PLASMA PROTEIN BINDING OF DELTAMETHRIN, CIS-PERMETHRIN AND TRANS-PERMETHRIN IN HUMAN AND RAT PLASMA

By

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(Under the Direction of James V. Bruckner)

ABSTRACT

Pyrethroids are widely used insecticides in the United States. These insecticides are extensively used in agriculture as well as to control insect vectors of human and animal diseases. Pyrethroids are regarded as relatively safe insecticides compared to organophosphate compounds. However, there are several studies that show that exposure to certain pyrethroids, either by oral, dermal or inhalation routes during unprotected handing or spraying, may result in clinical sings of neurotoxicity. Children are likely to be exposed to pyrethroids due to ingestion of soil or dust contaminated with pyrethroid spray. The main objectives of this study were to determine: 1) species dependent changes in the plasma protein binding of deltamethrin (DLM), cis-permethrin (CIS) and trans-permethrin (TRANS) in human and rat plasma; 2) age-dependent changes in plasma protein binding of DLM, CIS and TRANS in human and rat plasma; and 3) the role of albumin and lipoproteins in binding of DLM, CIS and TRANS. New solvent extraction method was developed, validated and used to characterize the plasma protein binding in human and rat plasma. DLM, CIS and TRANS were bound to both albumin and lipoproteins in the plasma. The binding of all three compounds was higher to albumin compared to

lipoproteins. The binding of DLM, CIS and TRANS to human and rat plasma was quantitatively different. Plasma protein binding of all three compounds was age dependent. We observed significantly higher free fractions in birth -1 week and >1 week - 4 weeks compared to adults in humans. In rat plasma, significantly greater free fraction was observed in post natal days (PND) 10 and PND 15 as compared to PND 90. Saturation binding studies were conducted in human and rat plasma to determine the values of Bmax (maximum binding capacity) and Kd (dissociation constant) for all three compounds in all age groups. The binding parameters obtained in our study will be used for the development of a physiologically-based pharmacokinetic model for children's risk assessment.

INDEX WORDS: Pyrethroid, Plasma protein binding, Deltamethrin, Cis-permethrin, Trans-permethrin

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DEDICATION

I would like to dedicate this dissertation to my family and friends who gave me strength and faith to succeed.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Background:

The flowers of Chrysanthemum cinerariaefolium and Chrysanthemum cineum produce a natural tan-colored syrupy extract pyrethrum, with well-known insecticidal properties (Casida 1973; Todd et al. 2003). The bioactive compounds of pyrethrum are called pyrethrins. Pyrethrum flowers were used to control body lice by Caucasian tribes and Persia in the early 1800.

Pyrethrins were first imported into the United States in the 1860's (Casida 1980; Schofield and Crisafulli 1980) but were first registered for use as insecticides in 1950's (EPA 2006). Since natural Pyrethrins break down quickly in the presence of sunlight, the synthetic analogs and derivatives of pyrethrins, called pyrethroids were developed (Casida 1980; Soderlund et al. 2002). Pyrethroids due to their increased photostability and enhanced overall insecticidal potency, are more efficient than pyrethrins for agricultural and public health use (Power and Sudakin 2007; Soderlund et al. 2002).

Since the 1970's these insecticides have been extensively used in agriculture as well as to control insect vectors of human and animal diseases. By the mid-1990's, pyrethroids ranked second only to organophosphates and represent 23% of the US dollar value of the world insecticide market (Soderlund et al. 2002). Over the last decade, the uses of pyrethroids in the United States has increased dramatically, partly due to the phase out of organophosphates (Amweg et al. 2005; Mirfazaelian et al. 2006) and partly due to their properties like low environmental persistence, relatively low mammalian toxicity, and selective insecticide activity.

Most commercial pyrethroids are available as mixtures of optical and geometrical isomers (Lee et al. 2002). The isomers can have different toxicities and insecticidal properties. Pyrethroids are also utilized on livestock, in food storage and transportation, and in residential settings. They are extensively in small and large animals to control a wide range of ectoparasites (Anadón et al. 2009).

Human Exposure:

The extensive use of pyrethroids has resulted in exposure of the human population, albeit at very low levels (Baker et al. 2000; Schettgen et al. 2002) through exposure to pyrethroids by eating foods containing residues of pyrethroids, inhalation, skin contact, and accidental swallowing or drinking of pyrethroid products. Wei et al. (2012) reported elevated levels of pyrethroid metabolites in the urine of flight attendants working on commercial aircraft disinfected by pyrethroids. Bar et al. (2010) reported the presence of 3-phenoxybenzoic acid (3PBA), a metabolite common to many pyrethroid insecticides, in more than 70% of the samples. Heudorf et al. (2001) found pyrethroids metabolites in 75% of urine samples from a large German population (1,177) of non-exposed (at work or home) children and adults.

