CHARACTERIZATION OF DISPOSITION OF DELTAMETHRIN, CIS-PERMETHRIN AND TRANS-PERMETHRIN AS A FUNCTION OF AGE IN SPRAGUE-DAWLEY RATS

by

MANOJ AMARANENI

(Under the Direction of James V. Bruckner)

ABSTRACT

Although the blood and tissue partition coefficients (PCs) for pyrethroids in adult rats have previously been published, no one has evaluated the influence of maturation on the systemic disposition of pyrethroids in rats. This series of studies addressed this deficit by testing the hypothesis that the distribution of pyrethroids in immature rats will be significantly different from that in adults. Steady-state levels of deltamethrin (DLM), cis-permethrin (CIS) and trans-permethrin (TRANS) were obtained using subcutaneously implanted Alzet pumps® in Sprague-Dawley (SD) rats. For all three compounds, fat exhibited far higher PC values than brain, liver and skeletal muscle in adult rats. Brain and liver exhibited the lowest PCs followed by skeletal muscle. For all three compounds, the tissue:plasma PCs in PND 15 pups were found to be significantly higher than in adult rats. Alternatively, there was no distinct pattern, in the differences in tissue:plasma PCs between PND 21 and adult rats.

An in situ brain perfusion technique was utilized to selectively investigate the role of plasma binding, membrane transporters and the blood brain barrier (BBB) on the
brain uptake of DLM. The data suggested that under normal physiological conditions, plasma binding along with the BBB plays a protective role in restricting the entry of DLM into the brain. At physiological albumin concentrations and low exposure levels, a passive, non-saturable transport of DLM predominates at the BBB. Brain uptake of DLM was determined as a function of age-dependent changes in plasma protein binding and the BBB integrity. Adult brain uptake of DLM from pediatric and adult rat and human plasmas did not vary significantly. When measured as a function of the BBB integrity, uptake in PND 15 and PND 21 pups was significantly greater than in adults.

The data presented here demonstrate that the steady-state tissue:plasma PCs for pyrethroids were inversely proportional to age. These differences become more pronounced as the age diminishes. At low exposure levels of DLM, ontogenic differences in plasma binding do not affect its brain uptake. However, increased brain entry of DLM in younger rats results from increased permeability of the BBB in these age groups.

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DEDICATION

I would like to dedicate my dissertation to my parents and my wife who gave me strength and faith to succeed.
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>x</td>
</tr>
<tr>
<td>ABBREVIATIONS</td>
<td>xii</td>
</tr>
<tr>
<td><strong>CHAPTER</strong></td>
<td></td>
</tr>
<tr>
<td>1. INTRODUCTION AND LITERATURE REVIEW</td>
<td>1</td>
</tr>
<tr>
<td>2. INFLUENCE OF MATURATION ON IN VIVO TISSUE TO PLASMA PARTITION COEFFICIENTS OF DELTAMETHRIN, CIS- AND TRANS-PERMETHRIN IN SPRAGUE-DAWLEY RATS</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>Abstract</td>
</tr>
<tr>
<td></td>
<td>Introduction</td>
</tr>
<tr>
<td></td>
<td>Materials and Methods</td>
</tr>
<tr>
<td></td>
<td>Results</td>
</tr>
<tr>
<td></td>
<td>Discussion</td>
</tr>
<tr>
<td></td>
<td>References</td>
</tr>
<tr>
<td>3. EFFECTS OF PLASMA PROTEIN BINDING AND MEMBRANE TRANSPORTERS ON THE BLOOD BRAIN BARRIER PERMEABILITY OF DELTAMETHRIN</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>Abstract</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
</tr>
<tr>
<td>----------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Introduction</td>
<td>90</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>92</td>
</tr>
<tr>
<td>Results</td>
<td>95</td>
</tr>
<tr>
<td>Discussion</td>
<td>95</td>
</tr>
<tr>
<td>References</td>
<td>101</td>
</tr>
<tr>
<td>4. BRAIN UPTAKE OF DELTAMETHRIN IN SPRAGUE DAWLEY RATS AS A FUNCTION OF AGE DEPENDENT DIFFERENCES IN PLASMA PROTEIN BINDING AND BBB PENETRATION</td>
<td>109</td>
</tr>
<tr>
<td>Abstract</td>
<td>110</td>
</tr>
<tr>
<td>Introduction</td>
<td>112</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>115</td>
</tr>
<tr>
<td>Results</td>
<td>121</td>
</tr>
<tr>
<td>Discussion</td>
<td>124</td>
</tr>
<tr>
<td>References</td>
<td>134</td>
</tr>
<tr>
<td>5. SUMMARY</td>
<td>161</td>
</tr>
<tr>
<td>APPENDIX</td>
<td>169</td>
</tr>
</tbody>
</table>
LIST OF TABLES

Table 2.1: *In Vitro* Rat Plasma to Red Blood Cell (RBC) Partition Coefficients for DLM (A), CIS (B) and TRANS (C)…………………………………………………………………………69

Table 2.2: DLM (A), CIS (B) and TRANS (C) Mean Concentrations and Tissue:Plasma Partition Coefficients for Adult Rat Tissues After 72-h Infusion…………………..71

Table 2.3: Comparison of DLM (A), CIS (B) and TRANS (C) Tissue:Plasma Partition Coefficients with those Previously Published for Adult Rats…………………………72

Table 2.4: DLM (A), CIS (B) and TRANS (C) Mean Concentrations and Tissue:Plasma Partition Coefficients for 21-day-old Rats After 48- and 72-h Infusions………………75

Table 2.5: DLM (A), CIS (B) and TRANS (C) Mean Concentrations and Tissue:Plasma Partition Coefficients for 15-day-old Rats After 48- and 72-h Infusions………………78

Table 2.6: Comparison of DLM (A), CIS (B) and TRANS (C) Steady-State Tissue:Plasma Partition Coefficients for Adults, 21-day, and 15-day-old Rats with those Estimated from Single Dose Toxicokinetic Studies……………………………………81

Table 4.1: Relative Increase in the Brain Uptake of Different Concentrations of DLM in PND 15 vs PND 21 vs Adults……………………………………………………………………158
**LIST OF FIGURES**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.1</td>
<td>Structure of Deltamethrin and Permethrin</td>
<td>31</td>
</tr>
<tr>
<td>Figure 1.2</td>
<td>Effect of Pyrethroids on Voltage Sensitive Sodium Channels in the CNS</td>
<td>32</td>
</tr>
<tr>
<td>Figure 1.3</td>
<td>General Metabolic Pathway of Pyrethroid Insecticides</td>
<td>33</td>
</tr>
<tr>
<td>Figure 1.4</td>
<td>Different Means of Transport Across the BBB</td>
<td>34</td>
</tr>
<tr>
<td>Figure 2.1</td>
<td>Experimental Approach for <em>In vivo</em> Tissue:Plasma PC Experiment</td>
<td>84</td>
</tr>
<tr>
<td>Figure 2.2</td>
<td>Comparison of Steady-State Tissue:Plasma PCs for DLM between PND 15, PND 21 and Adult Rats after 72-h Infusion</td>
<td>85</td>
</tr>
<tr>
<td>Figure 2.3</td>
<td>Comparison of Steady-State Tissue:Plasma PCs for CIS between PND 15, PND 21 and Adult Rats after 72-h Infusion</td>
<td>86</td>
</tr>
<tr>
<td>Figure 2.4</td>
<td>Comparison of Steady-State Tissue:Plasma PCs for TRANS between PND 15, PND 21 and Adult Rats after 72-h Infusion</td>
<td>87</td>
</tr>
<tr>
<td>Figure 3.1</td>
<td>Effect of HSA and CSA on the <em>In vivo</em> Brain Uptake of DLM</td>
<td>107</td>
</tr>
<tr>
<td>Figure 3.2</td>
<td>Effect of Mannitol on the <em>In vivo</em> Brain Association of DLM With and Without 4% HSA</td>
<td>108</td>
</tr>
<tr>
<td>Figure 4.1</td>
<td>Effect of Time of Incubation on <em>In vitro</em> Binding of DLM to Human Plasma</td>
<td>144</td>
</tr>
<tr>
<td>Figure 4.2</td>
<td>Effect of Time of Incubation on <em>In vitro</em> Binding of DLM to HSA</td>
<td>145</td>
</tr>
<tr>
<td>Figure 4.3</td>
<td>Effect of Time of Incubation on <em>In vitro</em> Binding of DLM to LDL</td>
<td>146</td>
</tr>
</tbody>
</table>
Figure 4.4: Effect of Time of Incubation on *In vitro* Binding of DLM to HDL...........147

Figure 4.5: Effect of Time of Incubation on *In vitro* Binding of DLM to Mixture of LDL and HDL..........................................................148

Figure 4.6: Effect of Binding to Physiological HSA Concentrations on the *In vivo* Brain Uptake of DLM..........................................................149

Figure 4.7: *In vitro* Binding of 1 μM $^{14}$C-DLM to Different Concentrations of HSA.....150

Figure 4.8: Effect of Binding to Physiological LDL & HDL Concentrations on the *In vivo* Brain Uptake of DLM..........................................................151

Figure 4.9: *In vitro* Binding of 1 μm $^{14}$C-DLM to Physiological Concentrations of LDL, HDL, and their Mixture..........................................................152

Figure 4.10: Effect of Binding to Lower, Normal and Higher Physiological LDL Concentrations on the *In vivo* Brain Uptake of DLM..........................................................153

Figure 4.11: *In vitro* Binding of 1 μM $^{14}$C-DLM to Different Concentrations of LDL.....154

Figure 4.12: Effect of Binding to Rat Plasma of Different Age Groups on *In vivo* Brain Uptake of DLM..........................................................155

Figure 4.13: Effect of Binding to Human Plasma of Different Age Groups on *In vivo* Brain Uptake of DLM..........................................................156

Figure 4.14: Effect of Maturity of BBB on *In vivo* Brain Uptake of DLM.................157

Figure 4.15: Concentration Dependent Uptake of DLM in PND 15, PND 21, and Adult SD Rats..........................................................159

Figure 4.16: Effect of Flow Rate on *In vivo* Brain Uptake of DLM.........................160
ABBREVIATIONS

3-PBA = 3-phenoxy benzoic acid
ABC = ATP-binding cassette
ANOVA = Analysis of Variance
AUC = Area under the Curve
BBB = Blood Brain Barrier
CEs = carboxylesterases
CIS = cis-permethrin
CNS = Central Nervous System
DLM = deltamethrin
\( f_u \) = fraction unbound
hCEs = human carboxylesterases
hCMEC = human cerebral microvessel endothelial cells
HDL = high density lipoprotein
HSA = human serum albumin
\( K_o \) = infusion rate
LD = loading dose
LDL = low density lipoprotein
Log P/Log \( K_{o/w} \) = octanol to water partition coefficient
OAT = Organic Anion Transporters
OATP = Organic Anion Transporting Polypeptide
OCT = Organic Cation Transporters
PBPK = physiologically based pharmacokinetic
PC = partition coefficient
PER = permethrin
P-gp = p-glycoprotein
PND = post-natal day
RBC = red blood cells
SC = subcutaneous
SD = Sprague-Dawley
TRANS = trans-permethrin
$t_{1/2} = \text{half-life}$
CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Background

Pyrethrum is a natural tan-colored syrupy extract with well-known insecticidal properties produced from the flowers of *Chrysanthemum cinerariaefolium* (Seed, 1973; Todd *et al.*, 2003). Pyrethrins are the bioactive compounds of pyrethrum. The use of pyrethrins in the United States dates back to the 1860’s (Casida, 1980; Schofield and Crisafulli, 1980) even though they were first registered for use as insecticides in the 1950’s (Agency., 2006). Isobutenyl, furan, and allyl substituents of pyrethrins are highly susceptible to photooxidation, making them not persistent enough for agricultural use (Casida, 1980). Since pyrethrins break down easily when exposed to sunlight, pyrethroids, which are synthetic analogs of pyrethrins were developed (Casida, 1980; Soderlund *et al.*, 2002). Combined with increased photostability and enhanced insecticidal potency, pyrethroids are far more effective in their agricultural and household usage compared to pyrethrins. Over the last decade, the use of pyrethroids has dramatically increased in the U.S., Canada, and the European Union, due to the U.S. EPA’s restrictions on use of organophosphates and due to their low environmental persistence and relatively lower mammalian toxicity (Power and Sudakin, 2007; Williams *et al.*, 2008). By the mid-1990’s, 23% of the worldwide insecticide market was pyrethroids (Casida and Quistad, 1998). Pyrethroids are often used in agricultural,
veterinary, medical and household settings (Soderlund et al., 2002). They are applied to a variety of crops for pest control and are also used to control vectors thereby promoting public health. In household settings, they are often used as knockdown agents to control houseflies and are also used in food storage areas. Medically, they are used for the topical treatment of scabies, mites and headlice infestations of pets and humans. Deltamethrin (DLM) is used to control mosquitoes in some tropical countries (Bradberry et al., 2005).

Human exposure to pyrethroids albeit at very low levels, can occur through a variety of routes like eating foods containing residues of pyrethroids, inhalation, skin contact and accidental ingestion of pyrethroid products (Baker et al., 2000; Schettgen et al., 2002). Pregnant women, infants, and children are widely exposed to pyrethroids (Schettgen et al., 2002; Whyatt et al., 2002). Pyrethroid metabolites were found in 75% of urine samples from a large German population without occupational exposure to the insecticide (Heudorf et al., 2004). Infants and children are more frequently exposed to pyrethroids compared to adults, due to their higher hand to mouth activity resulting in ingestion of dust or soil contaminated with pyrethroids.

**Structure of Pyrethroids**

Pyrethroids are obtained from the structural modifications of pyrethrins, which are esters of cyclopropanecarboxylic acid and a cyclopentenolone alcohol (Soderlund et al., 2002). Structural modifications were done either on the acid or alcohol moiety to enhance the photostability without the loss of insecticidal activity. Such modifications include the replacement of chlorines for methyl groups in the acid moiety and 3-
phenoxybenzyl for cyclopentenolone ring in the alcohol moiety (Soderlund et al., 2002). These modifications significantly increased the photostability without the loss of insecticidal activity. Pyrethroids were classified as Type I and Type II pyrethroids based upon their chemical structure and toxic signs. Type II pyrethroids differ from Type I pyrethroids in the presence of cyano substituent on the 3-phenoxybenzyl alcohol moiety (Figure 1.1). Type II pyrethroids have enhanced insecticidal potency due to the presence of the α-cyano substituent. Allethrin, permethrin, tetramethrin, and bifenthrin are examples of Type I pyrethroids. Deltamethrin, cypermethrin, and esfenvalerate are examples of Type II pyrethroids (Todd et al., 2003). Sixteen pyrethroids are registered for use in the U.S. in agricultural and consumer products (Bryant and Bite, 2003).

Pyrethroids are highly lipophilic compounds and thus have very low water solubility. Their log K<sub>o/w</sub> values are in the range of 5.7 - 7.6 (Laskowski, 2002). Due to their high octanol-water partition coefficients, pyrethroids partition into lipids and organic matter and bind with high affinity to sediments. Pyrethroids are highly soluble in organic solvents such as alcohol, chlorinated hydrocarbons, and kerosene (Todd et al., 2003).

**Mechanism of Action**

The principal site of action of pyrethroids is the voltage-sensitive sodium channels in the central nervous system (Figure 1.2). Pyrethroids exert neurotoxicity in mammals by delaying the closure of sodium channel gates, which results in repetitive discharge of nerve signals and eventually paralysis (Eells et al., 1992; Soderlund and Bloomquist, 1989; Soderlund et al., 2002). Type I and II pyrethroids differ in the prolongation of channel open times which contributes to the differences in the clinical manifestations.
after acute exposure (Ray, 2001). The duration of modification of sodium currents by type I pyrethroids lasts for a few milliseconds resulting in repetitive nerve discharges, whereas that of type II pyrethroids, lasts for several seconds resulting in stimulus-dependent nerve depolarization and blockage (Davies et al., 2007; Shafer et al., 2005; Vijverberg and Bercken, 1982).

Acute exposure of laboratory animals to high doses of pyrethroids results in two distinct toxic syndromes. Type I pyrethroids produce aggressive sparring, hyperexcitation, fine tremors progressing to whole-body tremor, and paresthesia, typically named as “T-syndrome”. Alternatively, Type II pyrethroids produce toxic signs such as excessive salivation, pawing and burrowing, coarse tremors progressing to choreoathetosis, and clonic seizures, collectively referred to as “CS-syndrome” (Lawrence and Casida, 1982; Ray et al., 2000; Shafer et al., 2005). In general, mammals are less susceptible to acute toxicity of pyrethroids due to their large body size, poor dermal absorption, and rapid detoxification mechanisms (Bradberry et al., 2005; Walters et al., 2009). The acute oral LD$_{50}$ for deltamethrin given in corn oil was reported to be 87 mg/kg in male rats (Myer, 1989), whereas for permethrin (45/55 cis/trans), it was reported to be 584 mg/kg (Metker et al., 1978). The average daily intake of permethrin by a 70 kilogram adult male is estimated to be about 3.2 micrograms per day (Todd et al., 2003).

**Metabolism of Pyrethroids**

Pyrethroids are metabolized primarily via two pathways: hydrolysis of the central ester bond by carboxylesterases; and oxidation at one or more sites in the acid or
alcohol moieties by cytochrome P450-dependent monooxygenases (Soderlund et al., 2002). These initial hydrolytic and oxidative products undergo conjugation with glucuronides, sulfates, or amino acids prior to excretion (Figure 1.3). CYP 2C6 and 2C11 in rats and CYP 2C9 and 3A4 in humans are the major CYP 450 isoforms that are involved in the oxidation of several pyrethroids (Godin et al., 2007; Scollon et al., 2009). Similarly, human carboxylesterase 1 (hCE1) and hydrolase A are the predominant carboxylesterases (CEs) that are involved in the hydrolysis of pyrethroids in humans and rats, respectively. Significant quantities of CEs are present in the serum of rats, whereas human serum is reported to have no esterase activity (Li et al., 2005). Therefore, blood may be an important tissue for hydrolysis of pyrethroids in rats, but not in humans (Godin et al., 2007). Immaturity of these metabolic enzymes is believed to contribute significantly to increased systemic exposure and neurotoxic effects of pyrethroids in younger age groups. Post-natal day 10 (PND 10) rats were more susceptible to neurotoxicity of DLM than PND 40 rats, due to limited metabolism in PND 10 rats (Anand et al., 2006). DLM and cis-permethrin (CIS) are primarily metabolized by oxidative pathways, whereas trans-permethrin (TRANS) is primarily metabolized by esterase mediated hydrolysis (Anand et al., 2006; Scollon et al., 2009). Usually, the trans-isomer of pyrethroids is more rapidly hydrolyzed than the cis-isomer (Yang et al., 2009). The presence of the cyano group on the alcohol moiety of type II pyrethroids makes these pyrethroids less prone to ester hydrolysis and P450 oxidation (Anand et al., 2006).
**Tissue:plasma Partition Coefficients**

Once a chemical is introduced into the body, the extent of partitioning into different tissues determines its fate and plays an important role in assessing its toxicological potential. The tissue:plasma partition coefficient (PC) is an important chemical-specific input parameter for physiologically based pharmacokinetic (PBPK) models which is used to predict the uptake and distribution kinetics of a chemical (Peyret *et al.*, 2010). Accurate tissue:plasma PCs help describe the distribution of a test compound in key tissues of the body. A PC can be defined as the ratio of the concentration of a chemical in two phases (i.e., tissue and plasma), once it reaches equilibrium. The systemic deposition of lipid-soluble chemicals is largely dependent upon their solubility in different organs (Poulin and Krishnan, 1995). It follows that these compounds will partition most readily into tissues with the highest lipid content (Parham *et al.*, 1997). Thus, a compound’s octanol:water PC is an important property used in computational approaches to estimate tissue:plasma PCs (Benfenati *et al.*, 2003; Basak *et al.*, 2004; DeJongh *et al.*, 1997; Poulin and Krishnan, 1995). Krishnan and his colleagues observed that tissue deposition of lipid soluble compounds is complex and subsequently took binding to blood proteins into account in their calculations to obtain more reliable results. This strategy, coupled with data on tissues’ neutral and phospholipid content, was utilized by Parham et al. to estimate tissue:blood PCs for all 209 polychlorinated biphenyl (PCB) congeners (Parham *et al.*, 1997). An algorithm was recently developed, which took into account deposition in neutral lipids and binding to plasma proteins, lipoproteins and hemoglobin, but not tissue proteins (Peyret *et al.*, 2010).
Tissue:plasma PCs may also be derived by direct measurement in vitro. PCs for the PBPK model for triadimefon (log $K_{ow}=3.2$) were obtained by measuring levels of the conazole fungicide in saline and the tissue fraction of a spiked homogenate (Crowell et al., 2011). Tissue:blood PCs were calculated by dividing the tissue:saline PCs by the blood:saline PC. A similar approach was employed involving ultrafiltration for pesticides. Pesticides with a log $K_{ow}\geq1.7$ could not be analyzed, as they were retained by the filtration units tested. A solid phase micro extraction (SPME) method was utilized for isolation of more lipophilic pesticides, but tissue:plasma PCs could not be determined for those with a log $K_{ow}\geq4$, due to excessive partitioning into the SPME fiber (Tremblay et al., 2012). The log $K_{ow}$ values for DLM and permethrin are 6.1 and 6.5, respectively (Todd et al., 2003). Rat tissue:blood PCs have been measured for a series of very lipophilic n-alkanes (Smith et al., 2005). The log $K_{ow}$ of dodecane (n-C$_{12}$), the longest-chain hydrocarbon measured was 6.1. The vial equilibration technique used, however, is dependent upon volatilization of the analyte. Pyrethroids’ volatility is insufficient for such vapor analyses.

Tissue:plasma PCs have been determined for lipid-soluble chemicals in several instances by direct measurement in vivo. It is common for PBPK modelers to utilize organ and plasma concentration data reported by other researchers to calculate PCs. This practice was used for construction of models for PCBs (Lutz et al., 1977), lindane (DeJongh and Blaauwboer, 1997), chlordecone (Belfiore et al., 2007), and polychlorinated dibenzo-p-dioxins and --furans (PCDD/Fs) (Maruyama et al., 2002).

Tissue:plasma PCs for pyrethroid PBPK models developed to date have not relied on steady-state measurements. A PBPK model for DLM developed by
Mirfazaelian et al. utilized time-course data from oral studies in rats. Tissue:plasma PCs were calculated as the ratio of the area under the tissue DLM concentration versus time curve to the plasma area under the curve (AUC) (Mirfazaelian et al., 2006). Another PBPK model for DLM developed by Godin et al. (Godin et al., 2010) used the computational procedure of Poulin and Theil (Poulin and Theil, 2000) to calculate PCs. This procedure was based on tissue composition and described DLM’s expected solubility in each tissue compartment. Similar to the approach of Mirfazaelian et al. approach, the only PBPK model for permethrin developed to date has utilized time-course data from oral studies in rats to estimate tissue:plasma PCs (Tornero-Velez et al., 2012). However, they adopted published PCs for deltamethrin (DLM), adapting some to fit their experimental kinetic data for CIS.

**Age-Related Differences in Disposition**

It is generally believed that infants and children are more susceptible to the toxic effects of pesticides than adults. Newborns undergo significant growth and maturational changes in physiological and biochemical processes. These changes have the potential to significantly affect the disposition of chemicals and thus their tissue dosimetry. The younger and more immature the subject is, the more different their pharmacokinetic processes are from that of an adult (Bruckner, 2000). Developmental changes in the gastro-intestinal (GI) tract such as changes in secretions, motility, metabolism and transport can influence the rate and extent of absorption of a chemical and thus its bioavailability (Strolin Benedetti et al., 2005). Maturational changes in membrane permeability, plasma protein binding, cardiac output, and body content of water and lipids may affect the distribution of a chemical in developing infants (Alcorn
and McNamara, 2003). In general, immature neonates exhibit high membrane permeability compared to adults. Plasma protein binding is lower in newborns and young infants (Renwick, 1998). Factors such as lower plasma protein concentrations, lower binding affinity and availability of competing endogenous substrates will result in decreased binding in neonates. This results in an increased free fraction of the chemical, thereby increasing its distribution into tissues. Newborns have higher total body water (80-90%) and lower fat content (10-15%) compared to older children and adults (Barrett et al., 2012; Lentner, 1981). Thus, volume of distribution of fat-soluble chemicals will be relatively lower in newborns than in adults. In newborns, many metabolic enzyme systems are immature and inefficient resulting in decreased clearance of the chemical. This results in a higher plasma concentration of the chemical. Even the renal clearance capacity of newborns is much lower when compared to adults. Because of this complex pattern of maturational changes in the pharmacokinetic processes, it is often difficult to predict the net effect of these changes on the disposition of the chemical (Bruckner, 2000). Increased unbound fraction, combined with the immature blood brain barrier (BBB) and higher cerebral blood flow leads to greater partitioning of potential neurotoxins into the brain, thereby increasing the susceptibility of neonates to neurotoxicity.

