distribution of deltamethrin for data sets from the current work, the work of Gray and Rickard (1982), Ruzo et al. (1978) and the work of Mirfazalein et al. (2007) (Fig 4.3 – 4.10) as compared to the published model structure of Mirfazalein et al. (2007). The modeling of deltamethrin by diffusion-limited kinetics appears to minimize the impact that the rate of hepatic clearance, and species differences in the metabolism of deltamethrin, have on peak brain concentration. This infers that our initial hypothesis was incorrect and that hepatic clearance does not significantly influence brain concentrations. These results imply that diffusion of deltamethrin into the liver and brain influence blood concentrations and ultimately influences the potency of individual pyrethroids.

SIGNIFICANCE OF RESEARCH RESULTS

Implications for Species Extrapolation

The results presented in Chapters II and III regarding the metabolism of deltamethrin and esfenvalerate are the first published reports comparing the metabolism of pyrethroids in laboratory animals and humans. These results identified species differences in the metabolism of pyrethroids that were previously unknown. These studies are also the first published studies to identify the major individual enzymes that are responsible for the

metabolism of pyrethroids. The result of which was identification of determinants of the major pathway a pyrethroid is likely to be metabolize by in rats and humans.

Clearance rates from the metabolism studies were utilized to develop PBPK models of exposure to deltamethrin. This allowed an extrapolation of the impact that the rate of hepatic clearance of deltamethrin has on exposure-dose relationships and relative potencies between rats and humans. The description of a PBPK models' kinetic structure can have a significant impact on predictions of exposure-dose relationships for pyrethroids. The previous model structure of Mirfazalien et al (2006) utilized a flow-limited description of the liver and brain compartments. The result of which is that the rate of hepatic clearance would have a significant impact on peak tissue concentrations in the blood and brain (see sensitivity analysis in Mirfazalein et al. 2006). The model presented in Chapter IV finds that a diffusion-limited description of these tissues may be more appropriate. This would have the effect of minimizing the impact of the species difference in the hepatic clearance of deltamethrin. In the diffusion-limited model, the rate of deltamethrin metabolism is limited by the diffusion into the liver.

Until now, it had been thought that the rate of hepatic clearance of pyrethroids significantly influenced the potency of individual pyrethroids, especially between cis- and trans-isomers of some pyrethroids. It was unclear how this related to individual chemicals. The diffusion-limited descriptions of the liver and the brain for deltamethrin in chapter IV result in the permeability of the pyrethroid into these tissues having a greater impact on peak brain concentrations than the rate of hepatic clearance (see sensitivity analysis Fig 4.11). This does not eliminate the pharmacokinetic influence on pyrethroid potency it simply alters how we might think of what impacts it. Due to the diffusion-limited description of tissue

compartments and the lipohilicity of deltamethrin, changes in metabolism, decreases for example, are going to result in greater distribution of deltamethrin. Based on the size and partition coefficients' of tissue compartments the majority of deltamethrin that is not metabolized will distribute to the fat and slowly perfused tissues resulting in comparatively small increases in brain concentrations (due to BBB influences on brain penetration).

The no observable adverse effect level (NOAEL) for deltamethrin is 1mg/kg after oral exposure in the rat (Crofton et al. 1995). This has been extrapolated by the EPA to a human reference dose (RfD) of 0.01mg/Kg/day by dividing by a 100-fold uncertainty factor (Federal Register 2004). That uncertainty factor can be broken into factors of 10 for species extrapolation and inter-individual variability. Based on a human model extrapolated from the rat PBPK model developed here, and a comparison of a 1mg/Kg oral dose, humans would be slightly more sensitive to deltamethrin exposure based on target tissue (brain) concentrations (Fig 4.12). Peak brain concentration is 1.8-fold higher in the human model than in the rat model and remains elevated for a longer period. This is likely due in large part to physiological differences between the brain of rats and humans. The human brain has significantly greater blood flow and volume compared to the rat (Table 4.1). These physiological differences may make humans more sensitive to neurotoxicants in general due to increased exposure to the brain. Thus even though humans metabolize deltamethrin more rapidly they appear as though they are more sensitive based on this analysis. In addition, modeling of deltamethrin with diffusion-limited kinetics reduces the impact that genetic polymorphisms or mutations, which can influence the activity of metabolizing enzymes, will have on pyrethroid potency. It would also be less likely that metabolic interactions during exposure to mixtures of pyrethroids would influence systemic clearance.

Modeling deltamethrin with diffusion-limited kinetics is a more health conservative/protective approach compared to utilizing flow-limited kinetics in which the more rapid rate of intrinsic hepatic clearance in humans for deltamethrin would have a more significant impact on brain concentrations. The 10-fold uncertainty factor for species extrapolation can also be further broken down. Renwick (1993) and the world health organization (WHO 1994) suggest a pharmacokinetic component of 4.0 and a pharmacodynamic component of 2.5. The EPA utilizes a factor of 3 for pharmacokinetics (Barnes and Dourson 1988). Based on the current analysis, humans would be nearly two fold more sensitive to a rat NOAEL dose of 1mg/kg (Chapter IV) on a brain concentration basis. This is in good agreement with a suggested three or four-fold uncertainty factor for pharmacokinetic differences between species.

FUTURE STUDIES

To best aid the risk assessment process for the pyrethroids, future studies should focus on modeling of other pyrethroids and developing a better understanding of determinants of brain distribution profiles for pyrethroids.