Lu et al. (2006) conducted a longitudinal study to assess the exposure of pyrethroid pesticides in elementary school-age children. During this study children's conventional diets were substituted with organic food items for 5 consecutive days. An association was found between the parents' self-reported pyrethroid use in the residential environment and elevated pyrethroid metabolite levels found in their children's urine. Naeher et al. (2010) reported substantially higher urinary 3-PBA levels in children living in Jacksonville (FL) suggesting higher pyrethroid pesticide exposures than the general populations of the United States. Morgan et al. (2007) described the potential exposures of 127 preschool children to the pyrethroid

insecticides from 127 homes and 16 daycare centers in six Ohio counties. Both cispermethrin and transpermethrin were detected in dust (100%) and hand wipes (>78%). The urinary metabolite 3-PBA was detected in 67% of the children's urine samples. They suggested dietary ingestion as the primary route of exposure.

Structures, Physical and Chemical Properties:

The six natural pyrethrins are pyrethrin I, pyrethrin II, cinerin I, cinerin II, jasmolin I, and jasmolin II. Pyrethrins are esters of cyclopropanecarboxylic acid and cyclopentenolone alcohol. Synthetic pyrethroids consist of one these domains along with variation in the alcohol portion of the compounds (Scollon et al. 2009; Soderlund et al. 2002). Individual pyrethroids are grouped into two classes, Type I (permethrin) and Type II (deltamethrin), based upon their physicochemical and toxicological properties. Althrin, the first commercial pyrethroid identified in 1949, was a type I pyrethroid (Bradberry et al. 2005). Type I pyrethroids are esters of primary or secondary alcohols whereas, type II pyethroids are esters of secondary alcohols that contain a cyano substituent in the 3-phenoxybezyl alcohol moiety (Nishi et al. 2006; Scollon et al. 2009; Soderlund et al. 2002). Although many new synthetic pyrethroids have been developed, less than twelve are currently used in the United States.

Pyrethroids are highly hydrophobic (log Kow in the range 5.7–7.6) and have a very low water solubility (of a few μg L−1) (Laskowski 2002). Pyrethroids are soluble in organic solvents like alcohol, chlorinated hydrocarbons, and kerosene (Todd et al. 2003). Pyrethroids tend to partition into lipids and organic matter due to their high octanol-water partition coefficients and high affinity to bind to sediment (Laskowski 2002).

Mechanism of Action:

Pyrethroids exert neurotoxicity in mammals and insects by acting on voltage sensitive sodium channels of nerve cells (Bradberry et al. 2005; Soderlund and Bloomquist 1989; Soderlund et al. 2002). Several studies have shown that pyrethroids delay the closure of sodium channel gates in mammalian and invertebrate neuronal membrane (Eells et al. 1992; Narahashi 1986). The initial influx of sodium during depolarization phase is protracted, resulting in a prolonged sodium tail current (Narahashi 1986; Soderlund and Bloomquist 1989; Vijverberg 1982). This results in repetitive discharge of nerve signals and eventually leads to paralysis. Based on the mode of action, type I pyrethroids produce repetitive nerve discharges whereas type II pyrethroids produce stimulus-dependent nerve depolarization and blockage (Soderlund and Bloomquist 1989). The duration of modified sodium currents by type I pyrethroids are believed to lasts for few milliseconds, while those of type II pyrethroids last for several seconds or longer (Davies et al. 2007).

Toxicity of Pyrethroids in Mammals:

In general, mammals are less susceptible to acute toxic effects of pyrethroids due their large body size, poor dermal absorption of pyrethroids, high body temperature and rapid and rapid conversion of these substances to nontoxic metabolites (Bradberry et al. 2005; Walters et al. 2009). The Acute oral toxicity of pyrethroids varies widely and is dependent on several factors like type of pyrethroid, ratio of stereo and optical isomers within a given pyrethroid formulation and type of administration vehicle used (Tan and Soderlund 2010). Acute oral LD_{50} for deltamethrin (DLM) given in corn oil was reported to be 87 mg/kg in male and 90 mg/kg in female rats (Myer 1989). Whereas, the value was > 5000 mg/kg when the dose was administered in aqueous suspension containing 1% methylcellulose (Varsho 1996). Similarly, the acute oral

LD_{50} for permethrin (45/55 cis/trans) neat in rats is 3,801 mg/kg. However, when the dose was given in corn oil the LD_{50} was 584 mg/kg (Metker et al. 1978).