Age-related differences in the sensitivity of immature and adult rats to the neurotoxicity of organophosphates have been clearly established. With regard to malathion, Lu et al. reported that newborns exhibit the highest sensitivity to malathion poisoning when compared to adults (Lu et al., 1965). It was also reported that lower levels of hepatic CEs and P450-mediated dearylation play a major role in the increased
sensitivity of juveniles to phosphorothionate toxicity (Atterberry et al., 1997). A similar observation has been made pertaining to high doses of DLM. PND 11 and 21 pups were found to be 16- and 7-times, respectively, more sensitive to acute neurotoxicity of DLM than adults (Sheets et al., 1994). Sheets et al. reported that this increased susceptibility of immature rats was most likely the result of lower capacity of metabolic enzymes, which becomes quite apparent at acutely toxic doses. Thus, dispositional factors rather than functional factors appear to contribute significantly to the relative sensitivity of younger age groups to pyrethroid neurotoxicity.

Relying on animal studies for developing risk assessment of pyrethroids in humans presents a problem. Not only are there significant differences in the enzymes involved in the metabolism of pyrethroids, but also the rate at which these enzymes mature is significantly different from humans. PBPK modeling is an important tool used in risk analysis to predict early life sensitivity to pyrethroids. This tool is particularly important when extrapolating across species and different age-groups. A PBPK model is a unique framework which incorporates various age-specific and species-specific pharmacokinetic factors that can impact the tissue dosimetry (Clewell et al., 2004). By incorporating the age-specific differences, it allows us to modify a validated PBPK model with adult pharmacokinetic data and use it for predicting concentration-time courses in young humans. Thus, this modeling approach can be used successfully to predict the relationship in humans between internal doses in the adult and internal doses during early life using chemical-specific parameters. In order to validate the accuracy of such predictions, proof-of-concept studies with representative pyrethroids should be conducted in rats. The parameters in a PBPK model can be categorized into
four types: exposure, physiological, partitioning, and metabolism (Clewell et al., 2007). It is important to obtain accurate measurements of physiological (tissue and fat volumes, circulation), metabolism and partition coefficient parameters to develop a reliable PBPK model.

**Objective and Significance**

Tissue:plasma PCs are key input parameters for PBPK models. Validated PBPK models can be used for reliable predictions of pyrethroid internal dosimetry in humans. Limited data are available in the literature from which to determine PCs for pyrethroids. An important implication of that data is that it is rather estimated and not accurately measured at steady-state. No studies till date have measured the tissue:plasma PCs for pyrethroids at steady-state or assessed differences in the distribution of these compounds between immature and adult rats.

Therefore, the overall objective of this project is to gain an insight into the systemic distribution of representative pyrethroid insecticides and to understand differences between the younger age-groups and adults in the pesticides disposition. The knowledge gained from these studies can be useful in risk assessment of pyrethroids in human sub-populations. Also the other scientists can use this information in the future to predict the distribution of other pesticides with similar physicochemical properties in different age-groups.

**Blood-Brain-Barrier**

The BBB is a selective ‘diffusion’ barrier, which restricts the entry of most compounds from blood to brain. The BBB plays an important role in maintaining the
homeostasis of the brain by regulating ionic and fluid movements between blood and brain. It also helps supply the brain with essential nutrients and protects the brain from circulating toxins and waste products. Endothelial cells, astrocyte end feet and pericytes constitute the cellular elements of the brain microvasculature that compose the BBB (Ballabh et al., 2004). The endothelial cells at the BBB are characterized by a continuous basement membrane, extensive tight junctions, absence of fenestrations and sparse pinocytotic vesicular transport (Sage, 1982). These characteristics differentiate the endothelia of cerebral capillaries from that of non-neural tissues. Because of the continuous tight junctions, the endothelium at the BBB behave like a plasma membrane (Sage, 1982). The tight junctions at the BBB are 50-100 times tighter than peripheral microvessels and prevent the entry of small hydrophilic molecules by limiting paracellular transport (Lawther et al., 2011). Therefore, the transport across the BBB is effectively limited to selective transcellular mechanisms. It is generally believed that the compounds with low molecular weight, high lipid solubility, low plasma protein binding, and a low degree of ionization at physiological pH diffuse freely across the BBB along their concentration gradient and attain rapid equilibrium. On the other hand, compounds with low lipid solubility, high polar surface area, high plasma protein binding, high molecular weight and a high degree of ionization at physiological pH have restricted and slow BBB permeability (Clark, 2003).

The presence of specific influx and efflux transporters on the apical and basolateral membranes of the endothelia further regulates the bidirectional transcellular transport across the BBB, thus providing a selective ‘transport’ barrier (Figure 1.4) (Abbott et al., 2006). Carrier mediated influx via facilitative diffusion is a passive process
that can transport many essential polar molecules such as glucose, amino acids and nucleosides into the central nervous system (CNS). Facilitative diffusion is an energy independent process, where the solute molecules bind to specific solute carriers (SLCs) and then move along their concentration gradients. On the other hand, efflux transporters (ABC transporters) are primarily active and energy-dependent transporters that intercept some of the passively diffusing solutes and pump them out of the endothelial cells (Abbott et al., 2010). It is the orientation and interplay of these transporters at the BBB that determines the direction of the transport (blood to brain or brain to blood) and the extent to which a chemical gains access to the CNS (Lee et al., 2001; Löscher and Potschka, 2005; Sun et al., 2003). Endocytosis is another important transport mechanism by which large molecules such as proteins and peptides enter the CNS. Endocytosis can be either receptor mediated (RME) or adsorptive mediated (AME) and contributes to selective uptake of macromolecules (Abbott et al., 2010). RME transports substances such as insulin, transferrin, and insulin like growth factor into the CNS and is highly energy dependent process (Lawther et al., 2011).

**Ontogeny of Blood-Brain-Barrier**

The BBB in newborns is immature and is poorly formed and leaky. The immature BBB in newborns contributes to higher brain levels of chemicals (Arya et al., 2006). Arya et al. reported that the brain uptake of glucocorticoids in neonatal rats is significantly higher than adults due to an immature BBB. The development of BBB is a gradual process in humans and it reaches adult capabilities by approximately 6 months of age (Watson et al., 2006). The fully differentiated (i.e., mature) BBB consisted of highly specialized endothelial cells, large number of pericytes imbedded in the
basement membrane, perivascular macrophages and overlapping astrocytic end feet (Engelhardt, 2003). Behnsen (1905) found that upon injection of very high concentrations of trypan blue, mice of 4 weeks of age incorporated more dye compared to mice of 5-8 weeks of age. This suggests that the BBB is more permeable in younger mice compared to adults. Clark et al. reported that the BBB permeability is high at birth and decreases in the first few weeks after birth (Clark et al., 1993). The BBB was not fully developed structurally and was not fully functional until about the time of weaning (Schulze and Firth, 1992). A clearly delineated basement membrane was absent in embryonic capillaries and was not fully developed until PND 16. Schulze and Firth suggested that maturation of interendothelial clefts is accompanied by establishment of a characteristic ratio of ‘narrow zone’ (complex tight junctions) to ‘wide zone’ and disappearance of membrane separations larger than 20 nm. While tight junctional contacts in immature BBB were described as having ‘touching’ outer leaflets, adult BBB appear to have ‘fused’ outer leaflets. Leaky interendothelial junctions in the developing brain results in high permeability. Apart from gradual decrease in unfused endothelial cell outer leaflets, junctional clefts also decrease and expanded junctional clefts (paracellular channels) virtually disappear with age in parallel to decreased permeability (Stewart and Hayakawa, 1987). Thus, developmental tightening of the BBB is primarily characterized by structural changes at tight junctional contacts. Like most other structures and physiological functions, the BBB is more mature at birth in humans than in rodents. Its rate of maturation in human infants is maximal during the third month of life. The rate of change progressively diminishes thereafter, until the fully developed BBB is achieved by 1 year of age (Statz and Felgenhauer, 1983).
Apart from the morphological changes, specific transporters at the BBB also undergo changes in their expression with age. Thus, ontogeny of transporters at the luminal and abluminal side of the membrane can affect the amount of chemical that enters the CNS. P-gp was first detected on PND 7, reaches 25% of the adult by PND 10 and then reaches plateau by PND 28 (Matsuoka et al., 1999). BCRP mRNA levels were high at birth and decreased to adult levels by PND 42. For OCT1, OCT2 and OAT1, mRNA levels peaked around PND 1 to 4 with limited expression before birth and throughout development. For some other transporters such as multi-drug resistance 1a (MDR1a), MRP3, OAT2 and OAT3, mRNA expression remained more or less constant throughout the development (de Zwart et al., 2008).

Since brain is the target organ of potential toxicity for pyrethroids, it is of great importance not only to accurately measure the permeability of pyrethroids, but also to understand various factors that contribute to their penetration into the brain. Even though DLM is a highly lipophilic compound (log K<sub>ow</sub> = 6.1), its entry into the brain is restricted by its high molecular weight (505.2) and high plasma protein binding (80%). Plasma protein binding plays a very important role in drug transport since only unbound drug is able to penetrate the membrane. Therefore, the free fraction of drug in the capillary blood is an important determinant of drug entry into the brain. There is a lack of understanding of the effect of plasma protein binding on brain uptake of pyrethroids and the absence of an accurate quantitative model that appropriately describes this effect. The plasma protein binding of many drugs is believed to be age dependent as mentioned in the previous section (1.6). Total plasma protein concentrations increased from 2.5 g/dL at birth to about 5.2 g/dL in adult rats and the corresponding albumin
concentration increased from 1.8 g/dL to 3.8 g/dL (de Zwart et al., 2008). Thus, the difference in plasma protein concentrations and the binding between newborns and adults can alter the brain uptake of pyrethroids. Thus, it is also of great significance to evaluate the effect of age-dependent changes in plasma protein binding and BBB maturity on the permeability of pyrethroids. Therefore, as a part of the overall project, BBB permeability of DLM will be determined as a function of age-dependent changes in BBB integrity and plasma protein binding.

**Hypothesis of Dissertation**

It is hypothesized that the distribution and brain uptake of pyrethroids in immature rats will be significantly different from that in adult rats due to differences in plasma protein binding and BBB permeability.

**Overview of Experimental Plan of Dissertation**

1. To determine *in vivo* tissue:plasma partition coefficients of DLM, CIS and TRANS in PND 15, PND 21 and adult rats.

2. To selectively investigate the role of a) plasma protein binding; b) membrane transporters; c) BBB – in limiting the uptake of pyrethroids into the brain.

3. To evaluate the brain uptake of DLM as a function of age-dependent changes in BBB integrity and plasma protein binding.
REFERENCES


Figure 1.1. Structure of Deltamethrin (A) and Permethrin (B). Adapted from Soderlund et al. (2002). Pyrethroids are esters of a cyclopropanecarboxylic acid and a cyclopentenolone alcohol. Addition of α-cyano group at the benzylic carbon atom gives type II pyrethroids such as deltamethrin.
Figure 1.2. Effect of Pyrethroids on Voltage Sensitive Sodium Channels in the CNS. Adapted from Shafer et al. (2005). Pyrethroids delay the inactivation of voltage sensitive sodium channels allowing more sodium ions to cross and depolarize the neuronal membrane.
Figure 1.3. General Metabolic Pathway of Pyrethroid Insecticides. Adapted from Kim et al. (2010). The initial metabolism of the parent compound results from the actions of either esterases at the central ester bond or CYP 450 mediated oxidation at one or more sites in the acid or alcohol moieties. The metabolites from hydrolytic or oxidative actions are further conjugated with sulfates or glucuronides prior to excretion.
Figure 1.4. Different Means of Transport across the BBB. Adapted from Abbott et al. (2010). Small, lipid soluble, non-polar molecules passively diffuse across the BBB. Presence of various efflux and influx transports on both the apical and basolateral membranes of endothelial cells can be observed from the figure.
CHAPTER 2

INFLUENCE OF MATURATION ON IN VIVO TISSUE TO PLASMA PARTITION COEFFICIENTS OF DELTAMETHRIN, CIS- AND TRANS-PERMETHRIN IN SPRAGUE-DAWLEY RATS

ABSTRACT

The major objective of this investigation was to develop a reliable approach for determining *in vivo* partition coefficients (PCs) for deltamethrin (DLM), *cis*-permethrin (CIS) and *trans*-permethrin (TRANS) in adult and young Sprague-Dawley (SD) rats. Adult, PND 21 and PND 15 rats were infused with a predetermined amount of DLM, CIS or TRANS via a sc osmotic pump for 48 or 72-h. Adult and PND 21 rats also received an oral loading dose. Systemic steady-state, or equilibrium was attained in each age-group within 72-h of the protocol. DLM, CIS and TRANS were all distributed to tissues according to their neutral lipid content, with adipose tissue exhibiting much higher tissue:plasma PCs than skeletal muscle, liver or brain. Liver:plasma and brain:plasma PCs were consistently lower than unity. Tissue:plasma PCs were generally higher for CIS than for DLM & TRANS, especially in immature rats. CIS is metabolized through much more slower oxidative processes, which may result in significantly higher circulating levels. This appears to saturate plasma binding and thus, might have resulted in greater tissue deposition of CIS. CIS tissue:plasma PCs were found to be inversely related to the rats’ age. This was also true for TRANS, although TRANS brain:plasma PCs were comparable in immature and mature animals. Interestingly, although DLM tissue:plasma PCs in PND 15 rats were significantly higher as compared to adults, PCs in PND 21 rats were consistently lower than that of adults. These results suggest that age-dependent partitioning may play an important role in determining the kinetics and ensuing risks of these commonly utilized insecticides.
INTRODUCTION

Pyrethroids, synthetic derivatives of naturally occurring pyrethrins, are active components of household pesticide sprays, insect repellents, pet shampoos and human lice and scabies treatments. These chemicals are also applied to agricultural fields, commercial nurseries, residential structures and lawns and gardens. Permethrin (PER), marketed as a mixture of its isomers CIS and TRANS, is the most frequently utilized insecticide in the U.S (Barr et al., 2010). Thus, it is not surprising that a large proportion of the general population in the U.S. and the E.U. are frequently exposed to such chemicals. Three-phenoxy benzoic acid (3-PBA), a metabolite common to a number of pyrethroids, was detected in 70% of over 5,000 people monitored in the U.S. in the National Health and Nutrition Examination Survey (NHANES) (Barr et al., 2010). Children exhibited significantly higher 3-PBA levels than adolescents or adults. Morgan et al. (Morgan, 2012) compiled data from 15 published studies of pyrethroid exposure of children in day-care centers and homes. Seven different pyrethroids were found in dusts, floor wipes, air and urine samples. PER was the most frequently detected. Ingestion from dusts and pets by hand-to-mouth activity was the most common source of exposure. Consumption of the very low levels in foods was of secondary importance (Lu et al., 2006). Substantially high doses of pyrethroids can be acutely neurotoxic, though the potency of different pyrethroids varies widely (Wolansky et al., 2006). The parent compounds are the proximate neuroactive moieties. Their primary mechanism of action on neurons is interference with voltage-gated sodium channels, resulting in stimulus-dependent nerve depolarization and block (Soderlund, 2012). The very high lipophilicity (i.e., Log Ps = 4.6 – 7.1) of pyrethroids significantly impacts their
pharmacokinetics. The chemicals appear to be poorly absorbed from the GI tract, despite their lipid solubility. Oral bioavailability of DLM in rats is reported to be just 14-16% (Anadon et al., 1996; Kim et al., 2008). DLM, CIS and TRANS are highly bound to rat and human plasma proteins and lipoproteins (Sethi et al., 2014). Systemic distribution of DLM and PER in rats is apparently governed largely by tissues’ fat content. Pyrethroids, as a chemical class, readily undergo metabolic inactivation via oxidation in the liver by cytochrome (CYP) P450s and hydrolysis by plasma and/or liver carboxylesterases (CEs) in rats and humans. Species- and/or age-specific metabolic rate constants have been published for DLM, CIS and TRANS (Anand et al., 2006; Godin et al., 2006; Tornero-Velez et al., 2012).

PCs are important determinants of the systemic deposition and ensuing biological effects of xenobiotics. A tissue:plasma PC can be defined as the ratio of concentrations of a chemical in two phases once equilibrium is attained. As partitioning into tissues substantially influences the pharmacokinetics of xenobiotics, PCs are an essential input parameter for physiologically based pharmacokinetic (PBPK) models. PBPK models are finding increasing use in predicting the internal dosimetry (i.e., plasma and tissue concentration time-courses) of drugs (Rowland et al., 2011; Shardlow et al., 2013) and other chemicals (Andersen, 2003; Lipscomb et al., 2012). PBPK models are also being developed to predict target organ dosimetry and associated health risks of commonly-used insecticides, including organophosphates and pyrethroids. Several first-generation models have been published for DLM and PER, two widely-utilized pyrethroids. In the initial two models for DLM, tissue:plasma PCs were calculated from ratios of areas under tissue concentration versus time curves (AUCs) to the plasma AUC in oral dosing
studies in rats (Mirfazaelian et al., 2006; Tornero-Velez et al., 2010). These tissue:plasma PCs for DLM were subsequently adopted for modeling CIS and TRANS (Tornero-Velez et al., 2012), due to a lack of PCs for PER. A computational approach was adopted to estimate PCs for a third DLM model (Godin et al., 2010). A significant problem with using these PC values is that the AUCs from in vivo single dose toxicokinetic studies were not determined under steady-state conditions. Most of the data used for estimating tissue:plasma PCs was obtained at relatively high doses compared to environmental exposure. Because of the low analytical sensitivity of pyrethroids, these high doses were required to obtain measurable tissue concentrations. Modelers must assume that accurate analytical methods were carefully performed by research groups. A likely, but frequently unrecognized problem with analyses of highly-lipophilic compounds is their pronounced adherence, or non-specific binding to, most plastic and glass devices. This was found to be true for deltamethrin and permethrin, when measuring their plasma protein binding (Sethi et al., 2014).

A number of other approaches have been used as well with varying success to determine tissue:plasma/blood PCs for pyrethroids and other highly lipophilic chemicals. Some researchers have advocated the use of computational methods in the interest of saving the time and cost of conducting animal studies. Poulin and Krishnan (Poulin and Krishnan, 1995b) assumed the solubility of a chemical in n-octanol corresponded to the sum total of its solubility in neutral lipids, phospholipids and water. The solubility of highly lipophilic compounds in water was considered negligible, and solubility in phospholipids 30% of that in neutral lipids. There was reasonable agreement between their predicted and empirical PCs for 23 organic chemicals for some human tissues but
not others. The predictions improved when solubility in olive or corn oil rather than n-octanol was used as a surrogate for neutral liposolubility (Poulin and Krishnan, 1995a). This approach worked well when it was utilized to calculate tissue:air and blood:air PCs for volatile organic compounds (VOCs) (Poulin and Krishnan, 1996a; Poulin and Krishnan, 1996b; Smith et al., 2005). Although tissue:blood PCs can readily be calculated when tissue:air and blood:air PCs are available, this approach cannot be used for pyrethroids, as their volatility is negligible. Payne and Kenny (Payne and Kenny, 2002) assessed the relative accuracy, strengths and shortcomings of 10 published algorithms for estimating tissue:blood PCs. Accuracy was problematic in most cases due to difficulties in modeling the effects of protein binding. DLM, CIS and TRANS, like other very lipophilic compounds, are highly bound to plasma proteins and lipoproteins (Sethi et al., 2014). More recently Krishnan and his colleagues (Peyret et al., 2010) have published what is termed a unified algorithm by integrating additional mechanistic algorithms, including estimations of hydrophobic interactions with neutral lipids and binding to plasma proteins.

Estimation of tissue:blood PCs by in vitro techniques offers a relatively inexpensive, time-saving alternative to in vivo approaches for non-volatile chemicals. Reasonably good agreement between in vitro and in vivo PCs has been reported for chemicals such as estradiol and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Murphy et al., 1995). Estradiol and TCDD were each dissolved in propylene carbonate (PCARB) and equilibrated for up to 5 hours with homogenized tissues and with blood. Tissue:PCARB values divided by blood:PCARB values yielded tissue:blood PCs. Reasonable agreement was observed for a series of barbituric acids, but the in vitro
data underestimated in vivo tissue distribution for the most lipophilic congeners (Ballard et al., 2003b). Albumin diffusion from tissue pieces to in vitro media was problematic for highly plasma-bound compounds (Ballard et al., 2003a). CIS and TRANS are 80-90% bound to albumin. The normal process of vascular perfusion of tissues does not occur in in vitro systems. Homogenization enhances the penetration of test chemical, but results in loss of structural integrity of cells and other potentially important cellular structures such as the blood-brain barrier. A method for estimating non-volatile pesticides involving ultrafiltration of tissue homogenates and pieces proved ineffective for compounds with a Log P ≥ 1.7, due to retention by the filtration units employed (Tremblay et al., 2012). A solid phase microextraction (SPME) technique was used for isolation of the more lipophilic pesticides, but tissue:blood PCs could not be determined for compounds with a Log P > 4, due to excessive partitioning into the SPME fiber. Log P values of most pyrethroids exceed 4. We found that adherence of pyrethroids to most glass, metals and plastics to be a serious problem that could be minimized by using clean silanized glass vials and LoBind® plastic pipettes (Sethi et al., 2014). In light of the uncertainties with each approach, it is clear that pyrethroid PBPK models developed thus far, lack reliable tissue:plasma PCs. Therefore, we decided to measure CIS and TRANS tissue:plasma PCs in vivo at steady-state in order to have more assurance of the reliability of input parameters for second generation PBPK models currently being developed for these chemicals.

Although Alzet pumps® were widely utilized for the delivery of various chemotherapeutic agents (Pun et al., 2004; Tamamura et al., 2003; Teicher et al., 1992) in general, they have not seen a lot of attention for use in achieving steady-state.
Alzet pumps® were used in the current study to maintain sustained delivery to reach steady-state levels. Alzet pumps® allow us to determine tissue:plasma PCs at environmentally relevant doses, which was a major drawback with the data reported previously. All the pyrethroid PCs estimated so far were based on studies conducted in adult rats. To our knowledge, no one has yet determined the tissue:plasma PCs of pyrethroids in younger rats. Miniature Alzet pumps® were successfully employed in the current study to achieve steady-state levels in younger rats.

The objectives of the current project were three-fold: (1) to develop an in vivo model from which measurements of relative amounts of pyrethroids could be directly determined at steady-state in adult rat tissues and plasma; (2) to utilize the model to accurately determine tissue:plasma PCs for brain, liver, skeletal muscle, and fat for DLM, CIS and TRANS; and (3) to determine whether these tissue:plasma PCs vary with the stage of maturity of the test subjects.

MATERIALS AND METHODS

Chemicals and Materials

Cis-permethrin (CIS) (99.3%) and Trans-permethrin (TRANS) (99.0%) were kindly provided by FMC Agricultural Products (Princeton, NJ). Deltamethrin (DLM) was donated by Bayer Crop Science (Research Triangle Park, NC). Glycerol formal was purchased from Acros Organics (NJ, USA). Acetonitrile (HPLC grade) and Spin-X® microcentrifuge filters were purchased from Fisher Chemical Co. (Pittsburgh, PA). Phosphoric acid (86%) was purchased from J.T. Baker (Phillipsburg, NJ). DNA Lo-bind® microcentrifuge tubes were obtained from Eppendorf (Hauppauge, NY), and d-SPE
(QuEChERS) kits were purchased from Agilent Technologies (Santa Clara, CA). Alzet® osmotic (Model 2ML1) and micro-osmotic (Model 1007D) pumps were supplied by the Durect Corporation (Cupertino, CA).

Animals

Adult male and timed-pregnant female Crl:CD SD rats were purchased from Charles River Laboratories (Raleigh, NC). The protocol for this study was approved by the University of Georgia Animal Care and Use Committee. Each rat was housed in a cage with a 12-h light/12-h dark cycle at ambient temperature (22°C) and relative humidity (55 ± 5%). The females were allowed to deliver, and the pups housed with their mothers until the offspring were 15 and 21 days old. The unsexed pups were housed with their mother during CIS and TRANS exposure regimens. Food (5001 Rodent Diet, PMI Nutrition International LLC, Brentwood, MO, USA) and tap water were provided ad libitum.