Development of PBPK models for other pyrethroids will help to validate the diffusion-limited structure of the deltamethrin PBPK model presented in this dissertation. Further modeling may also help to validate the use of computationally derived partition coefficients, which appear to have worked well in the current model with the exception of the brain. Discrepancy in computational and optimized partitioning into fat is also an area of continued uncertainty; however, the computational value only required a 4-fold reduction to fit the available data. This was also only required to model the later fat time points out to 500 hrs and did not influence the description of other tissue concentrations in the important

first 24 hrs after exposure. Discrepancy between the computational and optimized values could be due to a small amount of metabolism in the fat or potentially due to non-enzymatic degradation of deltamethrin in the fat over time. These are factors that could be examined in an *in vitro* setting and results could be applied to the model to increase confidence in the description of the fat compartment.

The brain, in which the partition coefficient had to be reduced nearly 300 fold to describe tissue concentrations, is of much more interest from a risk assessment standpoint because it is the target tissue for the neurotoxicity of pyrethroids. The drastic difference in brain partitioning of deltamethrin could be the result simply of the blood brain barrier reducing exposure to the brain through inhibition of diffusion. The basic cell membrane structure is the same in all tissues of the body and simple diffusion through the membrane should be similar in all tissues. Partitioning of a chemical is then determined by the affinity or solubility of the chemical in the composition of individual tissues. However, the capillary membrane of the blood brain barrier contains tighter junctions than those of other tissues. This acts to reduce the extravascular exposure of the brain to xenobiotics in the blood. Another possibility is that deltamethrin distribution into the brain is regulated by some yet to be described process.

In recent years, the influence of transporters on the distribution of xenobiotics into the brain has come into focus. Could export transporters be the reason for the lower than expected levels of deltamethrin in the brain? Could there be a correlation between the rate of transport and the rate of metabolism? Would this explain why it has been thought that the rate of metabolism correlated with potency? Would a structure activity relationship in transport be similar to those of metabolism for the pyrethroids? Gray et al. (1980) and Gray

and Rickard (1981) observed differences in brain distribution of cismethrin and deltamethrin compared to their distribution in other tissues. In those studies, based on equitoxic doses of deltamethrin and cismethrin, tissue concentration ratios were proportional to the dose ratios between chemicals in all tissues except in brain. This indicates that differences in metabolism are not affecting distribution into the brain. An unidentified factor however does appear to be influencing distribution into the brain. Does the difference in the tissue distribution ratios in the brain equate to differences in transport or simple differences in the thermodynamic properties of each chemical structure resulting in different diffusional characteristics. Deltamethrin is a larger more polar molecule compared to cismethrin. This does not however appear to affect their diffusion into other tissues of the body however as stated the BBB is different from capillary membranes in other tissues. So could transport explain these differences?

There is some circumstantial evidence to suggest that the pyrethroids may interact with the multidrug resistance protein, P-glycoprotein (P-gp). Lemaire et al. (2004) studied induction of P450s via activation of the human pregnane X receptor (PXR) by a number of pesticides including pyrethroids. They found that pyrethroids are capable of activating PXR. PXR is also involved in the regulation of P-gp (Geick et al 2001). P-gp is not only found in mammals but also insects and recently it has been found that some pesticide (including pyrethroid) resistant insect strains have significantly increased P-gp activity compared to non-resistant strains (Srinvas et al. 2005). Does this indicate that P-gp may have a significant role in the potency of pyrethroids in both insects and mammals? In another study however Bain and LeBlanc (1996) studied the interaction of a number of different pesticides with P-gp. Included in their studies were three pyrethroids. Their findings indicated that there was

very little interaction of the pyrethroids studied (esfenvalerate, fluvalinate and permethrin) with P-gp.

Another implication of P-gp transport of pyrethroids would be on absorption after an oral dose. P-gp is located not only in the brain endothelium of the BBB, but is also located in the liver and intestinal tract (Mei et al. 2004). Does P-gp play a role in the fecal excretion of the pyrethroids? The apparent lack of dose dependent absorption of deltamethrin in the bioavailability study in chapter IV and literature reports do not appear to suggest that intestinal transport is occurring.

These questions regarding pyrethroid transport if examined could aid the understanding of pyrethroid potency and aid extrapolation of exposure-dose-response relationships in risk assessment. If transporters are interacting with deltamethrin this could be utilized in the PBPK model to better explain distribution into the brain. If transporters are not involved in pyrethroid transport it likely indicates that pyrethroid absorption is mediated by solubility and vehicle dynamics while brain concentrations are largely dependent on the ability of each pyrethroid to cross the BBB. Other studies in our laboratory (unpublished) indicate that there are significant differences in brain permeability between pyrethroids that are not indicative of metabolic differences. In vitro examination of BBB permeability of pyrethroids would likely be highly useful in understanding the distribution of pyrethroids into the brain.

CONTRIBUTION TO THE FIELD OF PYRETHROID PHARMACOKINETICS

In summary, this dissertation has yielded a further understanding of the metabolism of pyrethroids in rats and humans. We have identified the enzymes that are responsible for the metabolism of pyrethroids in both rats and humans and pointed to likely determinants of

the pathway that will metabolize a pyrethroid in each species. We have also developed a refined PBPK model for deltamethrin, which will enable development and comparison to models for other pyrethroids. This will allow a determination of whether a common model structure can be applied to all pyrethroids. We have pointed out that the distribution of deltamethrin into the brain does not appear to be mediated solely by its concentration in the blood and that uncertainties remain regarding its distribution into and out of the brain. If correct this PBPK model structure also indicates that the rate of hepatic metabolism of the pyrethroids does not have as significant an effect on pyrethroid potency as has been thought. In addition, these results if validated may lead to the development of future pyrethroids designed to limit their brain distribution minimizing their mammalian toxicity while maintaining their insecticidal activity.

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