Experimental animals exposed to extremely high doses of pyrethroids display two distinct poisoning syndromes. "T-syndrome" is mainly induced by acute poisoning with type I pyrethroids. It is characterized by hyperexcitation, tremors, and skin parathesias, followed by prostration and paralysis. "CS-syndrome" is mainly induced by type II pyrethroids. The characteristic signs of this syndrome are excessive salivation, tremors, choreoathetosis (involuntary movements of body) and paralysis (Shafer et al. 2005; Verschoyle and Aldridge 1980). Wolansky et al. (2006) determined acute oral dose-response functions in adult male Long Evans rats exposed to type I (bifenthrin, S-bioallethrin, permethrin, resmethrin, tefluthrin), type II (β-cyfluthrin, λ-cyhalothrin, cypermethrin, deltamethrin, esfenvalerate) and mixed (fenpropathrin) pyrethroids. A dose-dependent decrease in motor activity was observed for all pyrethroids.

Male, adult Sprague–Dawley rats treated with ≥20 mg/kg DLM in glycerol formal via oral dose exhibited salivation, marked tremors and choreoathetosis. DLM was even more neurotoxic when injected IV in glycerol formal (Kim et al. 2007). McDaniel and Moser (1993) described a neurotoxicity study in Long-Evans hooded rats orally exposed to permethrin at 25, 75, or 150 mg/kg in corn oil. After 2 and 4 h following treatment, animals exhibited fine to whole-body tremor, aggressive sparring behavior, hyperthermia, and decreased motor activity.

Ishmeal and Litchfield (1988) studied the chronic toxicity of permethrin in rat and mice. Rats and mice were fed permethrin in their diet for 2 years and life time (up to 98 weeks) respectively. During the first two weeks of treatment, whole body tremors and hypersensitivity to noise was noted only in rats fed high doses of permethrin. No signs of toxicity were observed at

low doses. Mice exhibited no signs of toxicity even at high doses. Nemec (1998) observed postural changes, impaired mobility and gait, impaired aerial righting reflex, reduced grip strength, hypersensitivity to noise, decreased hindlimb extensor strength, piloerection, convulsions, popcorn seizures, and mortality in Sprague–Dawley rats exposed to DLM (at a dose of 800 ppm) in the diet for thirteen weeks.

El-Aziz et al. (1994) reported significantly reduced plasma testosterone levels in male rats administered DLM in oral doses of 1or 2 mg/kg for 65 days. Reduced testosterone levels were noted as early as day 14 and remained lower than the controls throughout 21 days of post-treatment observation. They also observed decreased weights of genital organs and sperm motility in treated animals. Some studies have suggested the role of permethrin and DLM in genotoxicity. A 28-day oral treatment with permethrin (125.7, 50.3, and 12.6 mg/kg) in male Wistar rats resulted in increased chromosomal aberration in a dose-related manner (Institóris et al. 1999). Hoellinger et al. (1987) observed a significant increase of micronuclei percentage in bone marrow of a female Sprague-Dawley rat administered cismethrin at oral dose of 31 or 40 mg/kg. Administration of DLM (5.6, 8.4, or 11.2 mg/kg) to adult female albino rats by i.p. (intraperitoneal) dose induced chromosomal aberrations and micronuclei in bone marrow cells. Deaths in domestic cats have been reported after erroneous treatment with (45-65%) permethrin products designed to be used as flea treatment for dogs (Meyer 1999; Richardson 2000).

He et al. (1991) described an increase in nerve excitability in 24 DLM sprayers following 3 day exposures during spraying. Following DLM exposure, there was a prolongation of supernormal period in median nerve of the exposed group. One case reported the death of a 30-year old male approximately 2 days after consumption of 30 ml of DLM (Peter et al. 1996). Vomiting, diarrhea, loss of consciousness and metabolic acidosis was observed in a 59-year old

male who attempted suicide by drinking approximately 600 ml of 20% permethrin emulsion (Gotoh et al. 1998).

Plasma Protein Binding:

After entering the circulatory system, drugs bind to plasma proteins in varying degrees. In general, such binding is reversible and a chemical equilibrium exists between the bound and unbound drug. Protein binding is an important factor impacting disposition, efficacy and safety of many drugs (Notarianni 1990; Olson and Christ 1996). The pharmacological response for a majority of drugs is related to the free drug concentration at the receptor site (du Souich et al. 1993). High levels of plasma protein binding may restrict the compound to the blood and, therefore limit its action at the target site (Trainor 2007). The extent of drug binding to plasma proteins can have a significant impact on pharmacokinetic parameters such as volume of distribution and clearance (Banker and Clark 2008; Musteata et al. 2006). Binding of insecticides to plasma proteins can significantly affect their distribution, metabolism and excretion (Gao et al. 2004).