Adult and PND 21 rats were anesthetized using Ketamine, Acepromazine, and Xylazine (KAX) cocktail @ 0.075 – 0.1 mL/100g body weight. Isoflurane was used to anesthetize PND 15 rats due to KAX toxicity. The hair was trimmed at the site of implantation on the dorsal surface of each unsexed pup and adult male rat. A small incision was made, and a large subcutaneous (sc) pocket created by dissecting the subcutaneous tissue. A DLM, CIS- or TRANS-loaded Alzet® pump was inserted into the pocket with the delivery port first. The incision was then closed using wound clips, and the rats returned to their cage. Animals were provided with food and water ad libitum during recovery and pyrethroid infusion.
**In vitro Plasma:Blood Partition Coefficient Determination**

Lo-bind® centrifuge tubes containing 100 µL of fresh control rat blood and 10 µL of 0.64 M sodium fluoride (NaF) were spiked with 5 µg DLM, CIS or TRANS and vortexed for 10 sec (Mini Vortexer®, VWR) (West Chester, PA). The tubes were incubated for 10, 30, 45 and 60 min in an orbital shaker at 37°C. The tubes were then centrifuged for 5 min at 4,342×g to separate plasma and red blood cells (RBC). DLM, CIS and TRANS in plasma and RBC were extracted and quantified using a modified high performance liquid chromatography (HPLC) method developed by Kim et al (Kim et al., 2006). Briefly, 50 µL of separated plasma were placed into a Lo-bind® tube and spiked with 8 µg of internal standard (DLM was used as internal standard for CIS and TRANS, and CIS was used as internal standard for DLM), and vortexed for 40 sec. Seventy-five µL of acetonitrile (ACN) were added and the resulting solution vortexed for 70 sec to denature the plasma proteins. The contents of the centrifuge tube were then centrifuged for 10 min at 15,871×g, and 100 µL of supernatant was transferred to a vial for HPLC analysis. The red blood cells (RBC) pellet was also spiked with 8 µg of DLM as an internal standard and vortexed for 40 sec. One hundred µL of cold distilled water were added and the suspension vortexed for 70 sec to lyse the RBC. Two hundred µL of ACN were added, and the contents were vortexed for 70 sec to denature the proteins. The tubes were then centrifuged at 15,871×g, and 150 µL of supernatant was taken for HPLC analysis. The analysis was performed using an Agilent 1100 series HPLC (Foster City, CA). The chromatographic separation was achieved using a Phenomenex® Luna C18 column. The mobile phase was methanol:HPLC water (97:3, v/v) operated at a constant flow rate of 1 mL/min. Concentration in blood was calculated as the sum total of
concentration in RBC and concentration in plasma. Plasma:blood PCs were calculated by dividing the concentration of DLM, CIS or TRANS measured in the plasma by that calculated in the blood.

**In vivo Tissue:Plasma Partition Coefficient Measurement**

An oral loading dose coupled with constant infusion of DLM, CIS or TRANS was employed, in order to achieve systemic steady-state or equilibrium of the chemical in adult and PND pups. Only constant infusion, however, was employed in PND 15 pups, due to toxicity seen with combined loading and infusion doses.

An Alzet® infusion pump containing DLM, CIS or TRANS was surgically implanted subcutaneously on adult rats’ dorsum, for constant infusions of 0.36 mg/h. Three to 4-h later, each adult was gavaged with a “loading dose” of 30 mg/kg of DLM, or 150 mg/kg of CIS or TRANS. After 72-h the adult rats were sacrificed by cervical dislocation and blood withdrawn by closed-chest cardiac puncture into a heparinized syringe. Whole blood was centrifuged immediately to separate plasma. Ten µL of 0.64M NaF were added for every 100 µL of plasma collected to inhibit esterases. The abdominal cavity was opened and sterile saline injected into the portal vein, in order to flush even more blood from the liver and other organs. Samples of whole brain, liver, thigh muscle and perirenal fat were excised, immediately flash-frozen with liquid nitrogen and stored at -80°C until analysis.

PND 15 and 21 pups with subcutaneous Alzet® pumps were infused with DLM or CIS at rates of 0.03 mg/h and 0.02 mg/h, respectively. PND 15 and 21 pups were infused with 0.05 mg TRANS/h. PND 21 pups were also administered a loading dose of
0.25 mg/kg DLM or CIS, or 1.15 mg/kg TRANS by gavage 3-4 h after the start of infusion. PND 15 pups did not receive a loading dose. After 48 and 72-h of infusion, groups of the pups were sacrificed by cervical dislocation. The maximum amount of blood possible was withdrawn by closed-chest cardiac puncture. Plasma was separated from whole blood as described above, and samples of whole brain, liver and abdominal muscle were excised and flash frozen like tissues from adults. Fat was not harvested from PND 15 and 21 pups due to lack of adipose tissue.

**Tissue Extraction and Analysis**

Brain and liver were homogenized in 2× volume of saline, while muscle and fat were homogenized in 3× volume of saline and water respectively, using a Tekmar Tissumizer® (Cincinnati, OH). Plasma and fat homogenates were extracted using a method developed by Gullick *et al* (Gullick *et al.*, 2014). Briefly, 100 µL of plasma or fat homogenate were extracted with 500 µL of an extracting solution comprised of internal standard (10 ng/mL DLM for CIS and TRANS analyses, and 50 ng/mL CIS for DLM analyses) in a mixture of 1% phosphoric acid and 99% ACN. The sample was mixed and centrifuged before drying the solvent and reconstitution with 6 µl toluene for analysis. Brain, muscle and liver samples were extracted using an improved bioanalytical method also developed by Gullick *et al* (Gullick *et al.*, 2016). Three hundred µl of tissue homogenate were extracted with 800 µl of an extracting solution comprised of internal standard (10 ng/mL DLM for CIS and TRANS analyses, and 50 ng/mL CIS for DLM analyses) in *n*-hexane-saturated acetonitrile. The sample was mixed and centrifuged. Sample clean-up of the supernatant was performed using
QuEChERS microcentrifuge tubes before drying the solvent and reconstitution with 6 µL of toluene for analysis by GC-negative chemical ionization-MS (GC-NCI-MS).

GC-NCI-MS analysis was performed using an Agilent 6890N gas chromatograph with a 5973 quadrupole mass selective detector and Enhanced Chemstation (Build 75 Agilent, 2003). Chromatographic separation was achieved using a Zebron® ZB5-MS GC column operating at 1 mL/min constant flow helium.

**Determination of Partition Coefficients**

For each animal, the steady-state concentration of DLM, CIS or TRANS in each tissue being studied was divided by the steady-state concentration in plasma, in order to determine the tissue:plasma PC. Means and standard deviations (SDs) were calculated with Microsoft Excel (Microsoft, Redmond, WA). All experiments were performed with a minimum of 4 animals unless otherwise stated. Statistical significance was evaluated between groups using either the Student’s t-test or one way ANOVA, followed by Tukey’s multiple comparison test, as indicated, with a significance level of p<0.05, using Graphpad Prism 6 software® (San Diego, CA).

**RESULTS**

**Partitioning of DLM, CIS and TRANS between Plasma and Blood**

The concentration of each compound in plasma and RBC was measured periodically by HPLC during 1-h incubation (*Table 2.1*). It is evident that the concentration of CIS and TRANS progressively decreased in the RBC and increased in the plasma during the 60 min incubation period, with the most pronounced changes occurring during the initial 30
min. Concentration of DLM in the RBC and plasma did not change over the incubation period. Concentrations of each compound are ~3 fold higher in plasma than RBC at 60 min. If it is assumed that plasma and RBCs account for 60% and 40% of the volume of rat blood, respectively, ~80% of each compound is present in the plasma.

**Partitioning of DLM, CIS and TRANS between Plasma and Tissues of Adult Rats**

Plasma concentrations of DLM, CIS and TRANS were measured periodically in adult rats in pilot experiments to determine when steady-state was attained. It is generally accepted that steady-state will be reached in ≤ 5 half-lives of a chemical. Since Anandon *et al.* (Anadón *et al.*, 1991) reported the half-life of permethrin in adult SD rats to be 12.4-h, steady-state should be achieved in approximately 62-h. We observed considerable intersubject variability in plasma CIS levels at 24 and 48-h, so it was decided to sacrifice adult animals after 72-h of the infusion protocol for plasma and tissue analyses. Mean plasma TRANS levels at 48 and 72-h were comparable, so the animals’ sacrifice for the tissue:plasma TRANS PC determination was also performed after 72-h. Similarly, the half-life (t½) of DLM, when given to adult SD rats in glycerol formal (GF) by gavage, was reported by Kim *et al.* (2008) to be 13.3- h, and the steady-state was expected to be achieved in approximately 67-h. Similar to TRANS, DLM mean plasma levels were comparable at 48 and 72-h. So the animals were sacrificed after 72-h of constant infusion.

CIS and TRANS exhibited the same pattern of tissue distribution and the same rank order of tissue:plasma PCs in adult rats (*Table 2.2B & 2.2C*). The CIS liver:plasma PC was lowest, followed in ascending order by the brain, skeletal muscle and fat. TRANS
PCs showed the same rank order, although TRANS levels in the liver of many subjects were below the limit of quantitation (LOQ). The fat:plasma PC for each isomer was far higher than that for the other tissues analyzed. The brain:plasma PCs for CIS and TRANS were quite similar. The CIS fat:plasma PCs, however, were about 1.5-fold higher than the TRANS PCs.

For DLM, the brain:plasma PC was lowest, followed by liver, skeletal muscle and fat (Table 2.2A). The brain:plasma and fat:plasma PCs for DLM were about 2 - 3 fold lower when compared to CIS and TRANS. However, DLM liver:plasma and muscle:plasma PCs were higher than that of CIS and TRANS. Similar to CIS and TRANS, after 72-h of constant infusion, fat accumulated far higher levels of DLM than other tissues.

**Partitioning of DLM, CIS and TRANS between Plasma and Tissues of Pups**

The duration of infusions of PND 15 and 21 rats did not substantially influence most tissue:plasma PCs. In both PND 21 and PND 15 pups, the 48 and 72-h tissue:plasma PCs for DLM were similar (Table 2.4A). In PND 21 pups, the 72-h brain:plasma PC for CIS was higher than that determined at 48-h (Table 2.4B). Although the 72-h muscle:plasma PC was also higher than that of 48-h, this difference failed to reach statistical significance. However, there was no change in liver:plasma PC at 48 and 72-h. The 72-h plasma TRANS concentrations in PND 21 pups were about half of that at 48 h, as were the tissue concentrations (Table 2.4C). The TRANS brain:plasma and muscle:plasma PCs remained about the same. Plasma, brain and liver concentrations of CIS remained relatively unchanged from 48 to 72-h in the PND
15 pups (Table 2.5B). Accordingly, the PCs remained much the same. Plasma, brain and liver TRANS levels decreased modestly in parallel, resulting in equivalent or comparable brain:plasma and liver:plasma PCs at 48 and 72-h (Table 2.5C). Nevertheless, skeletal muscle exhibited increased partitioning of CIS and TRANS between 48 and 72-h, with an ensuing increase in the muscle:plasma PC for both isomers. This is in contrast to DLM, where muscle:plasma PCs remained almost the same between 48 and 72-h (Table 2.5A).

In most cases, the results of the current study indicate that DLM, CIS and TRANS tissue:plasma PCs in younger pups were higher as compared to adult rats. While the brain:plasma and liver:plasma PCs for DLM in PND 15 pups were significantly higher as compared to adults, the muscle:plasma PC was not significantly different. Surprisingly, tissue:plasma PCs for DLM in PND 21 pups were consistently lower as compared to PND 15 pups and adults (Fig 2.2). Tissue:plasma PCs for DLM in PND 15 and PND 21 pups were consistently lower as compared to CIS and TRANS. CIS brain:plasma, liver:plasma and muscle:plasma PCs in PND 15 and PND 21 pups were significantly higher than in adults (Fig 2.3). Although the liver:plasma PC in PND 21 was significantly lower than PND 15 pups, no significant differences were observed in the brain:plasma and muscle:plasma PCs between PND 15 and PND 21 pups (Fig 2.3). The TRANS muscle:plasma PCs and likely the liver:plasma PCs were inversely proportional to age (Fig 2.4). The TRANS concentrations in the liver of PND 21 pups were below the LOQ and could not be measured. Interestingly, the PND 21 muscle:plasma PC did not differ significantly from that of adults. Unexpectedly, all three age-groups exhibited comparable TRANS brain:plasma PCs. It was not possible to
assess the influence of maturation on fat:plasma PCs, as there was no visible adipose tissue in the PND 15 or 21 pups.

**DISCUSSION**

Accurate tissue:plasma PCs are essential input parameters for PBPK models, as PCs describe the distribution of a test compound in key tissues of the body. There is a lack of reliable methods for the measurement of tissue:plasma PCs at steady-state. None of the PC values reported previously for pyrethroids were measured at steady-state. Also, relatively high doses were often employed due to the low analytical sensitivity of pyrethroids. These high doses are not environmentally relevant and may saturate the distribution and elimination processes. No one has yet established whether tissue distribution and PCs of pyrethroids vary significantly during maturation and development. In the current study, we successfully used Alzet pumps® to attain steady-state and accurately determine tissue:plasma PCs both in adult and younger rats.

The fat:plasma PCs of 45, 98 and 67, determined in the current investigation for DLM, CIS and TRANS respectively, were far higher than for other tissues monitored in adult rats. Tissues with a sizable fat content would be expected to accumulate the largest amounts of highly lipophilic chemicals such as pyrethroids, and thus exhibit relatively high tissue:plasma PCs. Rat adipose tissue is comprised of ~85% lipids, 13.5% extracellular water and 1.5% intracellular water (Rodgers et al., 2005). About 99.7% of the lipids are neutral lipids (Schmitt, 2008). Skeletal muscle contains just 2% lipids (Rodgers et al., 2005), but exhibited significantly higher DLM, CIS and TRANS PCs than brain or liver. This may be attributable to interspersed fat in the specimens of
muscle used for the determination. Unexpectedly, levels of DLM, CIS and TRANS in brain were similar to or lower than levels in plasma. It was previously reported that the DLM levels in brain were substantially lower than in blood (Kim et al., 2008; Mirfazaelian et al., 2006). Rat brain contains 4% lipids, of which more than half are composed of neutral and acidic phospholipids. The solubility of lipophilic chemicals in phospholipids is assumed to be just 30% of that in neutral lipids (Poulin and Krishnan, 1995b). The total lipid content of liver is even lower and its proportion of phospholipids higher than in brain (Schmitt, 2008). The liver of adult and PND 21 rats exhibited the lowest of any CIS PCs. Concentrations of TRANS in the liver of both age-groups were below the LOQ. Willemin et al. (Willemin et al., 2016) monitored the time-course of CIS and TRANS, but were also unable to quantify TRANS in the liver, kidney or testes of adult, male SD rats gavaged with 25 mg/kg of each isomer. Previous toxicokinetic studies with pyrethroids indicated that adipose tissue accumulates the highest levels of DLM, CIS and TRANS, followed in order by skin, skeletal muscle and other organs (Kim et al., 2008; Tornero-Velez et al., 2012).

Tissue:plasma DLM, CIS and TRANS PCs in adult rats obtained in the current steady-state study were compared in **Table 2.3** with tissue:blood PCs derived from the pharmacokinetic data of other investigators. Those PC values were calculated by dividing each research group’s published tissue AUCs by the corresponding blood/plasma AUCs. The DLM tissue:plasma PCs obtained from the current study were quite comparable to those previously reported. This can be attributed to the use of the same experimental approach. Both Mirfazaelian et al. and Kim et al. used male SD rats as test species and glycerol formal as vehicle to dissolve DLM. The pattern of
tissue:plasma PCs for CIS and TRANS obtained in the current project is also similar to those estimated by other investigators, but some differences should be noted. The differences are likely attributable, at least in part, to use of different doses and approaches to derive the values. Tornero-Velez et al. (Tornero-Velez et al., 2012) monitored blood and tissue CIS and TRANS concentrations in adult Long Evans rats sacrificed periodically after being given 1 or 10 mg/kg of a CIS/TRANS (40:60) mixture by corn oil gavage. Willemin et al. (Willemin et al., 2016) measured tissue and blood levels of CIS and TRANS in adult SD rats given 25 mg/kg of each isomer by corn oil gavage. These CIS brain:blood PCs are higher than the currently reported steady-state brain:plasma PC. The TRANS steady-state brain:plasma PC of 0.7 is about the same as the published PCs, with the exception of the inordinately high 1 mg/kg value. The CIS steady-state liver:plasma PC is somewhat lower than its previously reported counterparts. It should be remembered that, in general, blood concentrations will be lower as compared to plasma, due to its larger volume. Using AUCs from blood concentration time profiles may over estimate PCs, which might also be a reason for the higher PC values reported previously. CIS steady-state fat:plasma PC is quite comparable to the fat:blood PCs of Tornero-Velez et al. (Tornero-Velez et al., 2012), but lower than that of Willemin et al. (Willemin et al., 2016) Interestingly, all four TRANS fat:plasma/blood PCs are very similar.

Although, PCs are commonly assumed to be independent of dose, saturation of plasma binding and clearance mechanisms by high concentrations of pyrethroids may significantly alter their partitioning. Beliveau and Krishnan (Krishnan, 2000) observed increase in the unbound fraction ($f_u$) with increase in the blood concentration of four
volatile organic chemicals (VOCs). Protein binding constants typically rise in concert with chemicals' lipophilicity. CIS and TRANS are highly lipophilic (permethrin Log P = 6.3), bind hydrophobically to albumin and lipoprotein, and are 90% bound to human plasma at toxicologically-relevant concentrations (Sethi et al., 2014). Plasma binding becomes saturated when CIS and TRANS concentrations approach and exceed 300 ng/ml. Blood TRANS levels did not exceed 300 ng/ml in the in vivo experiments of Willemin et al. (Willemin et al., 2016) or Tornero-Velez et al., (Tornero-Velez et al., 2012) or in the current steady-state experiment. The TRANS brain:blood and fat:blood PCs based on these published data, with the exception of the 1 mg/kg value of Tornero-Velez et al., are similar to one another and to the corresponding steady-state TRANS PCs for adult rats (Table 3). The Willemin et al. CIS brain:blood, liver:blood and fat:blood PCs, however, are generally higher than the respective Tornero-Velez et al. PCs and our steady-state PCs. This may be attributable to Willemin et al.'s CIS blood levels of over 1,000 ng/mL, which would have exceeded the binding capacity of plasma. This blood level of 1,000 ng/mL CIS is much higher when compared to Tornero-Velez et al.’s 342 ng/mL and 32 ng/mL in the current study. Another important factor that could account for differences in the PCs between these studies is the lack of steady-state in the toxicokinetic studies. Time-course profiles for DLM, CIS and TRANS in brain and fat differ from those for blood, liver and kidney, reflecting differences in rates of tissue uptake and clearance. It is known that plasma binding of the pyrethroids is concentration-dependent and can govern their rate of tissue uptake (Amaraneni et al., 2016). The blood-brain barrier serves to limit and delay DLM uptake into the adult rat’s brain (Amaraneni et al., 2016). At steady-state, PCs are independent of the kinetics of
distribution into and clearance from tissues. Once steady-state is attained, the plasma $f_u$ is stable, allowing a constant rate of input into tissues. At steady-state, the plasma and tissue compartments are in equilibrium, with the exception of fat which continues to accumulate the highly lipophilic pyrethroids. Clearance will also be constant at a single steady-state concentration. Clearance *in vivo* would be expected to vary with concentration and become saturated due to the dose-dependent processes (plasma protein binding, metabolism) characteristic of pyrethroids.

To assess the influence of maturation on the distribution of pyrethroids, we measured the tissue:plasma PCs of DLM, CIS and TRANS in PND 15 and PND 21 pups. Tissue:plasma PCs for DLM were highest in the PND 15 rats as compared to both PND 21 and adult rats. The lower capacity of metabolic enzymes combined with lower plasma binding capacity might have resulted in greater distribution of DLM to the brain and liver. However, the muscle:plasma PC in this age group is lower than adults. This might be attributable to the lower fat content in muscle tissue in this age group. Surprisingly, tissue:plasma PCs in PND 21 pups were consistently lower when compared to PND 15 and adult rats. DLM in rats is rapidly metabolized by both hydrolysis by CEs and oxidation by CYP 1A1 and 3A2 (Godin et al., 2007). De Zwart et al. (de Zwart *et al.*, 2008) reported that plasma and liver CE activity, and CYP 1A1 and 3A2 activity in SD rats reach adult levels by PND 26. The lower tissue:plasma PCs found in PND 21 pups might be a result of rapid metabolism of lower doses of DLM (LD of 0.25 mg/kg vs 30 mg/kg in adults) in this age group. Sethi *et al.* (Sethi *et al.*, 2015) reported that the total plasma protein binding of DLM in PND 21 pups is not significantly different from that of adult rats. Rapid metabolism combined with increased plasma
protein binding might have contributed to lower tissue:plasma PCs for DLM in PND 21 pups.

Tissue:plasma PCs for permethrin’s isomers, notably CIS, generally decreased during maturation. Tissue concentrations of each isomer were consistently highest in the PND 15 rats in the current study. Elevated liver CIS levels in immature animals likely reflect diminished capacity to metabolize the pyrethroid and reduced ability to retain it in the bloodstream due to relatively low plasma protein levels. There are apparently no data on the age-dependence of permethrin biotransformation, although Anand et al. (Anand et al., 2006) described a progressive marked increase in CYP P450- and CE-mediated hepatic metabolism of DLM between PND 10 and 90 in SD rats. There was also a doubling of the total plasma protein level in SD rats between PND 10 and 60. Blood:air PCs for four VOCs were reported to be higher in mature than in immature rats (Mahle et al., 2007). Lerman et al. (Lerman et al., 1984) found a direct correlation between serum albumin and triglyceride levels in neonates, children and adults and blood:air PCs of a series of lipophilic anesthetics. Lower capacity of the plasma of immature humans and rodents to bind and retain lipophilic compounds undoubtedly contributes to higher liver levels and liver:plasma PCs. This situation should be compounded by elevation of systemic levels of lipophiles as a result of substantially reduced body fat stores in immature subjects. Unfortunately liver TRANS levels were too low in our steady-state experiment and in the in vivo study of Willemin et al. (Willemin et al., 2016) to calculate TRANS liver:plasma PCs for adult or PND 21 rats. Rapid metabolism of TRANS by CE mediated hydrolysis was responsible for levels below the LOQ.
Steady-state brain:plasma PCs measured for permethrin’s (PER’s) isomers did not exhibit a consistent pattern of change during rats’ maturation. The CIS brain:plasma PCs appeared to gradually increase with age, while the TRANS brain:plasma PC was essentially unchanged. One explanation for this dichotomy might be the presence of relatively high plasma CIS levels that saturate plasma binding in pups, resulting in greater diffusion from the blood to brain and other tissues. This may also account for the relatively high CIS muscle:plasma PCs we observed in pups. Greater permeability of the blood-brain barrier to pyrethroids could also contribute to elevated brain deposition and a higher brain:plasma PC in the immature animals. Conversely, increased myelination during development should tend to enhance CNS deposition of pyrethroids. An increase of 35% in brain lipid content has been measured in S-D rats between PND 16 and 24 (Galli and Cecconi, 1967). This is reflected in reported increases in brain:air PCs from birth to adulthood for lipophilic anesthetic gases in humans (Lerman et al., 1986) and VOCs in rats (Krishnan, 2000).

In Table 2.6, steady-state tissue:plasma PCs obtained from the current study in PND 15, PND 21 and adult rats were compared to those estimated from AUCs obtained from a single dose toxicokinetic study (Tanzir et al., Chen et al., to be submitted). We found that while some tissue:plasma PCs were comparable to each other, some were markedly different. It should be remembered that there were some differences among the doses and vehicles used in both the studies. Steady-state tissue:plasma PCs for DLM obtained from the current study were quite similar to those estimated from AUCs, especially in younger age groups (Table 2.6A). Steady-state plasma levels of 78 and 10 ng/mL in PND 21 and PND 15 pups respectively in the current study matched well with
the respective peak plasma concentrations of 47 and 21 ng/mL in the single dose study. This might probably be the reason for comparable tissue:plasma PCs obtained from both approaches. For CIS and TRANS, while steady-state brain:plasma PCs were comparable to those estimated from AUCs, steady-state muscle:plasma and fat:plasma PCs were somewhat lower than their counterparts (Tables 2.6 B&C). Steady-state liver:plasma PCs were quite lower compared to those estimated from AUCs. The peak plasma concentrations for CIS and TRANS (5 – 8 µg/mL) from single dose studies (600 mg/kg) were significantly higher than the respective steady-state plasma levels (30 – 80 ng/mL). Pronounced age-dependent differences in the tissue:plasma PCs estimated from AUCs were only evident in the liver tissue. Saturation of plasma protein binding and clearance mechanisms at high doses employed in the single dose study might be the reason for not seeing pronounced age-dependent differences in the tissue:plasma PCs estimated from AUCs.

In conclusion, we have developed a means of achieving steady-state for highly lipophilic chemicals in a rodent, in order to measure tissue:plasma PCs as accurately as possible in vivo. Our results indicate that the adipose tissue was the major repository for DLM, CIS and TRANS in the rat, followed by skeletal muscle, after 72-h of constant infusion. As a result, the tissue:plasma PC for fat was higher than for other tissues. Brain:plasma and liver:plasma PCs were lower than unity in adult animals. The liver concentrations of TRANS were below LOQ in adult and PND 21 rats, apparently due to rapid hydrolysis by carboxylesterases and oxidation by cytochrome P450s. Tissue:plasma PCs were found to be inversely proportional to age and decreased with increasing age. These differences become more pronounced as the age diminishes. A
few exceptions were observed in the PND 21 rats. It was not possible to assess the influence of maturity on partitioning of pyrethroids into fat, due to lack of visible adipose tissue in younger rats. The knowledge from these studies can be used by other researchers in the future to understand the disposition of other pesticides which have similar physicochemical properties.
REFERENCES


Table 2.1. *In Vitro* Rat Plasma to RBC Partition Coefficients for DLM (A), CIS (B) and TRANS (C)

### A  DLM

<table>
<thead>
<tr>
<th>Min</th>
<th>RBC</th>
<th>Plasma</th>
<th>PC&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg/mL&lt;sup&gt;a&lt;/sup&gt;</td>
<td>µg/mL&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>5.9</td>
<td>19.9</td>
<td>3.4</td>
</tr>
<tr>
<td>30</td>
<td>6.2</td>
<td>18.2</td>
<td>2.9</td>
</tr>
<tr>
<td>45</td>
<td>6.2</td>
<td>18.9</td>
<td>3.0</td>
</tr>
<tr>
<td>60</td>
<td>6.0</td>
<td>18.7</td>
<td>3.1</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mean concentration determined by a modified HPLC technique (n = 2).

<sup>b</sup>Calculated by dividing the concentration in plasma by concentration in RBC.

### B  CIS

<table>
<thead>
<tr>
<th>Min</th>
<th>RBC</th>
<th>Plasma</th>
<th>PC&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td></td>
<td>µg/mL&lt;sup&gt;a&lt;/sup&gt;</td>
<td>µg/mL&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>8.2</td>
<td>11.9</td>
<td>1.5</td>
</tr>
<tr>
<td>30</td>
<td>6.8</td>
<td>15.9</td>
<td>2.3</td>
</tr>
<tr>
<td>45</td>
<td>6.9</td>
<td>16.0</td>
<td>2.3</td>
</tr>
<tr>
<td>60</td>
<td>6.2</td>
<td>18.3</td>
<td>3.0</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mean concentration determined by a modified HPLC technique (n = 2).

<sup>b</sup>Calculated by dividing the concentration in plasma by concentration in RBC.
**C TRANS**

<table>
<thead>
<tr>
<th>Min</th>
<th>RBC µg/mL&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Plasma µg/mL&lt;sup&gt;a&lt;/sup&gt;</th>
<th>PC&lt;sup&gt;b&lt;/sup&gt;</th>
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<td>30</td>
<td>6.5</td>
<td>16.3</td>
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<tr>
<td>45</td>
<td>6.5</td>
<td>16.2</td>
<td>2.5</td>
</tr>
<tr>
<td>60</td>
<td>5.9</td>
<td>18.4</td>
<td>3.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean concentration determined by a modified HPLC technique (n = 2).

<sup>b</sup> Calculated by dividing the concentration in plasma by concentration in RBC.
Table 2.2. DLM (A), CIS (B) and TRANS (C) Concentrations and Tissue:Plasma Partition Coefficients for Adult Rat Tissues After 72-h Infusion

**A  DLM**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>DLM (ng/g)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>PC&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>55 ± 17</td>
<td>-</td>
</tr>
<tr>
<td>Brain</td>
<td>12 ± 5</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>Liver</td>
<td>27 ± 9</td>
<td>0.6 ± 0.3</td>
</tr>
<tr>
<td>Muscle</td>
<td>215 ± 71</td>
<td>3.2 ± 1.1</td>
</tr>
<tr>
<td>Fat</td>
<td>3,749 ± 2,357</td>
<td>45 ± 12</td>
</tr>
</tbody>
</table>

<sup>a</sup> Loading dose = 30 mg/kg po, Infusion rate = 0.36 mg/h for 72 h.
<sup>b</sup> Tissue concentrations determined by GC-MS are reported as the mean ± SD. (n = 7)
<sup>c</sup> PC values are reported as the mean ± SD. (n = 7)

**B  CIS & TRANS**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>CIS (ng/g)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>PC&lt;sup&gt;c&lt;/sup&gt;</th>
<th>TRANS (ng/g)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>PC&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>32 ± 13</td>
<td>-</td>
<td>22 ± 20</td>
<td>-</td>
</tr>
<tr>
<td>Brain</td>
<td>21 ± 10</td>
<td>0.6 ± 0.2</td>
<td>14 ± 11</td>
<td>0.7 ± 0.3</td>
</tr>
<tr>
<td>Liver</td>
<td>10 ± 6</td>
<td>0.3 ± 0.1</td>
<td>NM&lt;sup&gt;d&lt;/sup&gt;</td>
<td>NM&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Muscle</td>
<td>109 ± 83</td>
<td>2.0 ± 0.7</td>
<td>34 ± 9</td>
<td>2.8 ± 1.0</td>
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<tr>
<td>Fat</td>
<td>2,790 ± 877</td>
<td>98 ± 38</td>
<td>1,052 ± 333</td>
<td>67 ± 39</td>
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</tbody>
</table>

<sup>a</sup> Loading dose = 150 mg/kg po, Infusion rate = 0.36 mg/h for 72-h.
<sup>b</sup> Tissue concentrations determined by GC-MS are reported as the mean ± SD. (n = 9)
<sup>c</sup> PC values are reported as the mean ± SD. (n = 9)
<sup>d</sup> Not measurable due to low TRANS concentrations.
Table 2.3. Comparison of DLM (A), CIS (B) and TRANS (C) Tissue:Plasma Partition Coefficients with those Previously Published for Adult Rats.

A  DLM

<table>
<thead>
<tr>
<th>Study</th>
<th>Liver</th>
<th>Brain</th>
<th>Muscle</th>
<th>Fat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Current</td>
<td>0.6</td>
<td>0.2</td>
<td>3.2</td>
<td>45</td>
</tr>
<tr>
<td>Mirfazaelian et al.</td>
<td>0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.2</td>
<td>5.6</td>
<td>49</td>
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<tr>
<td>Kim et al.</td>
<td>0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.2</td>
<td>2.4</td>
<td>46</td>
</tr>
</tbody>
</table>

<sup>a</sup>In vivo Tissue:plasma PCs were calculated by dividing the AUC of tissue by AUC of plasma obtained from a single dose toxicokinetic study.

In Kim et al. (2010) study, plasma and tissue DLM concentration versus time profiles from 0 – 96 h were obtained for single oral dose of 10 mg/kg given to adult SD rats.

In Mirfazaelian et al. (2006) study, plasma and tissue DLM concentration versus time profiles from 0 – 48 h were obtained for single oral dose of 2 or 10 mg/kg given to adult SD rats.
### B  CIS

<table>
<thead>
<tr>
<th>Study</th>
<th>Brain</th>
<th>Liver</th>
<th>Muscle</th>
<th>Fat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Current</td>
<td>0.6</td>
<td>0.3</td>
<td>2.0</td>
<td>98</td>
</tr>
<tr>
<td>Tornero-Velez <em>et al.</em> 1 mg/kg</td>
<td>5.2(^a)</td>
<td>0.6</td>
<td>ND(^b)</td>
<td>126</td>
</tr>
<tr>
<td>Tornero-Velez <em>et al.</em> 10 mg/kg</td>
<td>2.2(^a)</td>
<td>0.5</td>
<td>ND(^b)</td>
<td>87</td>
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<tr>
<td>Willemin <em>et al.</em> 25 mg/kg</td>
<td>2.3(^a)</td>
<td>1.3</td>
<td>1.7</td>
<td>304</td>
</tr>
</tbody>
</table>

\(^a\) *In vivo* Tissue:blood PCs were calculated by dividing the AUC of tissue by AUC of blood obtained from a single dose toxicokinetic study.

\(^b\) Not determined in the corresponding study.

In Tornero-Velez *et al.* (2012) study, plasma and tissue CIS concentration versus time profiles from 0 – 48 h were obtained for single oral dose of 1 or 10 mg/kg permethrin given to adult Long Evans rats.

In Willemin *et al.* (2016) study, plasma and tissue CIS concentration versus time profiles from 0 – 48 h were obtained for single oral dose of 25 mg/kg CIS given to adult SD rats.
C TRANS

<table>
<thead>
<tr>
<th>Study</th>
<th>Brain</th>
<th>Liver</th>
<th>Muscle</th>
<th>Fat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Current</td>
<td>0.7</td>
<td>NM(^b)</td>
<td>2.8</td>
<td>67</td>
</tr>
<tr>
<td>Tornero-Velez et al. 1 mg/kg</td>
<td>4.9(^a)</td>
<td>0.5</td>
<td>ND(^b)</td>
<td>58</td>
</tr>
<tr>
<td>Tornero-Velez et al. 10 mg/kg</td>
<td>0.6(^a)</td>
<td>0.2</td>
<td>ND(^c)</td>
<td>56</td>
</tr>
<tr>
<td>Willemin et al. 25 mg/kg</td>
<td>0.6(^a)</td>
<td>NM(^a)</td>
<td>0.77</td>
<td>65</td>
</tr>
</tbody>
</table>

\(^a\)In vivo Tissue:blood PCs were calculated by dividing the AUC of tissue by AUC of blood obtained from a single dose toxicokinetic study.

\(^b\) Not measurable due to low TRANS concentrations.

\(^c\) Not determined in the corresponding study.

In Tornero-Velez et al. (2012) study, plasma and tissue TRANS concentration versus time profiles from 0 – 48 h were obtained for single oral dose of 1 or 10 mg/kg permethrin given to adult Long Evans rats.

In Willemin et al. (2016) study, plasma and tissue TRANS concentration versus time profiles from 0 – 48 h were obtained for single oral dose of 25 mg/kg TRANS given to adult SD rats.
Table 2.4. DLM (A), CIS (B), and TRANS (C) Mean Concentrations and Tissue:Plasma Partition Coefficients for 21-day-old Rats After 48- and 72-h Infusions\(^a\)

A DLM

<table>
<thead>
<tr>
<th>Tissue</th>
<th>48 h (ng/g)(^b)</th>
<th>PC(^c)</th>
<th>72 h (ng/g)(^d)</th>
<th>PC(^e)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>43 ± 28</td>
<td>-</td>
<td>78 ± 47</td>
<td>-</td>
</tr>
<tr>
<td>Brain</td>
<td>5 ± 3</td>
<td>0.1 ± 0.01</td>
<td>7 ± 2</td>
<td>0.1 ± 0.03</td>
</tr>
<tr>
<td>Liver</td>
<td>4 ± 1</td>
<td>0.1 ± 0.08</td>
<td>6 ± 2</td>
<td>0.1 ± 0.05</td>
</tr>
<tr>
<td>Muscle</td>
<td>34 ± 20</td>
<td>0.8 ± 0.2</td>
<td>79 ± 62</td>
<td>1.0 ± 0.7</td>
</tr>
<tr>
<td>Fat</td>
<td>ND(^f)</td>
<td>ND(^f)</td>
<td>ND(^f)</td>
<td>ND(^f)</td>
</tr>
</tbody>
</table>

\(^a\) Loading dose = 0.25 mg/kg po, Infusion rate = 0.02 mg/h.

\(^b\) Tissue levels determined by GC-MS are reported as the mean ± SD. (n = 6)

\(^c\) PC values are reported as the mean ± SD. (n = 6)

\(^d\) Tissue levels determined by GC-MS are reported as the mean ± SD. (n = 7)

\(^e\) PC values are reported as the mean ± SD. (n = 7)

\(^f\) Not determined due to lack of fat in pups.

No statistically significant differences (p>0.05) in between 48 and 72-h PCs.
### B CIS

<table>
<thead>
<tr>
<th>Tissue</th>
<th>48 h (ng/g)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>PC&lt;sup&gt;c&lt;/sup&gt;</th>
<th>72 h (ng/g)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>PC&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>60 ± 15</td>
<td>-</td>
<td>34 ± 10</td>
<td>-</td>
</tr>
<tr>
<td>Brain</td>
<td>38 ± 6</td>
<td>0.7 ± 0.1</td>
<td>34 ± 5</td>
<td>1.0 ± 0.2&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Liver</td>
<td>30 ± 14</td>
<td>0.7 ± 0.3</td>
<td>26 ± 10</td>
<td>0.7 ± 0.3</td>
</tr>
<tr>
<td>Muscle</td>
<td>240 ± 73</td>
<td>4.1 ± 1.3</td>
<td>244 ± 83</td>
<td>5.2 ± 1.2</td>
</tr>
<tr>
<td>Fat</td>
<td>ND&lt;sup&gt;d&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;d&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;d&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Loading dose = 0.25 mg/kg po, Infusion rate = 0.02 mg/h.<br>
<sup>b</sup> Tissue levels determined by GC-MS are reported as the mean ± SD. (n = 6)<br>
<sup>c</sup> PC values are reported as the mean ± SD. (n = 6)<br>
<sup>d</sup> Not determined due to lack of fat in pups.<br>

(*) denotes statistically significant differences (p<0.05) between 48 and 72-h PCs.
C TRANS

<table>
<thead>
<tr>
<th>Tissue</th>
<th>48 h (ng/g)(^b)</th>
<th>PC(^c)</th>
<th>72 h (ng/g)(^b)</th>
<th>PC(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>58 ± 13</td>
<td>-</td>
<td>28 ± 13</td>
<td>-</td>
</tr>
<tr>
<td>Brain</td>
<td>23 ± 4</td>
<td>0.4 ± 0.1</td>
<td>14 ± 6</td>
<td>0.6 ± 0.3</td>
</tr>
<tr>
<td>Liver</td>
<td>NM(^d)</td>
<td>NM(^d)</td>
<td>NM(^d)</td>
<td>NM(^d)</td>
</tr>
<tr>
<td>Muscle</td>
<td>79 ± 27</td>
<td>1.4 ± 0.5</td>
<td>33 ± 12</td>
<td>1.7 ± 0.6</td>
</tr>
<tr>
<td>Fat</td>
<td>ND(^e)</td>
<td>ND(^e)</td>
<td>ND(^e)</td>
<td>ND(^e)</td>
</tr>
</tbody>
</table>

\(^a\) Loading dose = 1.15 mg/kg po, Infusion rate = 0.05 mg/h.
\(^b\) Tissue levels determined by GC-MS are reported as the mean ± SD. (n = 5)
\(^c\) PC values are reported as the mean ± SD. (n = 5)
\(^d\) Not measurable due to low TRANS concentrations.
\(^e\) Not determined due to lack of fat in pups.

No statistically significant differences (p>0.05) in between 48 and 72-h PCs.
Table 2.5. DLM (A), CIS (B), and TRANS (C) Mean Concentrations and Tissue:Plasma Partition Coefficients for 15-day-old Rats After 48- and 72-h Infusions\(^a\)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>48 h (ng/g)(^b)</th>
<th>PC(^c)</th>
<th>72 h (ng/g)(^b)</th>
<th>PC(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>7 ± 5</td>
<td>-</td>
<td>10 ± 9</td>
<td>-</td>
</tr>
<tr>
<td>Brain</td>
<td>2 ± 2</td>
<td>0.2 ± 0.09</td>
<td>3 ± 2</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>Liver</td>
<td>9 ± 5</td>
<td>1.1 ± 0.4</td>
<td>12 ± 7</td>
<td>1.6 ± 0.6</td>
</tr>
<tr>
<td>Muscle</td>
<td>15 ± 16</td>
<td>2.1 ± 1.1</td>
<td>18 ± 9</td>
<td>1.8 ± 1.0</td>
</tr>
<tr>
<td>Fat</td>
<td>ND(^d)</td>
<td>ND(^d)</td>
<td>ND(^d)</td>
<td>ND(^d)</td>
</tr>
</tbody>
</table>

\(^a\) Infusion rate = 0.03 mg/h.
\(^b\) Tissue levels determined by GC-MS are reported as the mean ± SD. (n = 6)
\(^c\) PC values are reported as the mean ± SD. (n = 6)
\(^d\) Not determined due to lack of fat in pups.

No statistically significant differences (p>0.05) in between 48 and 72-h PCs.
B  CIS

<table>
<thead>
<tr>
<th>Tissue</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(ng/g)\textsuperscript{b}</td>
<td>PC\textsuperscript{c}</td>
</tr>
<tr>
<td>Plasma</td>
<td>81 ± 38</td>
<td>-</td>
</tr>
<tr>
<td>Brain</td>
<td>76 ± 43</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>Liver</td>
<td>87 ± 46</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td>Muscle</td>
<td>194 ± 139</td>
<td>2.5 ± 0.6</td>
</tr>
<tr>
<td>Fat</td>
<td>ND\textsuperscript{d}</td>
<td>ND\textsuperscript{d}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Infusion rate = 0.03 mg/h.
\textsuperscript{b} Tissue levels determined by GC-MS are reported as the mean ± SD. (n = 5)
\textsuperscript{c} PC values are reported as the mean ± SD. (n = 5)
\textsuperscript{d} Not determined due to lack of fat in pups.

(* ) denotes statistically significant differences (p<0.05) between 48 and 72-h PCs.
### C  TRANS

<table>
<thead>
<tr>
<th>Tissue</th>
<th>48 h (ng/g)(^b)</th>
<th>PC(^c)</th>
<th>72 h (ng/g)(^d)</th>
<th>PC(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>80 ± 23</td>
<td>-</td>
<td>52 ± 8</td>
<td>-</td>
</tr>
<tr>
<td>Brain</td>
<td>39 ± 6</td>
<td>0.5 ± 0.1</td>
<td>28 ± 5</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>Liver</td>
<td>92 ± 35</td>
<td>1.2 ± 0.4</td>
<td>83 ± 9</td>
<td>1.6 ± 0.4</td>
</tr>
<tr>
<td>Muscle</td>
<td>227 ± 104</td>
<td>2.8 ± 1.0</td>
<td>283 ± 33</td>
<td>5.5 ± 1.3*</td>
</tr>
<tr>
<td>Fat</td>
<td>ND(^f)</td>
<td>ND(^f)</td>
<td>ND(^f)</td>
<td>ND(^f)</td>
</tr>
</tbody>
</table>

\(^a\) Infusion rate = 0.05 mg/h.

\(^b\) Tissue levels determined by GC-MS are reported as the mean ± SD. (n = 4)

\(^c\) PC values are reported as the mean ± SD. (n = 4)

\(^d\) Tissue levels determined by GC-MS are reported as the mean ± SD. (n = 5)

\(^e\) PC values are reported as the mean ± SD. (n = 5)

\(^f\) Not determined due to lack of fat in pups.

(\(^*\)) denotes statistically significant differences (p<0.05) between 48 and 72-h PCs.
Table 2.6. Comparison of DLM (A), CIS (B), and TRANS (C) Steady-State Tissue:Plasma Partition Coefficients for Adults, 21-day, and 15-day-old Rats with those Estimated from Single Dose Toxicokinetic Studies.

**A DLM**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>15-day-old</th>
<th>21-day-old</th>
<th>Adults</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>0.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.1</td>
<td>0.9</td>
</tr>
<tr>
<td>Liver</td>
<td>1.6</td>
<td>0.1</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>1.6</td>
<td>0.1</td>
<td>0.8</td>
</tr>
<tr>
<td>Muscle</td>
<td>1.8</td>
<td>1.0</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>1.8</td>
<td>0.8</td>
<td>0.9</td>
</tr>
<tr>
<td>Fat</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>12.8</td>
</tr>
</tbody>
</table>

<sup>a</sup> *In vivo* Tissue:plasma PCs at steady-state measured from the current study.

<sup>b</sup>*In vivo* Tissue:plasma PCs calculated by dividing the AUC of tissue by AUC of plasma obtained from a single dose toxicokinetic study. Plasma and tissue DLM concentration versus time profiles from 0 – 72 h were obtained for corresponding single oral doses given to PND 15 (0.1 mg/kg), PND 21 (0.1 mg/kg) and adult (0.5 mg/kg) SD rats.

<sup>c</sup> Not determined due to lack of fat in pups.
### **B** CIS

<table>
<thead>
<tr>
<th>Tissue</th>
<th>15-day-old</th>
<th>21-day-old</th>
<th>Adults</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>1.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.0</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>1.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.3</td>
<td>1.0</td>
</tr>
<tr>
<td>Liver</td>
<td>1.4</td>
<td>0.7</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>2.7</td>
<td>1.7</td>
<td>1.6</td>
</tr>
<tr>
<td>Muscle</td>
<td>4.1</td>
<td>5.2</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>2.9</td>
<td>2.1</td>
<td>2.7</td>
</tr>
<tr>
<td>Fat</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10.6</td>
</tr>
</tbody>
</table>

<sup>a</sup> *In vivo* Tissue:plasma PCs at steady-state measured from the current study.

<sup>b</sup> *In vivo* Tissue:plasma PCs were calculated by dividing the AUC of tissue by AUC of plasma obtained from a single dose toxicokinetic study. Plasma and tissue CIS concentration versus time profiles from 0–24 h were obtained for corresponding single oral doses given to PND 15 (45 mg/kg), PND 21 (45 mg/kg) and adult (120 mg/kg) SD rats.

<sup>c</sup> Not determined due to lack of fat in pups.
**C  TRANS**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>15-day-old</th>
<th>21-day-old</th>
<th>Adults</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>0.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.6</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>0.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.6</td>
<td>0.4</td>
</tr>
<tr>
<td>Liver</td>
<td>1.6</td>
<td>NM&lt;sup&gt;c&lt;/sup&gt;</td>
<td>NM&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>1.4</td>
<td>1.1</td>
<td>0.9</td>
</tr>
<tr>
<td>Muscle</td>
<td>5.5</td>
<td>1.7</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>3.6</td>
<td>2.2</td>
<td>0.8</td>
</tr>
<tr>
<td>Fat</td>
<td>ND&lt;sup&gt;d&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;d&lt;/sup&gt;</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4.8</td>
</tr>
</tbody>
</table>

<sup>a</sup> **In vivo** Tissue:plasma PCs at steady-state measured from the current study.

<sup>b</sup> **In vivo** Tissue:plasma PCs were calculated by dividing the AUC of tissue by AUC of plasma obtained from a single dose toxicokinetic study. Plasma and tissue TRANS concentration versus time profiles from 0 – 24 h were obtained for corresponding single oral doses given to PND 15 (600 mg/kg), PND 21 (600 mg/kg) and adult (300 mg/kg) SD rats.

<sup>c</sup> Not measurable due to low TRANS concentrations.

<sup>d</sup> Not determined due to lack of fat in pups.
Figure 2.1. Experimental Approach for *In vivo* Tissue:Plasma PC Experiment
Figure 2.2. Comparison of Steady-State Tissue:Plasma PCs for DLM between PND 15, PND 21 and Adult Rats after 72-h Infusion. (*) denotes statistically significant differences (p<0.05) compared to adults. (#) denotes statistically significant differences (p<0.05) compared to PND 21 pups.
Figure 2.3. Comparison of Steady-State Tissue:Plasma PCs for CIS between PND 15, PND 21 and Adult Rats after 72-h Infusion. (*) denotes statistically significant differences (p<0.05) compared to adults. (#) denotes statistically significant differences (p<0.05) compared to PND 21 pups.
**Figure 2.4.** Comparison of Steady-State Tissue:Plasma PCs for TRANS between PND 15, PND 21 and Adult Rats after 72-h Infusion. (*) denotes statistically significant differences (p<0.05) compared to adults. (#) denotes statistically significant differences (p<0.05) compared to PND 21 pups.
CHAPTER 3

EFFECTS OF PLASMA PROTEIN BINDING AND MEMBRANE TRANSPORTERS ON
THE BLOOD BRAIN BARRIER PERMEABILITY OF DELTAMETHRIN

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ABSTRACT

It is important to assess the extent to which a chemical in the systemic circulation gains access to the brain, especially for neurotoxic compounds such as pyrethroids. We evaluated BBB permeability and uptake of DLM into the brain as a function of its free fraction in plasma, as well as the potential role of membrane transporters. $^{14}$C-DLM in HBSS buffer with 0.01% and 4% human serum albumin (HSA) concentrations was infused through the left common carotid artery @ 500 µL/min for 2 min, using a Harvard infusion pump. The left half of the brain was then collected, processed and analyzed for DLM by liquid scintillation counting. Infusion of DLM in 0.01% and 4% HSA resulted in left brain levels of 258.3 and 137.0 pmol/g respectively. Cyclosporine A (CSA) was coininfused with $^{14}$C-DLM to examine the potential role of transporters in brain uptake. CSA reduced the uptake of DLM from 258.3 to 175.1 pmol/g when infused in 0.01% HSA, whereas in 4% HSA, uptake was not significantly altered. These data suggest that unidentified influx transporters may play a role in the transport of high concentrations of free DLM across the BBB. Disruption of BBB by prior infusion of mannitol increased the uptake of DLM only in the absence of HSA. In summary, these data show the novel finding that DLM uptake into the brain is dependent on its free fraction in plasma and the binding to plasma proteins appear to limit the uptake of DLM and play a significant protective role under normal physiological conditions. The role of transporters in uptake may only be important at high concentrations of free DLM, which may not be observed at physiological HSA concentrations.
INTRODUCTION

Pyrethroids are synthetic analogs of pyrethrins, produced from the flowers of *Chrysanthemum cinerariaefolium* (Seed, 1973). Pyrethroids are highly lipophilic (Log $K_{ow} = 5.7-7.6$) compounds with very low water solubility (Laskowski, 2002). The phase out of organophosphates in the last decade made pyrethroids as the pesticides of choice in the U.S.A. By 1998, about 25% of the world wide insecticide market was constituted by pyrethroids (Casida and Quistad, 1998). This dramatic increase in the use of pyrethroids stems from their relatively low mammalian toxicity and low environmental persistence. Pyrethroids are often applied to agricultural fields and commercial nurseries to protect crops, residential structures and lawns to control pests. Certain pyrethroids are also utilized on livestock to control broad range of ectoparasites such as mites, headlice and scabies (Anadón *et al.*, 2009; Naeher *et al.*, 2009).