Effect of Age and Species on Plasma Protein Binding:

The plasma protein binding of many drugs are age dependent. This factor is more important during early developmental stages when plasma protein shows both quantitative and qualitative changes (Ehrnebo et al. 1971). In neonates, several factors like lower albumin levels, persistence of fetal albumin, and high concentration of exogenous and endogenous substrates (bilirubin, free fatty acids) in the plasma may result in decreased binding (Alcorn and McNamara 2003). It is widely accepted that plasma protein binding of drugs is generally lower in neonates than in adults. Meistelman et al. (1990) studied the effect of age on plasma protein binding of sufentanil. They observed a significantly higher free fraction in newborn than in infants (0.5 ±

0.3 yr), children (6.8 ± 3.0 yr), and adults (39.5 ± 9.0 yr). However, no significant difference in binding was observed between children and adults. Ehrnebo et al (1971) found low binding of ampicillin, α-azidobenzylpencillin, bezylpenecillin, phenobarbital and diphenyldantoin to human foetal and neonatal plasma as compared to adult plasma. Pacifici et al. (1987) examined plasma protein binding of furosemide in newborn infants and children. The unbound fraction of furosemide in newborns was significantly higher than adults. The unbound fraction reached the adult levels during first year of life.

Several studies have reported interspecies differences in plasma protein binding of drugs (Acharya et al. 2006; Matsushima et al. 1998). Matsushima et al. (1998) described the Plasma protein binding of tamsulosin hydrochloride in rats, dogs, and humans. The plasma protein binding of tamsulosin in humans was much higher (98.9–99.1%) than that in rats and dogs (79.0–80.6% and 90.2–90.3%, respectively). Lumpkin et al. (2003) reported higher binding of trichloroacetic acid in human as compared to rat plasma.

Plasma Proteins Involved in Binding:

Plasma contains several components that are capable of binding to drugs. However, previous studies indicate that insecticides predominantly bind to only two, albumin and lipoproteins, based upon concentration and binding affinity under physiological conditions (Maliwal and Guthrie 1981a; Skalsky and Guthrie 1978).

Albumin:

Albumin is the most abundant protein in the plasma, accounting for approximately 50% of the total proteins. It is a basic protein with a molecular weight of 66,500 Da. Albumin plays an important role in drug binding. It reversibly binds most compounds and thereby significantly affect the pharmacokinetics of the compound (Colmenarejo 2003). It exerts 80% of the colloid

osmotic pressure in the vascular compartment and thus aids in vascular fluid retention (Notarianni 1990). Albumin has two primary drug-binding sites, Sudlow I and II (Mandula et al. 2006). Kosa et al. (1997) pointed out that human albumin binding site I primarily binds to heterocyclic molecules (warfarin and phenylbutazone) via hydrophobic interactions. Site II preferably binds to aromatic carboxylic acids with a negatively charged group (diazepam and ibuprofen) at one end of the molecule away from a hydrophobic center (Hideto and Hirayama 2013).

Becker and Gamble (1982) described the binding of hexachlorobiphenyl to bovine serum albumin. They suggested that albumin possesses a cylinder like structure lined with hydrophobic residues that interact with hexachlorobiphenyl. Maliwal and Guthrie (1981) reported the binding of organophosphate, chlorinated hydrocarbon and carbamate insecticides to human serum albumin. The insecticides were bound with one high affinity and 4-6 moderate affinity binding sites to albumin. The affinity was inversely related to aqueous solubility of the compound. The binding was suggested to be hydrophobic in nature. However, (Cserháti and Forgács 1995) noted that albumin may bind to pesticides via hydrophilic forces occurring between the corresponding apolar substructures of pesticides and amino acid side chains of the protein. In an in vitro study, diazinon a highly bound (96.6%) organophosphate was found distributed to albumin (53.6 %) in human plama (Maliwal and Guthrie 1981b).