There are several incidents where both occupational and non-occupational exposures of general populations to pyrethroids have been documented. Non-occupational exposure occurs predominantly through the ingestion of fruits and vegetables contaminated with pyrethroids. It was reported that pyrethroid residues were found in food commodities consumed by elementary school-age children (Lu *et al.*, 2010). An association was observed between residential environmental levels of pyrethroids and their metabolite levels in children’s urine. Occupational exposure to pyrethroids occurs mainly through the dermal route (Bradberry *et al.*, 2005). Increased levels of 3-phenoxybenzoic acid (3-PBA), a metabolite of pyrethroids, were observed in Japanese pest control operators (Wang *et al.*, 2007).
Pyrethroids exert neurotoxicity by delaying the closure of voltage-sensitive sodium channels, thereby prolonging the sodium tail current (Soderlund et al., 2002). The duration of sodium channel opening and the subsequent clinical manifestations depend on the structure of pyrethroids. Type I pyrethroids lack the α-cyano group on the alcohol moiety and their major toxic signs are characterized by tremors and paresthesias (T-syndrome). Type II pyrethroids possess α-cyano substituent on the phenoxybenzyl alcohol moiety and include salivation and choreoathetosis (CS-syndrome) as their major signs of toxicity (Shafer et al., 2005).

The entry of most compounds into the central nervous system (CNS) is impeded by the presence of blood brain barrier (BBB), which is characterized by the presence of tight junctions between capillary endothelial cells, lack of fenestrations and limited pinocytotic transport (Begley and Brightman, 2003). The presence of these tight junctions close-off the paracellular diffusional pathway and presents a diffusional barrier for the transport of small, polar solutes. This forces most compounds to cross the BBB either by lipophilic transcellular diffusion or by specialized carrier mediated transport mechanisms. Compounds which are small (MW <400), lipophilic, non-polar containing 5 or less hydrogen bond donors or acceptors, and unionized are ideal compounds to cross the BBB by passive diffusion (Clark, 2003; Van De Waterbeemdm et al., 2001). Deltamethrin (DLM) is a highly lipophilic compound (Log K_{o/w} = 6.1) and is thus expected to cross the BBB readily by passive diffusion. However, this might not always be true due to the binding of these compounds to plasma proteins. It is generally accepted that plasma protein binding limits the brain uptake and that drug distribution to the brain is driven by the free, unbound concentration in circulating plasma (Buxton,
Moreover, a large number of membrane transporters expressed at the BBB are capable of either facilitating or restricting the brain uptake.

To our knowledge, no one has evaluated potential determinants of penetration for DLM or other pyrethroids across the BBB. There is a lack of understanding of the effect of plasma protein binding on brain uptake of DLM and the absence of an accurate quantitative model that appropriately describes this effect. The overall objective of this study was to understand and evaluate the role of plasma protein binding and membrane transporters in uptake of DLM into the CNS, so that the exact contribution of these parameters can be better predicted.

**MATERIALS AND METHODS**

**Materials**

Human Serum Albumin (HSA) and mannitol were purchased from Sigma-Aldrich (St. Louis, MO). Hanks Balanced Salt Solution (HBSS) buffer was purchased from Mediatech (Manassas, VA). Cyclosporine (CSA) was purchased from Sigma-Aldrich (Laramie, WY). Dimethyl Sulfoxide (DMSO) was purchased from Fisher chemicals (Fair Lawn, NJ). Radiolabeled \[^{14}\text{C}]\text{-DLM (57.9 mCi/mMole) was kindly donated by Bayer Crop Science (Research Triangle Park, NC).**

**Animal surgical preparation**

Brain uptake of DLM was measured using a modified *in situ* rat brain perfusion technique, originally developed by Takasato *et al* (Boje, 2001; Takasato *et al.*, 1984a). The University of Georgia Animal Care and Use Committee approved the protocol for
this study. Adult male Sprague-Dawley (SD) rats (300-350g) were purchased from Charles River Laboratories (Raleigh, NC). Upon receipt, all animals were inspected by a qualified animal technician. The rats were quarantined and their health monitored for one week at the University of Georgia’s AAALAC-accredited central animal facility. Each rat was housed in a cage with a 12-h light/dark cycle at ambient temperature (22ºC) and relative humidity (55 ± 5%). Food (5001 Rodent Diet, PMI Nutrition LLC, Brentwood, MO) and water were provided ad libitum. Rats were anesthetized with KAX (Ketamine, Acepromazine, and Xylazine) cocktail. The left common carotid artery was exposed and cannulated with a 25G needle affixed to PE-50 tubing for perfusion fluid administration. Prior to the start of the infusion, the heart was stopped by rapidly severing the cardiac ventricles to eliminate contributions of vascular perfusion from the systemic circulation. The left common carotid artery was then perfused with 2 mL of sterile saline to flush blood from the brain. This was followed by the infusion of corresponding perfusion fluids.

**Processing of brain**

Since the perfusion fluid was infused into the left common carotid artery, only the left cerebral hemisphere was processed for DLM quantitation. The isolated brain tissue was homogenized with a Tekmar Tissumizer IKA Ultra-Turrax homogenizer (Janke and Kunkel Laboratories, Cridersville, OH), by adding two volumes of ice-cold distilled water by weight. An aliquot (100 µL) of the brain homogenate was added to 4 mL of EcoLite scintillation cocktail (MP Biomedicals, Solon, OH). Uptake of DLM was quantified by measuring the radioactivity in each sample by liquid scintillation counting using a Beckman Coulter LS 6500 (Brea, CA) and normalizing to tissue weight.
**Effect of HSA and CSA on DLM associated brain levels**

The perfusion fluid consisted of 10 μM [\(^{14}\text{C}\)]-DLM and 0.01 or 4% HSA with or without the P-gp inhibitor CSA (25 μM) in HBSS buffer. All solutions were incubated for 15 min at 37°C prior to the infusion. In the CSA group, animals were pre-treated with CSA prior to the start of DLM infusion by perfusing 2 mL of sterile saline containing 25 μM CSA. The DLM containing perfusion fluid (including 25 μM CSA) was then infused at a constant rate of 500 μL/min for 2 min using a Harvard 22 syringe infusion pump (Harvard Apparatus, Holliston, MA).

**Role of the BBB in limiting DLM associated brain levels**

Mannitol (1.6 M) was infused at 0.25 mL/kg/sec for 30 seconds prior to the perfusion of DLM to disrupt the BBB (Brown et al., 2004). The perfusion fluid consisting of 1 μM [\(^{14}\text{C}\)]-DLM with or without 4% HSA in HBSS buffer was then infused at a constant rate of 500 μL/min for 2 min using a Harvard 22 syringe infusion pump.

**Statistical Analysis**

All experiments were performed with a minimum of three independent experiments unless otherwise stated. Statistical significance was evaluated between groups using either the Student’ t-test or one way ANOVA, followed by Tukey’s multiple comparison test, as indicated, with a significance level of p<0.05, using Graphpad Prism 6 software.
RESULTS

**Effect of HSA and CSA on DLM associated brain levels**

To assess the influence of plasma protein binding and to evaluate the relative contribution of carrier-mediated transport to BBB permeability of DLM, the brain uptake was measured from 0.01% and 4% HSA in the presence and absence of CSA (Fig 3.1). The brain uptake of DLM, when infused from 0.01% and 4% HSA was 258.3 pmol/g and 137.0 pmol/g, respectively. The uptake from 4% HSA was significantly lower as compared to 0.01% HSA (p<0.05). Interestingly, prior infusion of CSA reduced the uptake only from 0.01% HSA (P<0.05), whereas from 4% HSA, the uptake was not significantly altered (p>0.05).

**Role of the BBB in limiting DLM associated brain levels**

To understand the role of BBB and plasma protein binding in limiting the entry of DLM into the CNS, the uptake of DLM was measured with and without 4% HSA, in control and mannitol treated animals (Fig 3.2). As compared to control animals, prior treatment with mannitol significantly increased the uptake (p<0.05) from HBSS buffer, whereas from 4% HSA, the uptake was not altered significantly (p>0.05).

DISCUSSION

Compounds with high lipophilicity are long known to penetrate the BBB easily. Thus, DLM, which is a highly lipophilic compound (Log \( K_{o/w} = 6.1 \)) is expected to penetrate the BBB with relative ease. However, DLM peak brain concentrations and AUCs were found to be only 20% of plasma values (Kim et al., 2008). The measured brain:plasma PC for
DLM at steady-state was only 0.2 and quite low (Amaraneni et al., to be submitted). It was reported that ideally, a compound should have a Log \( K_{o/w} \) value in the range of 1-3, to have good brain permeation (Dishino et al., 1983; Van De Waterbeem et al., 2001). DLM has a molecular weight (MW) of 505.2 and a Log \( K_{o/w} \) value of 6.1, which might have satisfied the criteria for poor brain permeation. Apart from these two criteria, plasma protein binding also plays a major role in restricting the brain uptake of DLM. It was reported that DLM is about 90% bound to adult human plasma (Sethi et al., 2014). Only about 10% of DLM is free and available for diffusion across the BBB.

Conventionally, only unbound or free fraction of a compound is available for uptake into tissues (Pardridge, 2001). Pardridge et al. reported that the brain extraction of propranolol, measured using the brain uptake index technique, decreased when the albumin concentrations are increased (Pardridge and Landaw, 1984). Marathe et al. examined the effect of serum protein binding on the distribution of brain lidocaine in dogs. A strong and positive correlation was observed between brain to serum and serum free fractions (Marathe et al., 1991). Rowley et al. reported that the binding of the glycine/NMDA receptor antagonists to plasma protein limits their brain penetration (Rowley et al., 1997). Several other studies have reported good agreement between unbound fraction in the plasma and brain concentrations (Cory Kalvass and Maurer, 2002; Dubey et al., 1989).

An *in situ* brain perfusion technique was used in these studies to measure the brain DLM uptake. This technique was originally developed by Takasato et al (Takasato et al., 1984b). As opposed to brain uptake index (BUI) technique, perfusion time can be varied (10 – 600 sec) much longer than that of a single pass (1 – 2 sec), allowing the
BBB permeability to be measured over an extended range. The contributions from peripheral metabolism are eliminated by severing the heart and by pre-perfusing for 30-90 sec to wash out vascular constituents. Finally, not only is this technique sensitive, but also allows for absolute control over the perfusate composition, and thus, allows us to quantify the effect of different protein concentrations and inhibitors on brain uptake by varying the concentrations in the perfusate (Murakami et al., 2000). In the present study, the brain uptake of DLM from 0.01% HSA was significantly lower than from 4% HSA. These results suggest that brain influx for DLM correlated well with the free fraction in the plasma. These results are consistent with results from in vitro studies performed by Dr. Zastre (see Appendix Fig 2). It was demonstrated that accumulation into human cerebral microvessel endothelial cells (hCMEC/D3) increased with increasing DLM unbound fraction. We also conducted an experiment, in which a hypertonic solution of mannitol was infused into the carotid artery of anesthetized rats in order to assess the influence of physical disruption of the BBB on the subsequent brain uptake of DLM. Mannitol infusion produced about 2.2 fold increase in the brain uptake of DLM in the absence of 4% HSA. Virtually, 100% of the DLM is present as the free or unbound fraction under these conditions. Brain uptake of DLM was not significantly altered by mannitol pre-treatment when it was infused with 4% HSA. Only 20% of DLM is present as unbound fraction in the presence of 4% HSA. Albumin, of course, is too large a molecule (MW ~ 66,000) to cross the BBB, even when the tight junctions between the endothelial cells are disrupted by hypertonic mannitol (Brown et al., 2004). The observation that mannitol failed to significantly enhance brain uptake of DLM in the presence of 4% HSA, even when the BBB integrity is compromised, illustrates that
plasma protein binding limits uptake of this neuroactive insecticide, and plays a significant protective role under normal physiological conditions.

In addition to the diffusion barrier, the BBB also presents a transport barrier in the form of multiple carrier-mediated influx and efflux transporters at the brain capillary apical and basolateral membranes. While p-glycoprotein (p-gp) is the major efflux transporter located at the luminal membrane, organic anion transporting polypeptides (OATPs) and organic anion transporters (OATs) are the major solute carriers located at both the luminal and abluminal membranes (Abbott et al., 2006; Lee et al., 2001). It was reported that the uptake of DLM into caco-2 cells was not restricted by p-gp efflux but undergoes absorptive influx transport (Zastre et al., 2013). However, nothing is known about the role of p-gp or other influx transporters at the BBB. Interestingly, prior infusion of CSA significantly reduced the DLM uptake from 0.01% HSA, but not from 4% HSA. At 0.01% HSA, a higher free fraction of DLM is available to interact with the transporters, whereas at 4% HSA, the free fractions are not high enough for CSA to significantly impact the uptake. The fact that the DLM uptake was reduced but not increased by CSA casts doubt on the contributions of some unidentified influx transporters. However, specific transporter family or isoform involved in the transport could not be identified from these studies and requires further research. It should be remembered that despite being a major p-gp inhibitor, CSA is also known to be a promiscuous inhibitor of both influx and efflux transporters (Letschert et al., 2006). Our results are well supported by results from in vitro studies (Appendix). DLM uptake into hCMEC/D3 cells from 0.01% HSA was inhibited in the presence of CSA, which is an indication of a specific influx transport process.
In vitro results also demonstrate that substantial differences in the DLM accumulation into hCMEC/D3 cells can only be observed up to 1% HSA concentration. No significant difference was observed in the uptake between 2% and 4% HSA. Human albumin concentrations range from as low as 2.9% in neonates to as high as 4% in adults. From in vitro results, although it appears that differences in brain uptake of DLM would not be anticipated between human neonates and adults based on differences in albumin concentrations, confirmatory evidence from future in situ studies would be needed in this regard. Regardless of these findings, there is no data available on the effect of lipoprotein binding on the brain uptake of DLM, which is an important player in real life scenario. This may have additional implications related to the significance and physiological utility of these studies. Thus, it would be interesting to see how the binding of DLM to both albumin and lipoproteins together in the rat and human plasma, and how the age-related changes in these protein concentrations affect brain uptake. Apart from the ontogeny of plasma proteins, ontogeny of BBB penetration can also influence the brain influx. Therefore, the future studies will determine the brain uptake of DLM as a function of age-dependent changes in plasma protein binding and BBB penetration.

In conclusion, our results indicate that BBB plays some role in limiting the entry of DLM into the brain. Apart from BBB, plasma protein binding is also instrumental in restricting the entry of DLM into brain under normal physiological conditions. Thus, extensive binding to physiological albumin concentrations resulting in lower free fractions, is the primary reason why brain levels of DLM were lower than anticipated. Also, we found that p-gp does not play a role in limiting the DLM uptake into the brain. A low affinity influx transporter is suggested to contribute to DLM uptake, which becomes
significant only at high free fractions not typically observed under normal physiological conditions. The DLM influx into the brain is a function of its free fraction in the plasma.
REFERENCES


Figure 3.1. Effect of HSA and CSA on the *In vivo* Brain Uptake of DLM. The Left common carotid artery of adult male Sprague-Dawley rats was cannulated and infused (500 µL/min) with 10 µM $^{14}$C-DLM in 0.01% and 4% HSA solutions either alone or in combination with 25 µM CSA for 2 min. Results represent the mean ± SD ($n = 4 - 7$). (*) denotes statistically significant differences (p<0.05).
**Figure 3.2.** Effect of Mannitol on the *In vivo* Brain Association of DLM With and Without 4% HSA. The Left common carotid artery of adult male Sprague-Dawley rats was cannulated and infused (500 μL/min) with 1 μM ¹⁴C-DLM with and without 4% HSA. In the mannitol group, animals were pre-treated with 1.6 M Mannitol prior to infusion of DLM. Results represent the mean ± SD (n = 4 - 5). (*, and **) denotes statistically significant differences (p<0.05).
CHAPTER 4

BRAIN UPTAKE OF DELTAMETHRIN IN RATS AS A FUNCTION OF AGE
DEPENDENT CHANGES IN PLASMA PROTEIN BINDING AND BBB
PENETRATION

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3Manoj A., Jing P., Srinivasa M., Tanzir B.M., Brian S.C., Catherine A.W., and James V.B. To be submitted to Drug Metabolism and Disposition.
ABSTRACT

For potentially neurotoxic compounds such as pyrethroids, it is important to assess the extent to which a chemical in the systemic circulation gains access to the brain. This study investigated the role of age-dependent differences in plasma protein binding and BBB permeability on brain uptake of DLM using an in situ brain perfusion technique. We evaluated uptake of DLM into the brain from different physiological concentrations of HSA (2 – 5%). No significant differences were observed in the uptake of DLM among the groups tested. We measured uptake from plasma of PND 15, PND 21 and adult rats, and found no significant difference. Uptake of DLM from human plasma from birth – 1 week, 1 – 4 weeks, 4 weeks – 1 year, 1 – 3 years and adults was not significantly different among each group. We assessed the influence of individual lipoprotein constituents on the brain entry of DLM by measuring its uptake from physiological concentrations of LDL (100 mg/dL), HDL (50 mg/dL), and their mixture. The % DLM bound was similar among these groups and as was the uptake. We measured uptake from different LDL concentrations (50 – 400 mg/dL) to understand the influence of changes in LDL concentrations on brain uptake in hyper-cholesterolemia patients. The uptake into the brain was reduced with increasing LDL concentrations, although it was statistically significant only at 400 mg/dL. We also assessed brain uptake of DLM as a function of the maturation of the BBB. Uptake of three different concentrations of DLM from 4% HSA was measured in PND 15, PND 21 and adult rats. At all three concentrations tested, uptake in PND 15 and PND 21 pups was significantly greater than in adults. While PND 15 pups exhibited about a 2 – 4 fold increase in uptake compared to PND 21 and adult rats, PND 21 pups exhibited about a 2 fold increase in
uptake relative to adults. This data demonstrate that the brain uptake of environmental concentrations of DLM is not influenced by age dependent differences in plasma protein binding. However, these studies revealed that, at physiological albumin concentrations, uptake of DLM into the brain is a function of maturation of the BBB and is inversely proportional to age. The younger the rat, the greater the uptake. This increased uptake in younger rats may result from increased permeability of the BBB in this age group.
INTRODUCTION

Pyrethroids are the synthetic derivatives of pyrethrins obtained from the flowers of *Chrysanthemum cinerariaefolium*. Combined with the reduced use of organophosphates and their relatively lower mammalian toxicity, the use of pyrethroids has dramatically increased over the last decade (Power and Sudakin, 2007). The uses of pyrethroids are many fold and are often used as insecticides in agricultural, commercial and residential settings. Medically, they are used in the treatment of scabies, mites and headlice on pets and humans (Naeher *et al*., 2009). Several occupational and non-occupational exposures of large segments of the populace have been documented, although at low levels. The most frequent route of exposure is ingestion of pyrethroids from dust and through eating foods containing residues of these chemicals (Baker *et al*., 2000). About 75% of the urine samples sampled from a large German population were found to have pyrethroid metabolites (Heudorf *et al*., 2004). Flight attendants working on commercial flights disinfected with pyrethroids were found to have elevated urinary levels of pyrethroid metabolites compared to those that did not use pyrethroids (Wei *et al*., 2012). Low levels of these chemicals may be found in fruits and vegetables because of their application to crops (Lu *et al*., 2010).

Pyrethroids are classified as type I and type II pyrethroids based on their chemical structure and toxic signs from acute poisoning. Type II pyrethroids have an α-cyano substituent on the 3-phenoxybenzyl alcohol moiety. Pyrethroids primarily act on voltage-sensitive sodium channels in the central nervous system and exert neurotoxicity by delaying the closure of sodium channel gates (Soderlund *et al*., 2002). Type I pyrethroids delay the closure of sodium channels gates only for few milliseconds
resulting in repetitive nerve discharges, whereas the type II pyrethroids delay it for several seconds resulting in stimulus-dependent nerve depolarization and blockage (Shafer et al., 2005). This difference in the duration of modification results in characteristic clinical manifestations of type I and type II pyrethroids poisoning. Toxic signs of type I pyrethroid poisoning are characterized by hyperexcitation, fine tremors followed by whole body tremors and paresthesia, typically named as “T-syndrome”. Type II pyrethroids are characterized by excessive salivation, coarse tremors progressing to choreoathetosis and clonic seizures, collectively referred to as “CS-syndrome” (Ray, 2001).

The BBB characterized by extensive tight junctions and the absence of fenestrations, acts as a barrier which restricts the entry of most compounds from blood to brain (Ballabh et al., 2004). In general, compounds with high lipophilicity, small molecular size and low plasma protein binding diffuse freely across the BBB (Clark, 2003). Despite being highly lipophilic (Log P = 6.1), the entry of DLM into the brain is limited by its high molecular weight (505.2) and high plasma protein binding (80%). Only unbound compound is free to diffuse across the BBB. The brain uptake of DLM is influenced by their extent of plasma protein binding and is reduced with a decrease in its free fraction ($f_u$) (Amaraneni et al., 2016). Plasma proteins show age-dependent differences between neonates and adults, in terms of their concentration and binding affinity (Alcorn and McNamara, 2003). Neonates have lower plasma protein binding compared to adults resulting in higher free fractions. It is important to assess the influence of age-dependent differences in the plasma protein binding on the brain uptake of DLM. By examining the effect of binding to various concentrations of albumin
and lipoproteins in the physiological range, it is possible to understand how the brain uptake of DLM varies in individuals with disease conditions such as hypoalbuminemia or hypercholesterolemia.

Immature rats were more sensitive to the potential neurotoxicity of high doses of pyrethroids (Sheets et al., 1994). This can be attributed, in part, to the immaturity of the BBB along with other immature physiological and biochemical processes that alter the brain dosimetry. In general, the BBB in neonates is immature, poorly formed and leaky. The BBB undergoes several structural changes during development before becoming fully functional. Thus, the development of the BBB is a gradual process in humans, which is more immature and permeable at birth and becomes fully functional by approximately 6 months of age (Watson et al., 2006).

There is very little information available in the literature on the differences in the BBB permeability of pyrethroids between younger and adult age-groups. No studies have been conducted regarding how the binding of DLM to albumin and individual lipoprotein constituents such as LDL and HDL, affect its brain permeability. The effect of age-dependent differences in such plasma proteins and BBB penetration has also never been investigated. The objective of the current project was to examine the influence of age-dependent differences in the plasma protein binding and the BBB penetration on the brain uptake of DLM using an in situ brain perfusion technique. The effect of binding to albumin and lipoproteins constituents on the brain uptake was investigated. Such a determination of BBB permeability can be incorporated into physiologically based pharmacokinetic (PBPK) models for prediction of brain dosimetry in humans of different ages.
MATERIALS AND METHODS

Materials

Human Serum Albumin (HSA) was purchased from Golden West Biologicals (Temecula, CA). Low-density lipoproteins (LDL) and high-density lipoproteins (HDL) were purchased from Lee Biosolutions, Inc. (Maryland Heights, MO). Rat plasma of different age groups was purchased from Innovative Research, Inc. (Novi, MI). Hanks Balanced Salt Solution (HBSS) buffer was purchased from Mediatech (Manassas, VA). Radiolabeled [\(^{14}\)C]-DLM (57.9 mCi/mMole) was kindly donated by Bayer Crop Science (Research Triangle Park, NC). EcoLite scintillation cocktail was provided by MP Biochemicals (Solon, OH). Acetonitrile (HPLC-grade), hexamethyldisilazane (Reagent-grade), sodium fluoride (NaF) (purity, 99.0 %) were purchased from Sigma-Aldrich (St. Louis, MO). Isooctane (purity, 99.0 %) and 2-Octanol (Laboratory-grade) were purchased from Fisher Scientific (Pittsburgh, PA).

Human Plasma Samples

Anonymized plasma samples were obtained from Cincinnati Children’s Hospital Medical Center (CCHMC) Biobank in Cincinnati, OH. Refrigerated plasma samples that were not used for clinical studies were pooled, frozen at -20°C, and accumulated for each of the following age-groups: 0 – 1 week; 1 – 4 weeks; 4 weeks – 1 year; 1 – 3 years. Adult plasma was purchased from Innovative Research, Inc. (Novi, MI). Specimens from pediatric donors suffering from blood diseases were excluded. The health of the subjects was not otherwise verified. Gender and ethnicity were not
inclusion criteria. The use of the anonymous plasma samples for the current research project was reviewed and approved by the CCHMC Tissue Use Committee.

**In vitro binding studies of DLM**

Binding of DLM was determined by a three step solvent extraction method developed by Sethi *et al.* (Sethi *et al.*, 2014). Binding to different HSA concentrations (2 – 5%), physiological lipoprotein concentrations (100 mg/dL LDL, 50 mg/dL HDL, and their mixture), and different LDL concentrations (50, 200, and 400 mg/dL) was determined to correlate % free DLM to brain uptake. Briefly, a volume of 90 µL of corresponding biological matrix (HSA or lipoproteins) was spiked with 10 µL of 10 µM \[^{14}C\]-DLM in silanized glass vials. Samples were then incubated in an orbital shaker at 37°C with constant shaking (100 rpm) for 15 min for HSA and 30 min for lipoproteins. After 15 or 30 min, samples were removed and 200 µL of isooctane was added to each tube to extract the free (unbound) compound. The samples were vortexed for 30 sec and centrifuged at 10,000 rpm for 10 min. Three 50 µL aliquots were removed of the isooctane layer and transferred to scintillation vials containing 4 mL of EcoLite™ scintillation cocktail and vortexed for 10 sec. After removing the remaining isooctane, 300 µL of acetonitrile was added to each tube to extract the total bound compound. The tubes were then vortexed and centrifuged as mentioned above. A 50 µL aliquot was taken in triplicates and transferred to a scintillation vial with EcoLite™ and vortexed. The tubes were counted for radioactivity by LS 6500 Liquid Scintillation counter.

The *in situ* brain perfusion solutions were incubated for 15 – 30 min prior to infusion. To determine if this short time of incubation affects the binding of DLM. The
different biological matrices (human plasma, HSA, and lipoproteins) used in perfusion studies, were spiked with $[^{14}C]$-DLM and incubated for 15, 30, 60, 120, 180 and 240 min before the extraction. After the appropriate time of incubation, the above extraction procedure was repeated and counted for radioactivity. Since human plasma contains both albumin and lipoproteins, after performing isooctane extraction and before proceeding to acetonitrile extraction, tubes were extracted with n-octanol to determine the fraction bound to lipoproteins. After isooctane extraction was done, 200 µL of n-octanol was added to each tube, vortexed and centrifuged as above. Three 50 µL aliquots were then removed and added to the EcoLite cocktail in scintillation vials, vortex and counted for radioactivity.

**Animal surgical preparation**

Brain uptake of DLM was measured using a modified *in situ* rat brain perfusion technique, originally developed by Takasato *et al* (Boje, 2001; Takasato *et al.*, 1984). The University of Georgia Animal Care and Use Committee approved the protocol for this study. Adult male Sprague-Dawley (SD) rats (300-350 g) and pregnant female SD rats were purchased from Charles River Laboratories (Raleigh, NC). Upon receipt, all animals were inspected by a qualified animal technician. The rats were quarantined and their health monitored for one week at the University of Georgia’s AAALAC-accredited central animal facility. For immature BBB studies, the pups were allowed to reach 15 or 21 days of age before being used for experiments. Each rat was housed in a cage with a 12-h light/dark cycle at ambient temperature (22°C) and relative humidity (55 ± 5%). Food (5001 Rodent Diet, PMI Nutrition LLC, Brentwood, MO) and water were provided *ad libitum*. Rats were anesthetized with KAX (Ketamine, Acepromazine, and Xylazine)
cocktail. The left common carotid artery was exposed and cannulated with a 23G needle affixed to PE-50 tubing for perfusion fluid administration. In experiments with pups, due to the small diameter of the carotid artery, a 27G needle affixed to PE-10 tubing was used for perfusion fluid administration.

**Perfusion protocol**

Prior to the start of the infusion, the cardiac ventricles were rapidly severed to ensure the elimination of flow contributions from the systemic circulation. The left common carotid artery was then perfused with 2 mL and 0.5 mL of sterile saline in adults and pups respectively, to remove blood. This was followed by the infusion of the corresponding perfusion fluids. Once the infusion was stopped, the left common carotid artery was again perfused with corresponding volumes of sterile saline to remove unabsorbed DLM in the vasculature.

**Processing of brain**

Since the perfusion fluid was infused into the left common carotid artery, only the left cerebral hemisphere was processed for DLM quantitation. The isolated brain tissue was homogenized with a Tekmar Tissumizer IKA Ultra-Turrax homogenizer (Janke and Kunkel Laboratories, Cridersville, OH), by adding two volumes of ice-cold distilled water by weight. An aliquot (1000 µL) of the brain homogenate was added to 4 mL of EcoLite scintillation cocktail (MP Biomedicals, Solon, OH). Uptake of DLM was quantified by measuring the radioactivity in each sample by liquid scintillation counting using a Beckman Coulter LS 6500 (Brea, CA) and normalizing to tissue weight.
**Effect of binding to physiological HSA**

Uptake of DLM into brain from different physiological HSA concentrations was measured in adult SD rats. The perfusion fluid consisted of 1 µM $[^{14}\text{C}]$-DLM in 2%, 2.9%, 4% or 5% HSA solutions made in HBSS buffer. All solutions were incubated for 15 min at 37°C in an orbital shaker, prior to the infusion. The perfusion fluid was then infused at a constant rate of 500 µL/min for 2 minutes using a Harvard 22 syringe infusion pump (Harvard Apparatus, Holliston, MA).

**Effect of binding to physiological LDL and HDL**

The brain uptake of DLM from physiological concentrations of LDL (100 mg/dL), HDL (50 mg/dL) and their mixture (100 mg/dL LDL and 50 mg/dL HDL) was measured from adult SD rats. A control group was also included, where the uptake of DLM was measured from HBSS buffer alone. Brain uptake was also measured from the LDL concentrations of 50, 200, and 400 mg/dL to mimic uptake in individuals with hypocholesterolemia and hypercholesterolemia. The perfusion fluid consisted of 1 µM $[^{14}\text{C}]$-DLM in corresponding lipoprotein solutions made in HBSS buffer. All solutions were incubated for 30 min at 37°C in an orbital shaker prior to the infusion. The perfusion fluid was then infused at a constant rate of 500 µL/min for 2 minutes using a Harvard 22 syringe infusion pump (Harvard Apparatus, Holliston, MA).

**Effect of age-dependent differences in plasma protein binding**

The uptake of DLM from rat plasma and human plasma of different age groups was measured in adult SD rats. For uptake studies from rat plasma, the perfusion fluid consisted of 1 µM $[^{14}\text{C}]$-DLM in PND 15, PND 21 or adult rat plasma. For uptake studies
from human plasma, the perfusion fluid consisted of 1 µM $[^{14}\text{C}]$-DLM in human plasma of ages birth - 1 week, 1 - 4 weeks, 4 weeks - 1 year, 1 – 3 years, and adults. All solutions were incubated for 15 min at 37°C in an orbital shaker, prior to the infusion. The perfusion fluid was then infused at a constant rate of 500 µL/min for 2 minutes using a Harvard 22 syringe infusion pump (Harvard Apparatus, Holliston, MA).

**Effect of age-dependent differences in BBB penetration**

The uptake of different concentrations of DLM was measured in PND 15, PND 21 and adult rats. The perfusion fluid consisted of either 1, 10 or 50 µM $[^{14}\text{C}]$-DLM in 4% HSA solution made in HBSS buffer. All solutions were incubated for 15 min at 37°C in an orbital shaker, prior to the infusion. The perfusion fluid was then infused at a constant rate of 500 µL/min for 2 minutes using a Harvard 22 syringe infusion pump (Harvard Apparatus, Holliston, MA). The flow rate was reduced to 250 µL/min and the infusion time was increased to 4 minutes in PND 15 and PND 21 pups to make sure that the perfusion pressure in microvessels was not too high and the integrity of BBB was not compromised. A control study was conducted in adult rats to make sure that the change in flow rate did not alter the uptake of DLM. As part of the control study, 1 µM $[^{14}\text{C}]$-DLM from 4% HSA was infused in adult SD rats at a flow rate of 250 µL/min for 4 minutes.

**Data analysis**

All experiments were performed with a minimum of three independent animals unless otherwise stated. Statistical significance was evaluated between groups using either student’s $t$-test or one way ANOVA, followed by Dunnett’s or Tukey’s multiple
comparison test, as indicated, with a significance level of p<0.05, using Graphpad Prism 5 software.

RESULTS

Effect of incubation time on the binding of DLM

These *in vitro* experiments were conducted to determine the influence of incubation time on the binding of DLM to different biological matrices that were used in animal perfusion studies. No significant change in binding of DLM to human plasma was observed across the incubation times tested (Fig 4.1). The same pattern was observed in the binding of DLM to 4% HSA, except that the binding increased to 71% after 240 min of incubation (Fig 4.2). However, there was an initial increase in LDL binding from 48% to 63% after 30 min of incubation (Fig 4.3). No further changes were observed after 30 min. There were no significant time related changes in the binding of DLM to HDL (Fig 4.4) and the lipoprotein mixture (Fig 4.5). The percent bound remained the same throughout the incubation periods.

Impact of binding to physiological albumin concentrations

In order to assess the influence of physiological changes in albumin concentration on the brain uptake of DLM, experiments were conducted to measure uptake from 2 - 5% HSA. Brain uptake (pmol/g) of DLM from these experiments is shown in Fig 4.6. Interestingly, the uptake of DLM did not vary significantly between 2% and 5% HSA (p>0.05). This finding was supported by the fact that there was no significant difference in the binding of DLM between 2 – 5% HSA (Fig 4.7). The bound DLM increased from
41% in 2% HSA to 49% in 5% HSA. This increase was not significant enough to affect the uptake into the brain.

**Effect of binding to physiological lipoprotein concentrations**

To establish the effect of binding to individual lipoprotein constituents i.e. LDL and HDL on the BBB permeability of DLM, the uptake of DLM into the brain from physiological concentrations of LDL (100 mg/dL), HDL (50 mg/dL) and their mixture was measured (Fig 4.8). Even though the uptake from LDL and the mixture was slightly lower than that of HDL, the difference was not significant (p>0.05). The uptake from LDL, HDL, and the mixture was slightly lower as compared to HBSS buffer, but again not significantly different (p>0.05). It can be observed in Fig 4.9 that there was no significant difference in the binding of DLM to LDL (48%), HDL (51%), and the mixture (58%) (p>0.05). Thus, there was not much change in free fraction between these groups to affect the brain uptake.

To mimic the uptake in individuals with hypocholesterolemia and hypercholesterolemia, the brain uptake of DLM from 50, 200, and 400 mg/dL LDL was measured (Fig 4.10). The uptake of DLM decreased with increased LDL concentration. The lowest uptake was measured from 400 mg/dL, which was significantly different from that of 50 and 100 mg/dL (p<0.05). Even though the uptake from 200 mg/dL was lower than 50 and 100 mg/dL, the difference failed to reach statistical significance and so was between 50 and 100 mg/dL. In Fig 4.11, it can be seen that the binding of DLM was highest at 400 mg/dL (83%) and was significantly different from that of 50 (61%), 100 (55%) and 200 mg/dL (66%) concentrations.
Impact of age-dependent differences in plasma protein binding

To determine the influence of age-dependent differences in plasma protein binding on the brain uptake of DLM, the uptake was measured from human and rat plasma of different age groups. In Fig 4.12, it can be seen that the uptake of DLM from 15-day rat plasma (9.1 pmol/g) was not significantly different to that of 21-day (12.5 pmol/g) and adult rat plasma (9.7 pmol/g) (p>0.05). Sethi et al. (Sethi et al., 2015) reported that the total binding of DLM to adult (80%) and 21-day-old rats (79%) was only slightly higher than that of 15-day-old rats (73%).

Similar to rat plasma, the uptake from human plasma did not change much from birth - 1 week (13.6 pmol/g) to adults (13.5 pmol/g) (Fig 4.13). Thus, the difference in uptake from human plasma was not significantly different between the age-groups tested (p>0.05). Sethi et al. (Sethi et al., 2015) reported that the total binding of DLM to human plasma of ages birth – 1 week (73%) and 1 – 4 weeks (77%) was significantly lower compared to adults (90%). However, binding did not change significantly in other age groups. Interestingly, such differences were not observed in our in situ uptake studies.

Impact of age-dependent differences in BBB penetration

To examine how the differences in BBB penetration affect the brain entry of DLM, the uptake of different concentrations of DLM was measured by cannulating PND 15, PND 21, and adult SD rats (Fig 4.14). The uptake of DLM was significantly higher in PND 15 rats compared to PND 21 and adult rats at all concentrations tested (p<0.05). Similarly, PND 21 rats exhibited significantly greater DLM uptake compared to adults at
10 and 50 µM (p<0.05). Although this was also true at 1 µM DLM, the difference failed to reach statistical significance. The magnitude of difference in the uptake between PND 15 and adult rats was greatest at 1 µM concentration, while for 21-day rats, it remained constant at all concentrations tested (Table 4.1). A control study was conducted in adult rats to see if the change in flow rate affects the uptake into the brain. In Fig 4.16, it can be observed that the uptake was slightly decreased from 12.8 pmol/g to 9.3 pmol/g when the flow rate was reduced from 500 µL/min to 250 µL/min. However, this decrease was not statistically significant (p>0.05).

The brain uptake of increasing concentrations of DLM from 4% HSA in all the three age-groups is shown in Fig 4.15. These results suggest that the uptake of DLM from 4% HSA was linear at the concentration range tested in all the three age groups.

DISCUSSION

There are primarily three mechanisms by which a compound can enter the brain. They are passive diffusion, carrier-mediated (facilitative), and ATP-dependent (active) transport processes (Lee et al., 2001). The experiments from our previous work have indicated that DLM is not a substrate of p-glycoprotein (p-gp). An uncharacterized low affinity influx transporter is suggested to contribute to DLM uptake by the brain, which is significant only at higher free fractions of DLM, which is not typically observed either in adults or younger age groups (Amaraneni et al., 2016). Thus, the ontogenic differences in the transporters do not contribute to the age-dependent differences in the brain dosimetry of DLM. Therefore, two factors, namely lower plasma protein binding and an
immature BBB, are considered to be important determinants of greater transport of DLM across the BBB in younger age-groups.

The free fraction of a compound in plasma is an important determinant of the extent to which a compound diffuses freely across the BBB along its concentration gradient. Our previous work established that the \textit{in situ} brain uptake of DLM from 0.01\% HSA was significantly greater than that of 4\% HSA, confirming that brain uptake is dependent on the free fraction of DLM. Interestingly, the uptake of DLM into human cerebral microvessel endothelial cells (hCMEC/D3) did not vary significantly between 2\% - 4\% HSA \cite{Amaraneni2016}. The HSA concentrations in humans range from 4\% in adults and to 2.9\% in neonates at birth \cite{Sethi2015}. Therefore, we chose to measure the \textit{in situ} brain uptake of DLM from 2\% - 5\% HSA to see how the binding of DLM to the physiological range of HSA affects its brain uptake. This would tell us not only about the effect of age-dependent differences in HSA concentration on the brain uptake of DLM, but also how it varies in individuals with hypo- or hyper-albuminemia. Similar to our previous \textit{in vitro} findings, the uptake of DLM into brain did not vary significantly between 2\% and 5\% HSA. The bound DLM at 2\% HSA (41\%) was not significantly different from that of 5\% HSA (49\%). These results suggest that the age-dependent differences in HSA concentration do not significantly affect the brain uptake of low levels of DLM. Either extremely low levels of HSA (<2\%) or very high concentrations of DLM are required to impact the brain uptake significantly, both of which are rare to occur physiologically.

Since DLM is a highly lipophilic compound and exhibits a high degree of binding to lipoproteins in plasma, we focused on how the binding of DLM to individual lipoprotein
constituents such as LDL, HDL and their mixture affect its brain uptake. No studies have been undertaken to examine the influence of binding to individual lipoprotein constituents on the brain uptake of pyrethroids. These experiments were conducted at a normal physiological range of LDL and HDL. The brain uptake of DLM from physiological concentrations of LDL, HDL, and their mixture did not vary significantly from each other. Although the uptake from the lipoprotein mixture (50 mg/dL HDL and 100 mg/dL) was lower than the uptake from HDL (50 mg/dL), it was not statistically significant. It should be noted that the corresponding free fractions of DLM did not vary significantly between these groups. Unexpectedly, despite the same free fractions, the DLM uptake from physiological concentrations of lipoproteins was much higher compared to the uptake from physiological HSA concentrations. This might be attributed to the presence of lipoprotein receptors on the surface of brain capillary endothelial cells (ECs). It is also possible that DLM has a higher binding affinity for albumin than lipoprotein constituents due to simple partitioning into hydrophobic regions of lipoproteins. There is growing evidence that the BBB is equipped with LDL receptors for maintaining lipid and cholesterol homeostasis (Méresse et al., 1989). Pitas et al. (Pitas et al., 1987) demonstrated the presence of apoB,E (LDL) receptors in rat and monkey brains using immunocytochemistry. They also reported that apolipoprotein E and apo A-I were present in the human cerebrospinal fluid (CSF). LDL receptor mediated transcytosis is the primary mechanism for lipid transport and cholesterol homeostasis in the central nervous system. Dehouck et al. (Dehouck et al., 1997) used a coculture model of bovine brain capillary endothelial cells and rat astrocytes to demonstrate the transport of $^{125}$I-LDL and acetylated $^{125}$I-LDL through brain capillary EC monolayers.
They reported that LDL was transcytosed through the BBB via a receptor mediated mechanism. This mechanism is successfully employed in drug delivery to the brain using nanoparticles (Kreuter, 2001). Nanoparticles coated with polysorbates serve as an anchor for apo E and adsorb them onto their surface. These nanoparticles with apo E on their surface mimic LDL particles and interact with LDL receptors leading to their uptake by the ECs.

There has been a dramatic increase in hypercholesterolemic adults in the United States (Ford et al., 2010). Consequently, the use of cholesterol lowering drugs such as statins has also increased rapidly (Kit, 2014). Since hypercholesterolemia is usually associated with increased LDL levels, we examined the brain uptake of DLM from higher LDL concentrations to get a glimpse in patients with hypercholesterolemia. The uptake into the brain decreased with increasing LDL concentrations with the lowest uptake measured at 400 mg/dL. This can be correlated to the lowest free fraction of DLM at 400 mg/dL, which was almost half when compared to the other concentrations tested. These results corroborate our previous finding that the uptake of DLM into the brain is a function of its free fraction. However, caution must be employed when extrapolating these findings to human hypercholesterolemic patients. Since under normal physiological conditions, these elevated levels of LDL always coexist with albumin and other lipoproteins, it is difficult to assess the cumulative effect of binding to all of these plasma proteins on the brain uptake. At least, these results suggest that the brain uptake of DLM may be slightly lower in individuals with hypercholesterolemia as compared to those with normal LDL levels.
Plasma proteins show age-dependent differences in terms of their concentration and binding affinity. Neonatal animals have relatively low plasma protein binding capacity due to low albumin levels, lower binding affinity, and elevated levels of bilirubin, fatty acids and other substances that compete with xenobiotics for binding sites (Ginsberg et al., 2004). Plasma albumin levels in neonates are about 75% of adult human levels, while α₁ acid-glycoprotein concentrations are 75% of those in adults by 6 months (Alcorn and McNamara, 2003). Marked age-dependent differences in plasma binding may result in a significant effect on the pyrethroids’ BBB permeability and their brain dosimetry. In contrast, neonatal rats have about 50% of the adult plasma proteins, albumin and α₁ acid-globulins (de Zwart et al., 2008). Thus, the plasma protein binding capacity of neonatal and preweanling rats for pyrethroids is likely significantly lower than for their human counterparts. Therefore, we have chosen to measure the brain uptake of DLM from human and rat plasma of different age groups separately.

Sethi et al. (Sethi et al., 2015) reported that the total plasma binding of DLM was about 80% in both 21-day-old and adult rats, while it was approximately 75% in 15-day-old rats. Even though the difference in binding between 15-day-old and adult rats was reported to be statistically significant, the difference in the free fraction was not high enough to impact the brain uptake. We did not observe any significant difference in the in situ brain uptake of DLM from the plasma of 15-day, 21-day, and adult rats. Sethi et al. (Sethi et al., 2015) reported that significant differences in the binding of DLM to human plasma were observed only up to 4 weeks of age, after which the binding was reported to be similar to that of adults. The binding of DLM in human plasma of ages 1 week and 4 weeks was reported to be approximately 75%, which was lower compared
to adults (90%). We measured the uptake of DLM from human plasma of ages birth – 1 week, 1 – 4 weeks, 4 weeks – 1 year, 1 – 3 years, and adults. Interestingly, our results indicated that there was no significant difference in the brain uptake of DLM between any of the age groups tested. Together, these results suggest that the age-dependent differences in plasma protein binding do not significantly impact the brain uptake of DLM at low exposure levels.

There are only limited data on the stages of human development at which the permeability of the maturing BBB becomes comparable to that of adults. Some investigators conclude that recent morphological, biochemical and molecular data provide substantial evidence for the existence of well developed, effective barrier mechanisms in the fetus and newborn (Ek et al., 2012). Saunders et al. (Saunders et al., 2012) reported that intercellular tight junctions between cerebral endothelial cells are functionally effective as soon as they differentiate. These investigators do note that some barrier mechanisms may work to a lesser extent in maturing humans and laboratory animals, though other processes exhibit a higher rate of function.

Vascularization of the brain and BBB maturation have been studied in detail in the rat. Several researchers conducted early ultrastructural studies of embryonic and postnatal morphological changes of the cerebral microvasculature of rats (Bär and Wolff, 1972; Caley and Maxwell, 1970; Schulze and Firth, 1992). The investigators monitored maturation of the primary structural components of the BBB, including the vessel wall endothelium, basement membrane, pericytes and astrocytes. Structural integrity and maturity were reported by each research group to be achieved by PND 21. The magnitude of microvascular sprouting largely paralleled an increase in the cerebral
cortical mass during these 21 days (Donahue and Pappas, 1961; Rowan and Maxwell, 1981). Stewart and Hayakawa (Stewart and Hayakawa, 1987) described a progressive reduction in the number and width of clefts between adjacent cerebral capillary endothelial cells, which paralleled a decrease in the BBB permeability to horseradish peroxidase in maturing mice. The developmental tightening of the endothelial junctions was associated with an increasing thickness of the basement membrane and envelopment by pericytes. Basement membrane, pericytes and astrocytes signaling induce and maintain the integrity of the endothelial tight junctions (Liebner et al., 2011). Thus, these cells’ structural and functional development must occur in concert with that of the endothelium. Ferguson and Woodbury (Ferguson and Woodbury, 1969) monitored cerebral uptake of inulin in rats as an index of BBB penetration. Brain uptake of the water soluble compound progressively decreased with increasing age from PND 4-26. Leibnar et al. (Liebner et al., 2011) emphasized the BBB tightness is not “switched on” at a particular point during brain angiogenesis, but occurs as a gradual process during embryogenesis and postnatal maturation.

The immature BBB in neonatal rats resulted in higher levels of glucocorticoids compared to adults (Arya et al., 2006). Upon injection of trypan blue, mice of 4 weeks of age incorporated more dye compared to mice of 5-8 weeks of age (Ribatti et al., 2006). Kyu-bong Kim et al. reported that brain DLM concentration versus time profiles are age-dependent and that the youngest rats exhibited the highest brain concentrations over time, upon ingestion of DLM (Kim et al., 2010). The BBB was not fully developed structurally and was not fully functional until about the time of weanling. Interendothelial junctions in the immature BBB are characterized by membrane separations greater than
20 nm and are leaky (Schulze and Firth, 1992). These leaky interendothelial junctions in developing brain results in high permeability. The maturation of the BBB is accompanied by the disappearance of these membrane separations and establishment of complex tight junctions. Thus, the structural changes at tight junctional contacts underlie the developmental tightening of the BBB. We measured the brain uptake of DLM in 15-day, 21-day and adult rats by cannulating their carotid artery.

We have controlled for the free fraction and measured the uptake of three different concentrations of DLM by cannulating PND 15, PND 21 and adult rats. At all the concentrations tested, the youngest rats exhibited the highest uptake of DLM. Interestingly, the uptake of 1 µM DLM in 15-day-old rats was 3.7 fold higher than that of adults, while for 10 and 50 µM DLM, it was 2.2 and 2.5 fold higher respectively. Similarly, the magnitude of difference in the uptake between PND 15 and PND 21 rats was 2.1 and highest at the lowest DLM concentration, while it remained constant at about 1.5 fold difference at the other two DLM concentrations. It was possible that the uncharacterized influx transporter at the BBB was saturated at higher DLM concentrations and thus, had a constant contribution to the DLM uptake. The uptake in PND 21 rats is about 1.5 fold higher than that of adults at all the concentrations tested. These results suggest that, the younger the rats, the greater is the uptake. The linear uptake of DLM in the concentration range tested in all the three age groups indicates that passive diffusion is the main transport process involved.

Several studies in pediatric patients involving intravenous drug injection and CSF monitoring have failed to provide definitive information on the age-dependence of permeability changes of the BBB or brain-CSF barriers (Heideman et al., 1989; Kellie et
al., 2002; Lowe et al., 2001). Jacobs et al. (Jacobs et al., 1986) administered Imipenem and Cilastatin by i.v. infusion and assessed CSF drug concentrations. No correlation was seen between age and the extent of CSF penetration. In an early study, Widell (Widell, 1958) measured concentrations of several proteins in the CSF of 98 normal children aged 0 – 13 years. Total protein levels diminished during the first 9 months of life. Similar findings were subsequently reported by other investigators (Statz and Felgenhauer, 1983; Wong et al., 2000). Both groups of clinicians found that CSF protein concentrations decreased rapidly during the initial 6 months postnatally. Shah et al. (Shah et al., 2011) measured CSF protein levels in a large group (n = 375) of infants only 56 days of age and younger. Levels were quite variable but lowest during the first 2 - 4 weeks after birth. An inverse linear relationship was reported between entry of sodium fluorescein into the CSF and age of subjects up to 6 months old (Misra et al., 1987). The largest drop in CSF fluorescein levels occurred during initial 2 weeks of life. Increased BBB permeability to unconjugated bilirubin is an important contributor to neonatal jaundice (Brito et al., 2014). Higher brain bilirubin levels were found in PND 2 than in PND 14 piglets given the compound intravenously (Lee et al., 1995). In summary, functional maturation of the BBB as a barrier to substances in the blood appears to occur earlier in humans than rats. If it is assumed that maximal barrier function is largely achieved in both rats and humans within 3 to 4 weeks of parturition, this interval represents a considerably larger proportion of the rat’s period of development.