Ontogeny of Albumin:

Florence et al. (1995) measured serum concentrations of 10 acute-phase proteins in healthy term and preterm infants from birth to age 6 months. Albumin levels were significantly lower in newborn (35 g/L) and infants (35.6-40.1 g/L) compared to adults (45.1 g/L) throughout the period. Nayak and Mital (1977) studied the ontogeny of albumin during normal physiologic

growth and development in humans. Increases in serum albumin levels were observed from 3.5 g/L at 16 weeks of intrauterine age to 38 g/L at 4 weeks of post-natal age. Holt et al. (1983) reported significantly lower plasma albumin levels in neonates (35.7 ± 3.3 g/L), and infants (40.0 ± 2.9 g/L) from those of adults (44.6 ± 1.6 g/L). However, albumin levels in children (42.0 ± 2.9 g/L) were not significantly different from adult. Similar results were reported by Meistelman et al. (1990), where they observed significant differences in plasma albumin levels of neonates (38.5 ± 3.0 g/L), and infants (37.2 ± 4.2 g/L) from those of adults (43.9 ± 3.5 g/L). Children (40.8± 4.2 g/L) and adults had similar albumin levels in their plasma.

Lipoproteins:

Lipoproteins are macromolecular complexes of lipids and proteins and are the primary vehicles responsible for the transport of hydrophobic material through the aqueous circulatory system (Wasan et al. 2008). Adsorption may be mediated by hydrophobic or electrostatic interactions. The hydrophobic interaction may be mediated by the apoprotein, whereas electrostatic interactions may occur between the phospholipid charged groups and oppositely charged solutes. Lipoproteins are divided into five groups: chylomicrons, high density lipoproteins (HDL), low density lipoproteins (LDL), very low density lipoproteins (VLDL) and intermediate density lipoproteins (IDL). Although plasma concentrations of lipoproteins are relatively low they can account for greater than 90% binding in the plasma (Wasan et al. 2008). Lipoproteins reversibly bind to nonionic and basic lipophilic drugs (Martínez-Gómez et al. 2006).

Changes in lipoprotein concentrations can alter the pharmacokinetic and pharmacodynamics of drugs that bind to lipoproteins. Gardier et al. (1993) examined the distribution of cyclosporine A in the plasma of patients awaiting heart transplantation and the

influence of plasma lipid constituents on the pharmacokinetics of cyclosporine. Patients with high cholesterol levels demonstrated increased cyclosporine association with LDL and an increased cyclosporine induced toxicity compared to normolipidemic controls. Vadiei et al. (1990) reported higher plasma concentrations and induced renal toxicity of amphotericin B in hyperlipidemic obese Zucker rats compared to lean litter-mates.

Insecticides are thought to partition into the lipid core rather than binding to specific site on lipoproteins (Maliwal and Guthrie 1981b). Maliwal and Guthrie (1982) reported rapid transfer of chlorinated hydrocarbons among VLDL, LDL and HDL after in vitro incubation in human plasma. The distribution was independent of the amount taken up by the lipoproteins. Maliwal et al. (1981) described the binding of chlorinated hydrocarbon, carbamate and organophosphate insecticides to human LDL and HDL. The lack of discrete binding sites on HDL and LDL suggested that the transport function of lipoproteins was mainly due to the partitioning of these insecticides into lipoproteins. The binding affinities of the insecticides were inversely related to the water solubility of these compounds.

Vomachka et al. (1983) examined the uptake, distribution and transfer of 2, 4, 5, 2', 4', 5'-hexachlorobiphenyl in human and rat plasma. They found that after intravenous administration of 2, 4, 5, 2', 4', 5'-hexachlorobiphenyl to the rat, for a short time following injection the LDL fraction served as the primary transport vehicle. However, after 24 hours there was there was a shift in the distribution of the hexachlorobiphenyl from LDL to HDL and the remaining protein-rich fraction. It was suggested that lipoproteins compete with albumin for binding to hexachlorobiphenyl.

Ontogeny of Lipoproteins:

Lane and McConathy (1986) evaluated changes in the serum lipids and apolipoproteins following feeding in the first 4 weeks of life. They reported maximum levels of apolipoproteins and serum lipids occurred at 14 days followed by a decline to lower levels at 28 days. Berenson et al (1981) studied serum lipids and lipoprotein in newborns, infants and school age children. They reported a rapid increase in α-lipoproteins after birth reach maximum levels by two year of age. The β-lipoproteins and triglycerides increase in early infancy and reach a maximum level around six months of age. Higher levels of α-lipoproteins were found in children as compared to adults. The developmental changes in plasma lipoproteins in levels and composition in rats during the first week of the life are comparable to those described in humans (Garcia-Molina et al. 1996).