In conclusion, our results indicate that the brain uptake of DLM is a function of age and is significantly higher in younger age groups. The more immature the animal, the
less efficient is the BBB in limiting the uptake of DLM into the brain. At low exposure levels of DLM, the ontogeny of plasma proteins does not significantly affect its brain uptake. Likewise, changes in physiological concentrations of albumin or lipoproteins would not have any significant impact on the brain uptake of low levels of DLM. Extreme hypoalbuminemic conditions or very high concentrations of DLM would be needed to have a significant effect on the brain uptake.

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REFERENCES


Figure 4.1. Effect of Time of Incubation on *In vitro* Binding of DLM to Human Plasma.

One µM $^{14}$C-DLM in adult human plasma was incubated for 15, 30, 60, 120, and 240 min at 37°C in an orbital shaker, before extracting with isooctane and acetonitrile. Results represent the Mean ± SD (n = 3). (*) represents statistically significant difference (p<0.05) compared to 15 min.
Figure 4.2. Effect of Time of Incubation on *In vitro* Binding of DLM to HSA. One µM $^{14}$C-DLM in 4% HSA was incubated for 15, 30, 60, 120, and 240 min at 37°C in an orbital shaker, before extracting with isoctane and acetonitrile. Results represent the Mean ± SD (n = 3). There was no statistically significant difference in the % bound DLM compared to 15 min (p>0.05).
Figure 4.3. Effect of Time of Incubation on \textit{In vitro} Binding of DLM to LDL. One µM $^{14}$C-DLM in 100 mg/dL LDL was incubated for 15, 30, 60, 120, and 240 min at 37°C in an orbital shaker, before extracting with isooctane and acetonitrile. Results represent the Mean ± SD (n = 3). There was no statistically significant difference in the % bound DLM compared to 15 min (p>0.05).
Figure 4.4. Effect of Time of Incubation on *in vitro* Binding of DLM to HDL. One µM $^{14}$C-DLM in 50 mg/dL HDL was incubated for 15, 30, 60, 120, and 240 min at 37°C in an orbital shaker, before extracting with isooctane and acetonitrile. Results represent the Mean ± SD (n = 3). There was no statistically significant difference in the % bound DLM compared to 15 min (p>0.05).
Figure 4.5. Effect of Time of Incubation on *In vitro* Binding of DLM to Mixture of LDL and HDL. One µM $^{14}$C-DLM in the mixture of LDL and HDL (50 mg/dL HDL + 100 mg/dL LDL) was incubated for 15, 30, 60, 120, and 240 min at 37°C in an orbital shaker, before extracting with isooctane and acetonitrile. Results represent the Mean ± SD ($n = 3$). There was no statistically significant difference in the % bound DLM compared to 15 min (p>0.05).
Figure 4.6. Effect of Binding to Physiological HSA Concentrations on the *In vivo* Brain Uptake of DLM. The LCA of adult male SD rats was cannulated and infused (500 µL/min) with 1 µM $^{14}$C-DLM in 2 - 5% HSA solutions for 2 min. Results represent the Mean ± SD (n = 3 - 4). There was no statistically significant difference in the uptake among the groups tested (p>0.05).
Figure 4.7. *In vitro* Binding of 1 µM $^{14}$C-DLM to Different Concentrations of HSA (2 – 5%) Following 15 min Incubation at 37°C in an Orbital Shaker. Results represent the Mean ± SD (n = 3). There was no statistically significant difference in the % bound DLM among the groups tested (p>0.05).
**Figure 4.8.** Effect of Binding to Physiological LDL & HDL Concentrations on the *In vivo* Brain Uptake of DLM. The LCA of adult male SD rats was cannulated and infused (500 µL/min) with 1 µM $^{14}$C-DLM in 50 mg/dL HDL or 100 mg/dL LDL or their mixture for 2 min. Uptake of 1 µM $^{14}$C-DLM was measured from HBSS buffer alone in the control group. Results represent the Mean ± SD ($n = 3 - 4$). There was no statistically significant difference in the uptake among the groups tested ($p>0.05$).
**Figure 4.9.** *In vitro* Binding of 1 µM $^{14}$C-DLM to Physiological Concentrations of LDL (100 mg/dL), HDL (50 mg/dL) and their Mixture Following 30 min Incubation at 37°C in an Orbital Shaker. Results represent the Mean ± SD (n = 3). There was no statistically significant difference in the % bound DLM among the groups tested (p>0.05).
Figure 4.10. Effect of Binding to Lower, Normal and Higher Physiological LDL Concentrations on the *In vivo* Brain Uptake of DLM. The LCA of adult male SD rats was cannulated and infused (500 µL/min) with 1 µM $^{14}$C-DLM in 50, 200 & 400 mg/dL of LDL solutions respectively for 2 min. Results represent the Mean ± SD ($n = 3 - 6$). (*) represents statistically significant differences ($p<0.05$) compared to 50 mg/dL.
**Figure 4.11.** *In vitro* Binding of 1 µM $^{14}$C-DLM to Different Concentrations of LDL (50 - 400 mg/dL) Following 30 min Incubation at 37°C in an Orbital Shaker. Results represent the Mean ± SD (n = 3). (*) represents statistically significant differences (p<0.05) in the % bound DLM compared to 50 mg/dL.
Figure 4.12. Effect of Binding to Rat Plasma of Different Age Groups on *In vivo* Brain Uptake of DLM. The LCA of adult male SD rats was cannulated and infused (500 µL/min) with 1 µM $^{14}$C-DLM in rat plasma of PND 15, PND 21 and PND 90 for 2 min. Results represent the Mean ± SD (n = 3 - 4). There was no statistically significant difference in the uptake among the groups tested (p>0.05).
Figure 4.13. Effect of Binding to Human Plasma of Different Age Groups on *In vivo* Brain Uptake of DLM. The LCA of adult male SD rats was cannulated and infused (500 µL/min) with 1 µM $^{14}$C-DLM in human plasma of corresponding age groups for 2 min. Results represent the Mean ± SD ($n = 3 - 5$). There was no statistically significant difference in the uptake among the groups tested ($p>0.05$).
Figure 4.14. Effect of Maturity of BBB on *in vivo* Brain Uptake of DLM. The LCA of PND 15, PND 21 and PND 90 SD rats was cannulated and infused (500 or 250 µL/min) with 1, 10 & 50 µM ^14^C-DLM in 4% HSA solution for 2 - 4 min. A flow rate of 250 µL/min and an infusion time of 4 minutes was employed in 15-day and 21-day old rats. Results represent the Mean ± SD (n = 2 - 8). (*) denotes statistically significant differences (p<0.05) compared to adults and (#) denotes statistically significant differences (p<0.05) compared to PND 21.
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**Table 4.1.** Relative Increase in the Brain Uptake of Different Concentrations of DLM in PND 15 vs PND 21 vs Adults.
Figure 4.15. Concentration Dependent Uptake of DLM in PND 15, PND 21, and Adult SD Rats. The LCA of PND 15, PND 21 and PND 90 SD rats was cannulated and infused (500 or 250 µL/min) with 1, 10 & 50 µM $^{14}$C-DLM in 4% HSA solution for 2 - 4 min. A flow rate of 250 µL/min and an infusion time of 4 minutes was employed in PND 15 and PND 21 rats. Results represent the Mean ± SD (n = 2 - 8). Same data as Fig 4.14 but plotted differently.
Figure 4.16. Effect of Flow Rate on *In vivo* Brain Uptake of DLM. The LCA of adult male SD rats was cannulated and infused with 1 µM $^{14}$C-DLM in 4% HSA @ 250 µL/min for 4 min. Results represent the Mean ± SD (n = 3). There was no statistically significant difference in the uptake among the groups tested (p>0.05).
CHAPTER 5

SUMMARY

Despite published data describing the toxicokinetics of pyrethroids in rats, no one has characterized the systemic distribution of pyrethroids at steady-state. In vivo tissue:plasma partition coefficients (PCs) reported previously in the literature for pyrethroids have been based on experimental time-course data from the single dose studies. The tissue:blood PCs were calculated as the ratio of the tissue AUC to the blood AUC. These toxicokinetic studies were often conducted at relatively high doses, which may not be environmentally relevant. Also, the major drawback is that the PC values reported were not measured at steady-state. To our knowledge, no studies have addressed whether tissue distribution and PCs of pyrethroids vary significantly during maturation and development. The series of studies presented here attempted to bridge this gap in knowledge by characterizing age-dependent differences in the distribution of pyrethroids in Sprague-Dawley (SD) rats. The overall hypothesis of this work was that the distribution of pyrethroids in immature rats will be significantly different from that in adults. To address this hypothesis, we first conducted pilot studies to determine the time it takes for the plasma levels to reach steady-state with our model system. Steady-state was attained in adult and in PND 15 and PND 21 rats between 48- and 72-h of constant infusion using an Alzet pump®. It is generally held that steady-state will be reached within ≤ 5 half-lives (t½) of a chemical. For DLM, CIS & TRANS, the t½ was reported to
be around 13-h. We first conducted experiments to measure steady-state *in vivo* tissue:plasma PCs of DLM, CIS and TRANS in adult male SD rats. For all three compounds, adipose tissue exhibited much higher PC values than brain, liver, and skeletal muscle. This is not surprising since fat would be expected to accumulate large amounts of highly lipophilic pyrethroids (Log P = 4.6 - 6.1). Unexpectedly, brain exhibited relatively low PCs, as did liver. Skeletal muscle PCs were somewhat higher compared to brain and liver. At the given dose ($K_0 = 0.36$ mg/h and LD = 150 mg/kg), we could not detect TRANS in the liver tissue, as the levels were below LOQ. We postulated that the rapid hydrolysis of TRANS by plasma and liver carboxylesterases (CEs) in rats were responsible for this observation.

In the second set of studies conducted to assess the influence of maturation on the systemic distribution of pyrethroids, we determined *in vivo* tissue:plasma PCs of DLM, CIS and TRANS in PND 15 and PND 21 pups. For all three pyrethroids, the tissue:plasma PCs were highest for PND 15 pups as compared to adults, with very few exceptions. Diminished capacity to metabolize pyrethroids in this age group results in higher plasma levels, which can saturate relatively low plasma protein levels. Thus, high circulating levels of pyrethroids combined with lack of adipose tissue results in higher tissue:plasma PCs in this age group. Greater permeability of the BBB to pyrethroids results in greater brain:plasma PC in PND 15 pups. The differences in tissue:plasma PCs become less pronounced between PND 21 and adult rats. De Zwart *et al.* (2008) reported that CE activity in both liver and plasma, and CYP 1A1 & 3A2 activity in liver, reaches adult levels by day 26. CIS tissue:plasma PCs for PND 21 pups were significantly higher than for adults, probably due to relatively slower metabolism by CYP
mediated oxidation resulting in higher plasma levels saturating the protein binding. Conversely, DLM and TRANS were rapidly metabolized by CE mediated hydrolysis resulting in relatively lower circulating levels. Sethi et al. (2015) reported that there was no significant difference in the plasma protein binding of DLM, CIS and TRANS between PND 21 pups and adult rats. Thus, rapid hydrolysis combined with increased plasma protein binding in PND 21 pups resulted in tissue:plasma PCs for DLM and TRANS that were either comparable or lower than in adults. Finally, these data support the hypothesis that the distribution of pyrethroids in younger rats will be significantly different from that in adults.

The neurotoxicity of high acute doses of pyrethroids is well characterized. Accurate measurement of brain uptake is vital to predict target organ dosimetry. To our knowledge, few studies have been conducted to characterize uptake of pyrethroids into the brain. Highly lipophilic pyrethroids are expected to diffuse freely across the BBB. However, previous toxicokinetic studies have reported that peak brain concentrations of DLM were only about 20% of plasma values. Also, brain:plasma PC value at steady-state was measured as 0.2 from our PC studies in adult rats. This suggests that parameters such as plasma protein binding, efflux membrane transporters such as p-gp may govern the transport of DLM across the BBB. No one has yet illustrated the effect of these parameters on the brain uptake of DLM. We evaluated this gap of knowledge by selectively investigating the role of plasma protein binding, membrane transporters and BBB in limiting the entry of DLM into the brain.

We first confirmed the effect of free fraction on the brain uptake of DLM. We measured the brain uptake of DLM from 0.01% and 4% HSA using an in situ brain
perfusion technique. We found that the uptake from 0.01% was significantly higher than that from 4%. Then, we investigated the role of BBB in limiting the entry of DLM into the brain and obtained exciting results. We disrupted the BBB by prior treatment of mannitol and then measured the uptake of DLM from HBSS buffer and 4% HSA in control and mannitol pre-treated animals. Interestingly, even upon the disruption of BBB, the uptake from 4% HSA did not differ significantly from that of control. In the absence of 4% HSA, the uptake from HBSS buffer increased significantly in mannitol treated animals. The BBB thus appears to play some role in opposing the entry of DLM into the brain. In addition to the BBB, plasma protein binding is also a key player in limiting brain association of DLM and thus, plays a significant protective role under normal physiological conditions. We then investigated the role of membrane transporters in facilitating or inhibiting brain uptake of DLM. We measured the uptake of DLM from 0.01% and 4% HSA in the presence and absence of CSA. Surprisingly, CSA reduced DLM uptake from 0.01% HSA significantly, while uptake from 4% HSA was not significantly altered. This finding illustrates that some unidentified influx transporters contribute to the influx of free DLM across the BBB at high concentrations. These transporters apparently do not play a role under normal physiological conditions, where a passive, non-saturable permeation predominates. This data also demonstrates a significant finding that under physiological albumin concentrations and at low exposure levels, ontogenic differences in membrane transporters do not affect the brain transport of DLM. Since CSA is a promiscuous inhibitor of transporters, it was not possible to identify and characterize a specific transporter family or isoform involved in the
transport. This will require further research. These findings concurred with previous *in vitro* data (Appendix Fig. 2B).

Results from PC studies have indicated that the partitioning of pyrethroids into younger rat’s brain is significantly higher than in adult rats. In general, newborns and young infants have lower plasma protein binding as compared to adults. Sethi *et al.* (2015) reported that the plasma protein binding of pyrethroids in PND 15 pups was significantly lower than in adult rats. Relatively lower plasma protein binding results in higher free fractions, which can readily cross the BBB. Also, in immature animals and neonates, the BBB is not fully functional and is characterized by leaky endothelial junctions. Therefore, we attempted to assess the influence of age-dependent changes in plasma protein binding and BBB integrity on the brain uptake of pyrethroids.

We first assessed the potential influence of maturation of plasma protein binding on the brain entry of DLM, again using the *in situ* perfusion model. We measured the uptake of DLM from 2%, 2.9%, 4%, and 5% HSA and found no significant difference among these groups. This finding was not surprising because there was not a significant difference in the free fraction of DLM in this range of protein concentrations. Here, 2.9% and 4% HSA correspond to albumin concentrations in neonates and adult humans, respectively. These results provided us an insight that age-dependent differences in plasma protein binding of DLM might not be sufficient to significantly affect its uptake. We subsequently measured brain uptake of DLM from PND 15, PND 21 and adult rat plasma, and found no significant difference in uptake. We also measured the uptake of DLM from human pediatric plasma from ages birth – 1 week, 1 – 4 weeks, 4 weeks – 1 year, 1 – 3 years, and adults. As with rat plasma, there was no
significant difference in the uptake of DLM among these groups. The difference in the free fraction of DLM among the above groups tested was not sufficiently different to alter brain deposition.

We also measured brain uptake of DLM from physiological concentrations of LDL (100 mg/dL), HDL (50 mg/dL), and their mixture, in order to characterize their influence on binding and ensuing brain uptake. Interestingly, the uptake did not vary significantly among these groups. Not surprisingly, the free fraction of DLM did not differ significantly either among the groups tested. Under conditions with the same free fraction of DLM, uptake from physiological concentrations of individual lipoprotein constituents was far higher than that from 4% HSA. This might be attributed to the presence of lipoprotein receptors on the surface of brain capillary endothelial cells. It is also possible that DLM has a higher binding affinity for albumin than lipoprotein constituents due to simple partitioning into hydrophobic regions of lipoproteins. We also measured the brain uptake of DLM from different LDL concentrations (50 – 400 mg/dL) to characterize potential effects on brain uptake in individuals with hypo- and hyper-cholesterolemia. Clearly, lowest uptake was observed from the highest LDL concentration tested.

The final set of experiments were conducted to study permeability of the immature BBB to DLM and its role in brain uptake. We measured the uptake of 1, 10 and 50 µM DLM from 4% HSA in PND 15, PND 21 and adult rats using the in situ perfusion model. The nature of this particular study focused on the relationship between the BBB penetrability and the brain uptake of DLM, while keeping the free fraction constant at physiological conditions. At all concentrations tested, the brain uptake of DLM in PND 15 pups was significantly higher than that of PND 21 and adult rats. Also,
the uptake in PND 21 pups was much higher as compared to adults. Studies have shown that the rat BBB was not fully developed structurally and was not fully functional until about the time of weanling. Interendothelial junctions in the immature BBB are characterized by membrane separations greater than 20 nm and are relatively leaky. Such junctions in developing rat brain could result in high permeability to DLM. Finally, these findings support our hypothesis that the brain uptake of pyrethroids in immature rats will be significantly different from that in adult rats due to differences in plasma protein binding and BBB permeability. However, we should be very careful while extrapolating these findings in rats to humans, since the functional maturation of the BBB as a barrier to substances in the blood appears to occur earlier in humans than rats.

In conclusion, studies described in this dissertation filled gaps in knowledge that exist in understanding the differences in the distribution of pyrethroids in immature and adult rats. For the most part, our results clearly showed that the steady-state tissue:plasma PCs for pyrethroids were inversely proportional to age. This was particularly true for PND 15 pups, which exhibited far higher PCs than adults. A few exceptions were observed in PND 21 pups. The knowledge gained from these studies can be used for risk assessment of pyrethroids in human sub-populations (adults vs neonates). Other researchers in the future can refer to these findings to understand age-dependent differences in the disposition of other pesticides which have similar physicochemical properties.

*In situ* brain perfusion studies in this dissertation facilitated reliable measurement of the uptake of DLM into the brain. The data from these studies demonstrated that the
brain uptake of DLM in the youngest rats is significantly higher than that of mature rats. However, at low exposure levels, such differences in the uptake were not the result of ontogenic differences in the expression of membrane transporters or plasma protein binding. Instead, a more permeable BBB in maturing rats was the reason for significantly higher brain uptake in this age group. The data obtained from these studies can be useful in predicting the brain dosimetry of pyrethroids in immature human sub-populations.

Future studies should be conducted to expound upon the findings presented here. Since the distribution of pyrethroids is associated with its tissues’ lipid content, it would be interesting to study the distribution of these highly lipophilic compounds in an obese rat model. Obesity is often associated with hyperlipidaemia and increased triglyceride and fat mass. Due to these reasons, distribution of pyrethroids in these subpopulations may be quite different from that in lean populations. Additionally, based on our findings related to the contribution of transporters towards the brain uptake of DLM, a P-gp knockout rat model can be used in the future to definitively demonstrate that DLM or other pyrethroids are not substrates of this major transporter. The same technology can be used to identify specific transporter that contribute to the movement of pyrethroids across the BBB. Studies that characterize the role of lipoprotein receptors in facilitating the transport of pyrethroids across the BBB can be very informative as well.
APPENDIX

PLASMA PROTEIN BINDING LIMITS THE BLOOD BRAIN BARRIER PERMEABILITY OF THE PYRETHROID INSECTICIDE, DELTAMETHRIN⁴

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ABSTRACT

Previous pharmacokinetic studies of deltamethrin (DLM) have revealed that brain levels of this highly lipophilic pyrethroid insecticide are only 15-20% of plasma levels. Experiments were performed to assess determinants limiting CNS access including plasma protein binding and the efflux transporter, P-gp. A human brain microvascular endothelial cell line, hCMEC/D3, was utilized as a model in vitro system to evaluate blood-brain barrier (BBB) permeation. Incubation of DLM with a series of human serum albumin (HSA) concentrations showed that unbound (fu) DLM ranged from 80% with 0.01% HSA to ~20% at the physiologically-relevant 4% HSA. A positive correlation (R=0.987) was seen between fu and cellular uptake. Concentration-dependent uptake of DLM in 0.01% HSA was non-linear and was reduced at 4°C and by the P-gp inhibitor cyclosporine (CSA), indicative of a specific transport process. Cellular accumulation of [3H]-paclitaxel, a P-glycoprotein (P-gp) substrate, was increased by CSA but not by DLM, suggesting that DLM is neither a substrate nor an inhibitor of P-gp. The concentration-dependent uptake of DLM from 4% HSA was linear and not significantly impacted by temperature or CSA. In situ brain perfusion studies monitoring brain association of DLM at 0.01% and 4% HSA confirmed the aforementioned in vitro findings. This study demonstrates that brain uptake of DLM under normal physiological conditions appears to be a passive, non-saturable process, limited by the high protein binding of the pyrethroid.

KEYWORDS: Blood-Brain Barrier, Insecticide, Pyrethroid, P-glycoprotein, Transport
1.0 INTRODUCTION

Pyrethroids are the most frequently utilized insecticides in the U.S., since the phase out of organophosphates (Williams et al., 2008). Pyrethroids are used outdoors in many agricultural, urban construction and landscaping settings, as well as indoors in homes and other structures. Certain pyrethroids, such as permethrin, are used to treat ticks, mites and lice on pets and humans (Frankowski et al., 2010). Thus, it is not surprising that large segments of the population are exposed to this class of pesticide. In fact, 3-phenoxybenzoic acid, a metabolite common to many pyrethroids, was detected in the urine of the majority of over 5,000 persons monitored in the general U.S. population (Barr et al., 2010). Pyrethroid exposures most frequently result from the inadvertent ingestion of dusts, hand-to-mouth activity with pets and consumption of very low levels in foods (Lu et al., 2010; Morgan, 2012).

Although most pyrethroids exert relatively low mammalian toxicity, high doses can be acutely neurotoxic. Their primary mechanisms of action are interference with the closure of neuronal voltage-gated calcium and sodium channels (Cao et al., 2011; Soderlund, 2012). The duration of the closure delay and modification of sodium currents by deltamethrin (DLM) may last for several seconds, resulting in stimulus-dependent nerve depolarization and blockage. This results in typical toxic signs of salivation and hyperexcitability, possibly progressing to choreoathetosis (i.e., CS syndrome).

It would be anticipated that these highly lipophilic compounds should readily enter and accumulate in the brain. However, DLM peak brain concentrations and areas under plasma concentration-time curves were found to be only 15-20% of plasma and blood values (Kim et al., 2008; 2010). This suggests that the CNS deposition of DLM is
governed by other parameters that are not well understood. It has been well established that binding to albumin and other plasma proteins can significantly reduce the free fraction ($f_{u}$) of drugs available for absorption into the CNS. *In situ* brain extraction of a series of benzodiazepines in rats, for example, is significantly influenced by their extent of plasma binding (Jones et al., 1988; Lin and Lin, 1990). Metabolism and systemic clearance also reduce the amount of free chemical available for CNS uptake. DLM and other pyrethroids are extensively oxidized by rat and human hepatic microsomal cytochrome P450s and hydrolyzed by hepatic and plasma carboxylesterases (Anand et al., 2006; Ross et al., 2006; Scollon et al., 2009). Transport mechanisms are prevalent within BBB endothelial cells, including a number of Solute Carrier and ATP Binding Cassette transporters. P-gp, an ATP-dependent efflux transporter, is located on the apical membrane of endothelial cells of the BBB. Although recent studies from our laboratory demonstrated that P-gp does not appreciably limit DLM intestinal permeability using the Caco-2 cell line model (Zastre et al., 2013), it is unknown if P-gp limits CNS deposition via the BBB. To our knowledge, no one has evaluated potential determinants of flux for DLM or other pyrethroids across the BBB.

The overall objective of this investigation was to gain an understanding of the transfer of the pyrethroid, DLM, from the blood into the brain. Emphasis was placed on clarifying the role of processes generally believed to limit access, in particular, protein binding and whether it is a substrate for influx or efflux transporters, notably P-gp. A human brain microvessel endothelial cell line, hCMEC/D3, was selected as a model *in vitro* system. These cells display many characteristics of brain endothelium *in vivo*, including tight junction formation and expression of transporters (Weksler et al., 2005;
Zastre et al., 2009). This in vitro model was supplemented by in situ brain DLM uptake experiments in rats.