The triglyceride and total cholesterol levels in a newborn infant is one-half to one-third those of the adult (Lloyd 1973). Srinivasan et al. (1976) studied serum lipoprotein profiles in children ages 5-14 years. They reported little change in α-lipoprotein levels with age. However, significant increases in pre-β-lipoprotein levels and β-lipoprotein levels were noted between 11 and 14 years. Asayama et al. (1990) reported sex related differences in HDL_2 concentrations between ages 11-15 years. HDL_2 levels increased with age in females, whereas in males, levels increased up to ages 6-10 years but tend to decline thereafter. HDL_3 levels were higher in adults than in children.

Role of Albumin and Lipoproteins in Transport of Insecticides:

Several studies have highlighted the role of albumin and lipoproteins, in transport of insecticides in various animals. Most of these studies are based on distribution or binding of insecticides to various plasma fractions (Maliwal and Guthrie 1981b; Skalsky and Guthrie 1977).

Skalsky and Guthrie (1978) determined the binding of DDT, and dieldrin in human plasma. Albumin and β-lipoprotein exhibited the highest affinity for DDT and dueldrin in vitro. Binding was demonstrated to be low affinity, hydrophobic in nature. Mohammed et al. (1990) described the distribution of toxaphene, DDT and PCB in plasma of adult Sprauge-Dawley rats and in human plasma. They observed up to 52 % of these compounds in albumin and ~37 % associated with lipoproteins. Maliwal and Guthrie (1981) studied binding of chlorinated hydrocarbon, carbamate and organophosphate insecticides to human LDL and HDL. They suggested the interaction to be due to partitioning rather than saturable binding. Further, they found the distribution was related to lipid content and composition of lipoproteins.

There are limited conflicting reports of plasma protein binding of pyrethroids in the literature. In one study Cui et al. (2006) observed significant binding of cypermethrin to bovine serum albumin and bovine hemoglobin. However, Aqel et al. (2002) noted no significant interaction of permethrin with human serum albumin after 1 hour of incubation. These differences may be due to interspecies variations or different measurement techniques. Kosa et al. (1997) reported differences in the binding pattern of diazepam to bovine, rabbit and rat albumin from those of human albumin. Several studies have indicated that with increasing polarity, plasma protein binding of a drug decreases. Pyrethroids, due to their non-polar and lipophilic nature, are anticipated to bind to both albumin and lipoproteins.

The overall objective of this research project is to gain insight into the plasma protein binding of representative pyrethroid insecticides. The interplay between affinity of pyrethroid insecticides for the plasma protein and maximum plasma protein binding capacity will determine the amount of free chemical available in the system. An example in this case is the study conducted by Lumpkin et al. (2003) to determine the effect of plasma protein binding of

trichloroacetic acid (TCA) availability in human, rat and mice. The researchers observed greater plasma protein binding of TCA in humans that reduced the availability of TCA for uptake by liver and other tissues. The information can subsequently be used to refine physiologically-based pharmacokinetic (PBPK) models. Recently Buur et al. (2007) developed a PBPK model that linked plasma protein binding to disposition of sulfamethazine and Flunixin meglumine in swine. Such models are needed to predict the internal dosimetry (i.e., time-course in the bloodstream, central nervous system, liver, skeletal muscle, fat and other tissues) of common pyrethroids for different exposure scenarios. The data obtained from our study will subsequently be used as input parameters for construction of PBPK models to predict target organ doses of selected pyrethroids and their associated health risks. Gaining an understanding of these processes will reduce uncertainties in the PBPK models' predictions of internal dosimetry and neurotoxicity.

Specific Aims:

1. Develop a method for quantification of binding of deltamethrin (DLM), cispermehtrin (CIS) and transpermethrin (TRANS) to plasma proteins.
2. To investigate a) Species differences; b) Characterization of pyrethroid binding to major plasma components; c) Calculation of binding parameter – for use in human and rat PBPK models.
3. To determine age dependent changes in plasma protein binding of DLM, CIS and TRANS in human and rat plasma.

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CHAPTER 2

DEVELOPMENT OF A METHOD TO DETERMINE PLASMA PROTEIN BINDING OF DELTAMETHRIN, CIS-PERMETHRIN, AND TRANS-PERMETHRIN IN HUMAN AND RAT PLASMA

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Abstract:

Pyrethroids are widely used insecticides in the United States. The extensive use of these insecticides has resulted in human exposure, although at very low levels. While high dose toxicities have been extensively studied in animals, human and animal health effects of low environmental exposures are unknown. There have been limited and conflicting reports in the literature about the plasma protein binding of pyrethroids. Traditional methods failed to determine their plasma binding due to problems like non-specific adsorption and limited solubility of these compounds in the polar solvents. Preliminary experiments showed a significant portion (~ 55%) of the compounds adhered to plastic vials. To avoid non-specific binding silanized glass vials were used. A solvent extraction method employing radiolabeled pyrethroids was developed to quantify plasma protein binding of deltamethrin (DLM), cispermethrin (CIS) and transpermethrin (TRANS). The absolute recoveries of all three compounds ranged from 91-96%. Both inter- and intra-day precision for all three pyrethroids in human and rat plasma was below 15 % R.S.D. DLM, CIS, and TRANS were found ~90% bound to human plasma and ~80% to rat plasma at a concentration range of 0.25µM - 0.75µM. Binding was inversely proportional to concentration > 750 nM. The solvent extraction method proved to be rapid and accurate to determine plasma protein binding of three pyrethroids.

Introduction:

Pyrethroids are the most commonly used insecticide in the United States since the phase-out of organophosphates (Lao et al. 2012). Pyrethroids are used to control insects in nearly all agricultural crops, home/garden environments and nurseries. They are also used in construction sites (pre-construction termiticides), various urban structural and landscaping sites (Spurlock and Lee 2008). Pyrethroids are also widely used for indoor pest control (Barr et al. 2008). Certain

pyrethroids are also used to treat mites, mosquitos, human head lice and scabies (Go et al. 1999). Because of the wide spread use, these pyrethroids are found in fruits, vegetables and grains at low levels (Pang et al. 1995; Pang et al. 2006; Torres et al. 1996). Permethrin (PER), commonly a mixture of *cis* and *trans* isomer is the most widely used insecticide in United States the (Barr et al. 2008).

Traditionally, pyrethroids are grouped into two classes, Type I and II, based upon their physicochemical and toxicological properties. Type I pyrethroids lack a cyano-phenoxybenzyl moiety at the alpha position, while type II have a cyano moiety (Crofton and Reiter 1988; Nasuti et al. 2003). In insects, pyrethroids produce their effect by causing impairment of motor function (Wolansky and Harrill 2008). At high doses, similar effects are observed in mammals (Shafer et al. 2005). Exposures to pyrethroids have been documented in several human studies, including infants, children and pregnant women (Becker et al. 2006; Berkowitz et al. 2003; Fortin et al. 2008; Schettgen et al. 2002; Whyatt et al. 2002). Type I pyrethroids (permethrin) produce signs like aggressive sparring and increased sensitivity to external stimuli, parasthesias and fine tremor (Anand et al. 2006; Ray et al. 2000). Type II pyrethroids (deltamethrin) produce salivation, hyperexcitability, choreoathetosis, and seizures as the major signs of poisoning (Anand et al. 2006; Miyamoto et al. 1995).

There are limited conflicting reports of the plasma protein binding of pyrethroids in the literature. One study (Cui et al. 2006) observed significant binding of cypermethrin to bovine serum albumin and bovine hemoglobin. However, another study (Abu-Qare and Abou-Donia 2002) noted no significant interaction of permethrin with human serum albumin after 1 hour of incubation. These differences may be due to interspecies variations or different measurement techniques.

Plasma protein binding can have a significant impact on the pharmacokinetics and pharmacodynamics of a drug (Jusko and Gretch 1976; Lin et al. 1987). Plasma protein binding is an important factor that affects drug disposition, efficacy and safety (Notarianni 1990; Olson and Christ 1996). The knowledge of plasma protein binding is useful for the pharmacokinetic modeling of drugs in the discovery, preclinical and clinical stages of development (Banker and Clark 2008; Taylor and Harker 2006). The extent of drug binding to plasma proteins can have a significant impact on pharmacokinetic parameters such as clearance and volume of distribution (Banker and Clark 2008; Musteata et al. 2006). Only the unbound fraction of drug in plasma is available for pharmacodynamic action, distribution and elimination. Thus, accurate measurement of unbound plasma drug concentrations is essential to understand important pharmacokinetic and pharmacodynamic characteristics of a drug (Wright et al. 1996).