2.0 MATERIALS AND METHODS

2.1 Materials: Cell culture media and supplements, which include EBM-2 media and vascular endothelial growth factor, insulin-like growth factor 1, epidermal growth factor, fibroblast growth factor, hydrocortisone, ascorbate, and gentamycin supplements, were obtained from Lonza (Allendale, NJ). Rat tail collagen type 1 and trypsin/EDTA were purchased from BD Biosciences (San Jose, CA) and Mediatech (Manassas, VA), respectively. Fetal bovine serum (FBS), mannitol, and human serum albumin (HSA) were purchased from Sigma-Aldrich (St. Louis, MO). Cell culture flasks, plates, and dishes were obtained from Greiner Bio-one (Monroe, NC). Cyclosporine A (CSA) was purchased from Amresco (Salon, OH). Radiolabeled [\(^{14}\)C]-DLM (54.1 mCi/mmol) was kindly donated by Bayer CropScience (Research Triangle Park, NC). [\(^{3}\)H]-Paclitaxel (PTX) (23 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA), while iso-octane and acetonitrile were purchased from EMD Chemicals (Billerica, MA).

2.2 Cell Culture: hCMEC/D3 cells were kindly donated by Dr. Babette Weksler, Weill Cornell Medical College. Cells were maintained at 37°C, 5% CO₂, and 95% humidified air in EBM-2 media supplemented with vascular endothelial growth factor, insulin-like growth factor-1, epidermal growth factor, fibroblast growth factor, hydrocortisone, ascorbate, gentamycin, and 2.5% FBS as previously described (Weksler et al., 2005). Cells were seeded onto rat-tail collagen type-1 coated flasks and 24-well plates.
2.3 Protein Binding of DLM: Protein binding of DLM with HSA was measured using a modification of a procedure previously reported (Sethi et al., 2014). Briefly, 1.0 \( \mu M \) \(^{14}\)C-DLM was incubated with increasing concentrations of HSA (0.01, 0.1, 1.0, and 4.0% w/v) dissolved in PBS at 37\( ^{\circ} \)C for 15 min. Two volumes of iso-octane were added and vortexed for 1 min, followed by centrifugation at 14,000xg for 5 min to separate aqueous and organic layers. The organic layer was removed, and the free (unbound) fraction it contained was quantified using a liquid scintillation counter (LS 6500, Beckman Coulter, Brea, CA). The protein-bound fraction was quantified by subjecting the aqueous layer to protein precipitation using 3 volumes of acetonitrile. The sample was vortexed for 1 min and centrifuged at 14,000xg for 5 min. The supernatant was removed and quantified by liquid scintillation counting. To establish the effect of DLM concentration on protein binding, increasing concentrations of \(^{14}\)C-DLM ranging from 0.1 – 10 \( \mu M \) with either 0.01% or 4.0% HSA was assessed as described above.

2.4 Cellular Uptake Experiments: The uptake of 1.0 \( \mu M \) \(^{14}\)C-DLM by hCMEC/D3 cells from a series of HSA concentrations (0.01, 0.1, 1.0, and 4.0% w/v) was assessed at 37\( ^{\circ} \)C for 3 min (Linear range of uptake – Suppl. Fig. 1). Prior to uptake, the cells were washed twice with pre-warmed HBSS (Mediatech) containing 10 mM HEPES pH = 7.2 (hereafter, referred to as transport buffer), and pre-incubated for 15 min with transport buffer. Subsequently, 1.0 \( \mu M \) \(^{14}\)C-DLM in HSA containing transport buffer was added to the wells. After the uptake time, the cells were washed 3 times with ice-cold PBS and lysed (1% Triton X-100, 50 mM Tris, 250 mM NaCl pH=8.0). The amount of DLM was quantified using liquid scintillation counting, and the results were normalized to total protein using a BCA protein assay kit (Thermo Scientific, Rockford, IL).
A similar protocol was used to establish whether DLM is a substrate or inhibitor for P-gp-mediated efflux. Inhibitor properties were assessed by determining the uptake of the P-gp substrate $[^{3}H]$-PTX (4 nM) with or without the P-gp inhibitor, CSA (25 μM), and DLM (10 μM) at 37°C for 30 min in transport buffer. For substrate properties of DLM for P-gp, the uptake of 1.0 μM $[^{14}C]$-DLM was monitored in the presence of the P-gp inhibitor CSA (25 μM) from 0.01% and 4% HSA (low and high protein binding extremes, respectively). The amount of PTX or DLM was quantified using liquid scintillation counting and normalized to total protein.

2.5 Kinetic Analysis of DLM Transport: The concentration-dependent uptake of DLM was determined to evaluate its transport kinetics for estimation of the Michaelis–Menten constant ($K_m$) and maximal transport velocity ($V_{max}$) in hCMEC/D3 cells. The uptake (3 min) of increasing concentrations of $[^{14}C]$-DLM (0.1-10 μM) in transport buffer containing 0.01% or 4% HSA was performed as described above. The total uptake rate into hCMEC/D3 cells is the sum of all saturable (specific; nonlinear) and non-saturable (nonspecific; linear) components. Two approaches were utilized to estimate the non-saturable contribution including measuring the concentration-dependent uptake of DLM at 4°C or in the presence of 25 μM CSA (37°C uptake). Both were included since over estimation may be associated with reduced temperatures decreasing passive diffusion and membrane fluidity (Poirier et al., 2008). The concentration of DLM for the analysis was adjusted based on the free fraction available for uptake that was determined as described above. Kinetic analysis was performed by applying a one-site Michaelis–Menten equation with a nonsaturable component using Graphpad Prism 6 software.
Equation 1: \[ V = \frac{V_{\text{max}}[S]}{K_m+[S]} + k_{\text{ns}}[S] \]

Where \( V \) is the total rate of uptake, \( V_{\text{max}} \) is the maximum uptake rate, \( K_m \) is the Michaelis-Menten constant, \([S]\) is the substrate concentration, and \( k_{\text{ns}} \) is the coefficient for nonspecific uptake by diffusion.

2.6 In Situ Brain Perfusion:

2.6.1 Animal surgical preparation: Brain-associated DLM levels were measured using a modified in situ rat brain perfusion technique, originally developed by Takasato et al (Boje, 2001; Takasato et al., 1984). The University of Georgia Animal Care and Use Committee approved the protocol for this study. Male Sprague-Dawley (SD) rats (300-350g) were purchased from Charles River Laboratories (Raleigh, NC). Each rat was housed in a cage with a 12-h light/dark cycle at ambient temperature (22ºC) and relative humidity (55 ± 5%). Food (5001 Rodent Diet, PMI Nutrition LLC, Brentwood, MO) and water were provided ad libitum. Rats were anesthetized with KAX (Ketamine, Acepromazine, and Xylazine) cocktail. The left common carotid artery was exposed and cannulated with a 25G needle affixed to PE-50 tubing for perfusion fluid administration. Prior to the start of the infusion, the heart was stopped by rapidly severing the cardiac ventricles to eliminate contributions of vascular perfusion from the systemic circulation. The left common carotid artery was then perfused with 2 mL of sterile saline to flush blood from the brain. This was followed by the infusion of corresponding perfusion fluids.

Since the perfusion fluid was infused into the left common carotid artery, only the left cerebral hemisphere was processed for DLM quantitation. The isolated brain tissue
was homogenized with a Tekmar Tissumizer IKA Ultra-Turrax homogenizer (Janke and Kunkel Laboratories, Cridersville, OH), by adding two volumes of ice-cold distilled water by weight. An aliquot (100 µL) of the brain homogenate was added to 4 mL of EcoLite scintillation cocktail (MP Biomedicals, Solon, OH). Uptake of DLM was quantified by measuring the radioactivity in each sample by liquid scintillation counting using a Beckman Coulter LS 6500 (Brea, CA) and normalizing to tissue weight.

**2.6.2 Effect of HSA and CSA on DLM associated brain levels:** The perfusion fluid consisted of 10 µM [¹⁴C]-DLM and 0.01 or 4% HSA with or without the P-gp inhibitor CSA (25 µM) in HBSS buffer. All solutions were incubated for 15 min at 37°C prior to the infusion. In the CSA group, animals were pre-treated with CSA prior to the start of DLM infusion by perfusing 2 mL of sterile saline containing 25 µM CSA. The DLM containing perfusion fluid (including 25 µM CSA) was then infused at a constant rate of 500 µL/min for 2 min using a Harvard 22 syringe infusion pump (Harvard Apparatus, Holliston, MA).

**2.6.3 Role of the BBB in limiting DLM associated brain levels:** Mannitol (1.6 M) was infused at 0.25 ml/kg/sec for 30 seconds prior to the perfusion of DLM to disrupt the BBB (Cosolo et al., 1989). The perfusion fluid consisted of 1 µM [¹⁴C]-DLM with or without 4% HSA in HBSS buffer was then infused at a constant rate of 500 µL/min for 2 min using a Harvard 22 syringe infusion pump.

**2.7 Statistical Analysis.** All experiments were performed with a minimum of three independent experiments unless otherwise stated. Statistical significance was evaluated between groups using either Student’s *t*-test or one way ANOVA, followed by Tukey’s
multiple comparison test, as indicated, with a significance level of p<0.05, using Graphpad Prism 6 software® (San Diego, CA).

3.0 RESULTS

3.1 Impact of Protein Binding on DLM Cellular Uptake

Protein binding experiments were performed with increasing concentrations of HSA to establish the free fraction of DLM. The percent DLM unbound decreased with increasing concentration of HSA ranging from ~80% with 0.01% HSA to ~20% at the physiologically-relevant concentration of 4% HSA (Fig. 1A). The free fraction of DLM (~80%) with 0.01% HSA was unchanged over a range of DLM concentrations (Fig. 1B). At 4% HSA, an increase in the free fraction from ~20% to ~40% was observed only at high concentrations of DLM ranging from 1 to 10 µM (Fig. 1C). To establish whether a relationship exists between the extent of DLM uptake by hCMEC/D3 cells and the free fraction, cellular uptake of DLM from increasing concentrations of HSA was determined. Figure 1D demonstrates a progressive decrease in DLM uptake by hCMEC/D3 cells with increasing concentration of HSA. A strong correlation (R=0.987) was found between the free fractions of DLM and the extent of DLM uptake by hCMEC/D3 cells (Fig. 1E).

3.2 Role of P-glycoprotein

The functional activity of P-gp in our hCMEC/D3 model system was demonstrated by an increased cellular accumulation of the P-gp substrate PTX in the presence of the P-gp inhibitor CSA (Fig. 2A). In contrast, DLM had no effect on PTX accumulation. To elucidate the potential role of P-gp in limiting DLM accumulation in the brain, DLM
uptake was assessed in the presence of 25 µM CSA. The uptake of DLM was significantly reduced by CSA in the presence of 0.01% HSA, corresponding to a high free fraction of DLM (Fig. 2B). However, CSA had no effect on DLM uptake in the presence of 4% HSA (low DLM free fraction) (Fig. 2B). The presence of CSA had no effect on the extent of DLM protein binding with either 0.01% or 4% HSA (Suppl. Fig. 2).

3.3 Concentration-Dependent Uptake of DLM

To establish the transport kinetics, the concentration-dependent uptake of DLM by hCMEC/D3 cells was assessed at the two extremes of protein binding/free fraction (i.e. 0.01% and 4% HSA). Although the concentrations evaluated ranged from 0.1 to 10 µM, Fig. 3 represents only the available free fraction of DLM at each concentration that was calculated based on Fig. 1B and C.

The cellular uptake of DLM from 4% HSA (low free fraction) demonstrated linear kinetics (Fig. 3A). In contrast, DLM exhibited non-linear kinetics with 0.01% HSA (high free fraction) (Fig. 3B). Furthermore, the extent of DLM uptake was greater from 0.01% HSA than 4% HSA. The concentration-dependent uptake of DLM was evaluated at 4°C to reduce energy-dependent processes in the cell. Uptake of DLM at 4°C was substantially lower than at 37°C and linear over the DLM concentration range with 0.01% HSA (high free fraction) (Fig. 3B). No difference was observed for DLM uptake at 4°C and 37°C with 4% HSA (low free fraction) (Fig. 3A). Similarly, the inclusion of CSA had no effect on DLM uptake from 4% HSA, but reduced uptake over the concentration range in the presence of 0.01% HSA.
The linearity of DLM transport with 0.01% HSA at both 4°C and with the inclusion of CSA allows for an estimation of the non-saturable (passive) uptake component. To estimate the saturable transport kinetics of DLM, equation 1 was fitted to the total and non-saturable uptake components. A saturable curve (dotted line) was obtained for DLM with $V_{\text{max}}$ of 327 +/- 12.2 pmol/mg protein/min and $K_m$ of 2.3 +/- 0.3 µM using 4°C for the non-saturable component (Fig. 3C). Similar values were obtained using CSA (solid line) as the non-saturable component with a $V_{\text{max}}$ of 110 +/- 13.2 pmol/mg protein/min and a $K_m$ of 1.4 +/- 0.3 µM.

3.4 In Situ brain perfusion of DLM

The extent of brain associated DLM from a perfusate containing 0.01% HSA (high free fraction) was significantly greater than from 4% HSA (low free fraction) (Fig. 4A). Similarly to the in vitro studies, the presence of CSA significantly reduced brain association of DLM from 0.01% HSA but not from 4% HSA. Fig. 4B illustrates the extent of brain association of DLM with and without 4% HSA after BBB disruption using mannitol. In the absence of protein (HBSS group), pre-treatment with mannitol significantly increased the brain association of DLM compared to control. In the presence of 4% HSA, the pre-treatment with mannitol did not have a significant impact on brain association of DLM.

4.0 DISCUSSION

The BBB is an effective regulator of the entry of endo- and xenobiotics into the brain. There is generally a correlation between the rate at which a compound enters the brain and its lipophilicity (Clark, 2003; Levin, 1980). Even though DLM is a highly
lipophilic compound (LogP = 6.1), brain levels are relatively low compared to the plasma compartment (Kim et al., 2008; 2010). Several research groups have described parabolic relationships between the lipophilicity of drugs and their brain uptake/pharmacological activity (Dishino et al., 1983; Rowley et al., 1997). Moderately lipophilic compounds exhibit the highest uptake, but a point of diminishing return is reached, beyond which higher LogP values result in decreasing BBB penetration. DLM and other pyrethroids satisfy two criteria for poor membrane permeability, namely molecular masses >500 and logP > 5 (Lipinski et al., 2001). Non-specific membrane protein and lipoprotein binding, coupled with lipid partitioning also serve to reduce the flux of highly-lipid soluble compounds through the BBB, by virtually trapping them there (Liu et al., 2011; Nau et al., 2010; Waterhouse, 2003).

Another important factor in the extent to which xenobiotics cross the BBB is the unbound fraction in plasma (Kalvass and Maurer, 2002; Kalvass et al., 2007; Tanaka and Mizojiri, 1999). Binding to albumin reduces the amount of compound free to permeate the BBB (Tanaka and Mizojiri, 1999). Since DLM has a high degree (90%) of protein and lipoprotein binding in plasma (Sethi et al., 2014), only a small fraction (unbound) of an applied dose would be considered available for transport across endothelial cells into the brain. Results of the current investigation demonstrate that the extent of binding of DLM to HSA is an important determinant of disposition by brain endothelial cells in vitro. It is noteworthy that DLM uptake by hCMEC/D3 cells was quite low in the presence of 4% HSA, a concentration frequently present in vivo.

In situ brain perfusion is one of the most sensitive and physiologically-relevant techniques for estimation of CNS uptake of drugs and other chemicals (Bickel, 2005).
The rat brain perfusion model has been successfully used to accurately measure uptake of a variety of neurotoxic chemicals and neuroactive drugs (Kumar et al., 2007; Rabin et al., 1993). Rowley et al. (1997) used the model to assess BBB penetration of a series of anticonvulsants, demonstrating that albumin binding reduced brain uptake, as did excessive lipid solubility. Similarly, brain extraction increased as the unbound fraction and lipophilicity increased for a series of benzodiazepines (Jones et al., 1988; Lin and Lin, 1990). In the present study, uptake of DLM into the brain of rats was significantly lower from perfusate with a physiological HSA concentration than from perfusate with a low HSA concentration. These findings are consistent with our in vitro results that demonstrated greater hCMEC/D3 accumulation with increasing DLM unbound fraction.

Hypertonic mannitol was infused in order to assess the influence of the BBB on limiting brain uptake of DLM. Electron microscopy has demonstrated that mannitol disrupts the BBB by reducing endothelial cell size and opening endothelial tight junctions (Brown et al., 2004; Cosolo et al., 1989). Mannitol infusion produced almost a 3-fold increase in the brain association of DLM in the absence of HSA demonstrating that the BBB is an effective barrier limiting brain penetration of DLM. In the presence of 4% HSA, brain association of DLM was not significantly altered by mannitol pretreatment. Albumin, and correspondingly DLM bound to albumin, is too large a molecule (Molecular Weight ~66,000) to cross the BBB, even when the tight junctions between the endothelial cells are disrupted by hypertonic mannitol (Brown et al., 2004). Therefore, our findings at 4% HSA illustrate that plasma protein binding limits brain association of this neurotoxic insecticide and plays a significant protective role under normal physiological conditions.
Our observations of DLM transport into hCMEC/D3 cells are similar in several respects to those with the *in vitro* intestinal transport cell line model, Caco-2 (Zastre et al., 2013). There was little evidence of P-gp-mediated efflux of DLM and no effect on accumulation of P-gp substrates in either cell type, indicating that this pyrethroid is not a P-gp inhibitor or substrate. Kinetic analysis of DLM uptake revealed a nonlinear curve at a high DLM free fraction in hCMEC/D3 cells that was analogous to the non-linearity observed in Caco-2 cells in the absence of HSA. CSA was capable of reducing the uptake of DLM by hCMEC/D3 cells as well as brain uptake using the *in situ* brain perfusion model. CSA significantly reduced the brain uptake of DLM from 0.01% HSA, where higher free fractions are available to interact with the transporters. With 4% HSA, the free fractions are not high enough for CSA to significantly impact uptake. Although CSA is a well-established P-gp inhibitor, it is also known to be a promiscuous inhibitor of both influx and efflux transporters (Letschert et al., 2006; Tang et al., 2002). Inhibition of DLM uptake by CSA was also found in an intestinal cell line, Caco-2 (Zastre et al., 2013). The reduction of DLM uptake at high unbound fractions, at 4°C, and in the presence of CSA are all indicative of a specific influx transport process. Kinetic analysis of the DLM uptake by hCMEC/D3 cells from 0.01% HSA resulted in a $K_m$ of 1.3 μM using 4°C as the non-saturable component, which is similar to the $K_m$ in Caco-2 cells of 1.5 μM. Both the BBB and the intestinal tract express many of the same transporters, and the similarity in the $K_m$ values suggests a common transporter maybe involved (Sai and Tsuji, 2004). However, our findings do not identify which transporter family or isoform may be involved in DLM influx transport. Conversely, DLM uptake from 4% albumin exhibited linearity, and the uptake at 4°C or with CSA was not inhibited. The
brain uptake of DLM from 4% albumin also exhibited linearity in the in situ brain perfusion experiment. These in vitro findings corroborated by in situ results reflect a passive, non-saturable BBB transport process predominating under normal physiological conditions.

Pronounced variations in albumin concentrations may result in changes in the fraction of DLM unbound and ultimately in the extent of BBB penetration. Our results demonstrate that substantial DLM uptake into hCMEC/D3 cells progressively increases when albumin concentrations are less than 1%. Plasma albumin levels in humans vary with age, ranging from 3.3% in neonates to 4.5% in adults (Kanakoudi et al., 1995; Lerman et al., 1984). Although ontogenic differences in the expression of transporters have been characterized (Daood et al., 2008; de Zwart et al., 2008), marked displacement of DLM from albumin binding sites and/or disease conditions producing hypoalbuminemia would likely need to be present for age-dependent differences in carrier-mediated processes to influence BBB permeability to DLM. Age-dependent differences in BBB penetration, or DLM uptake by the brain may not be prominent when there are normal physiological levels of albumin.

An additional variable in DLM sequestration in vivo may be lipoproteins. Sethi et al. (2014) reported that DLM and permethrin, another highly lipophilic pyrethroid, were bound by both albumin and lipoproteins. Thus, lipoprotein binding may also be a significant determinant of the plasma free fraction available for brain uptake. It therefore appears worthwhile to establish how the binding of DLM to both albumin and lipoproteins in human plasma may impact brain uptake of DLM, and whether age-related changes in their plasma concentrations could be of importance. Future studies
will be conducted to measure brain uptake of DLM from human plasma of neonates and adults, using the in situ rat brain perfusion technique employed in the current investigation.

In conclusion, our results indicate that the efflux transporter P-gp does not appreciably contribute to the lower than anticipated accumulation of DLM in the brain. Although a low affinity transporter is suggested to contribute to DLM uptake by the brain, this is only significant at high free fractions of DLM not observed under normal physiological conditions or in younger age groups. The extensive binding and low penetration of DLM with physiological concentrations of albumin suggest that the modest brain levels of DLM observed in vivo are due to poor BBB permeation by the highly lipophilic compound and its limited free fraction available for diffusion.

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Fig. 1. Relationship between HSA concentration, DLM binding, and uptake of DLM by hCMEC/D3 cells. (A) Extent of protein binding of 1.0 μM $[^{14}\text{C}]$-DLM with increasing concentrations of HSA for 15 min at 37°C. (B) Protein binding with increasing concentrations of $[^{14}\text{C}]$-DLM at 0.01% HSA for 15 min at 37°C. (C) Protein binding with increasing concentrations of $[^{14}\text{C}]$-DLM at 4% HSA for 15 min at 37°C. (D) Accumulation of 1.0 μM $[^{14}\text{C}]$-DLM by hCMEC/D3 cells with increasing concentrations of HSA at 37°C for 3 min. (E) Correlation between the extent of DLM uptake by hCMEC/D3 cells at varying HSA concentrations with the unbound fraction of DLM. All results are expressed as the mean +/- SD (n = 3).
**Fig. 2.** Assessment of the P-gp substrate and inhibitor properties of DLM in hCMEC/D3 cells. (A) Uptake by hCMEC/D3 cells of $[^3]$H]-paclitaxel (4 nM) with or without the P-gp inhibitor, CSA (25 μM), and DLM (10 μM) at 37°C for 30 min. Results are expressed as the mean +/- SD (n = 3). (★) denotes statistically significant differences (p<0.05). (B) Uptake of 1.0 μM $[^{14}]$C]-DLM by hCMEC/D3 cells with 0.01 and 4% HSA at 37°C for 3 min with and without CSA (25 μM). Results are expressed as the mean +/- SD (n = 3). (★) denotes statistically significant differences (p<0.05).
Fig. 3. Concentration-dependent uptake of DLM by hCMEC/D3 cells. (A) Uptake (3 min) of increasing concentrations of $[^{14}\text{C}]-\text{DLM}$ in transport buffer containing 4% HSA at (●) 37°C, (○) 4°C, and (□) with 25 µM CSA at 37°C. Results are expressed as the mean +/- SD (n=6). (B) Uptake (3 min) of increasing concentrations of $[^{14}\text{C}]-\text{DLM}$ in transport buffer containing 0.01% HSA at (●) 37°C, (○) 4°C, and (□) with 25 µM CSA at 37°C. Results are expressed as the mean +/- SD (n=6). (C) The saturable DLM uptake was estimated by curve fitting the total (37°C) and non-saturable uptake components from 4°C (Dotted line) or from CSA (Solid line).
Figure 4: (A) Effect of HSA and CSA on the *in vivo* brain association of DLM. The Left common carotid artery of adult male Sprague-Dawley rats was cannulated and infused (500 µL/min) with 10 µM [¹⁴C]-DLM in 0.01% and 4% HSA solutions either alone or in combination with 25 µM CSA for 2 min. Results represent the mean ± SD (n = 4 - 7). (✉) denotes statistically significant differences (p<0.05). (B) Effect of mannitol on the *in vivo* brain association of DLM with and without 4% HSA. The Left common carotid artery of adult male Sprague-Dawley rats was cannulated and infused (500 µL/min) with 1 µM [¹⁴C]-DLM with and without 4% HSA. In the mannitol group, animals were pre-treated with 1.6 M Mannitol prior to infusion of DLM. Results represent the mean ± SD (n = 4 - 5). (✉) denotes statistically significant differences (p<0.05).
Supplementary Fig. 1. Time dependent accumulation of 1.0 µM [¹⁴C]-DLM by hCMEC/D3 cells with 0.01% HSA at 37ºC. Results are expressed as the Mean ± SD (n = 3).
Supplementary Fig. 2. (A) Effect of CSA (25 µM) on the protein binding of 1.0 µM [¹⁴C]-DLM with 0.01% HSA at 37ºC for 15 min. Results are expressed as the Mean ± SD (n = 3). (B) Effect of CSA (25 µM) on the protein binding of 1.0 µM [¹⁴C]-DLM with 4% HSA at 37ºC for 15 min. CSA (25 µM) was added simultaneously to the DLM/protein mixture as described in the methods. Results are expressed as the Mean ± SD (n = 3).