Several methods including ultrafiltration, equilibrium dialysis, ultracentrifugation, high performance liquid chromatography and gel filtration, are commonly used to determine protein binding (Barre et al. 1985; Taylor and Harker 2006; Waters et al. 2008). However, with lipophilic drugs these methods are of limited use because of nonspecific adsorption (Lee et al. 2003; Taylor and Harker 2006). Several studies have reported that the polymer used to construct devices and filter membrane used to measure plasma protein binding can be susceptible to non-specific binding of test compounds (Banker et al. 2003; Fois and Ashley 1991; Lee et al. 2003; Taylor and Harker 2006). Therefore, for highly lipophilic drugs, modifications to traditional methods has been attempted to reduce or prevent non-specific binding. Lee et al. 2003, observed a significant decrease in non-specific binding for etoposide, hydrocortisone, propranolol, and vinblastine when equilibrium dialysis membrane was pretreated with 5% tween 80 or 5% benzalkonium chloride. Taylor and Harker (2006) devised a modified ultracentrifugation method

in order to overcome the non-specific binding problems associated with corticosteroids. (Henricsson 1987) used stainless steel equilibrium dialysis chambers to minimize the problem of non-specific binding of cyclosporine to polytetrafluoroethylene (Teflon) chambers. Other techniques like red blood cell partitioning methods (Roos and Hinderling 1981; Trung et al. 2006), solid phase microextraction (Vaes et al. 1996; Yuan and Pawliszyn 2001) and use of solid-supported lipid (Transil) membranes (Schuhmacher et al. 2000) have been applied to the determination of plasma protein binding of highly bound drugs. Unfortunately, for pyrethroids these techniques cannot be utilized because of limitations such as adsorption, poor solubility of the drug in the buffer, or the necessity of using large amounts of plasma. Since these methods have their own limitations, new technologies for accurate determination of plasma protein binding of pyrethroids must be developed.

The objective of the current investigation was to develop a new method to determine plasma protein binding for three common pyrethroids: Deltamethrin (DLM), Cispermethrin (CIS) and Transpermethrin (TRANS). An important goal was to validate the method using other highly bound, lipophilic compounds and compare them with the literature values.

Materials and Methods:

Materials:

Radiolabeled [^{14}C]-deltamethrin (54.1 mCi/mmol) was supplied by Bayer CropScience (Stilwell, KS), [^{14}C]-Cis-permethrin (61 mCi/mmol) and [^{14}C]-trans-permethrin (61 mCi/mmol) was provided by Symbiotic Research (Mount Olive, NJ). Radiolabeled [^{14}C]-diazepam (55 mCi/mmol) and [^{3}H]-Cyclosporine (20 Ci/mmol) were purchased from American Radiolabeled Chemicals (St.Lous, MO). Standard DLM (purity, 98.8 %), CIS (purity, 99.0 %), and TRANS (purity, 99.3 %) were kindly provided by FMC Agricultural Product Group (Fresno, CA, USA).

Acetonitrile (HPLC-grade), hexamethyldisilazane (Reagent-grade), sodium fluoride (NaF) (purity, 99.0 %) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Isooctane (purity, 99.0 %) and 2-Octanol (Laboratory-grade) were purchased from Fisher Scientific (Pittsburgh, PA, USA). All clean glassware was silanized with 5% hexamethyldisilazane 24 hours prior to the start of experiments.

Plasma:

Frozen, heparinized, pooled adult human and rat plasma were obtained from Innovative Research (Novi, MI, USA). All plasma samples were stored at -80°C until use. All plasma samples were filtered through 0.45 µm Millipore® filters to remove precipitated fibrinogen.

Plasma Protein Binding Measurements:

Binding to selected pyrethroids was determined by three step solvent extraction method (Fig. 2-1). Stock solutions of ^{14}C radiolabelled DLM, CIS and TRANS were prepared at final concentration of 2500 nM. A volume of 80 µl of human or rat plasma was spiked with 10 µl of ^{14}C radiolabelled DLM, CIS or TRANS (250 nM to 100 uM) in silanized glass vials. Plasma samples were then treated with 10 µl of 0.64 M NaF to inhibit carboxylestrase. Samples were incubated in an orbital shaker (110 rpm) at 37°C for 3 hours. Pilot experiments revealed that all three pyrethroids binding reached equilibrium by 3 hours. Samples were mixed with 200 µl of isooctane and vortexed for 30 sec. The isooctane layer, containing the unbound fraction was removed and mixed with 3 ml of scintillation fluid in a liquid scintillation vial. The total amount of radiolabeled pyrethroid present in these 200 µl was considered the unbound fraction. The remaining plasma was mixed with 200 µl of 2-octanol and vortexed for 30 sec. The total amount of radiolabeled pyrethroid present in these 200 µl was considered associated with lipoproteins. The 2-octanol layer, containing the lipoprotein-bound DLM, CIS or TRANS, was removed and

mixed with 3 ml of scintillation fluid in a liquid scintillation vial. The remaining plasma was then mixed with 200 µl of acetonitrile and vortexed for 30 seconds. The total amount of radiolabeled pyrethroid present in these 200 µl was considered the protein (albumin) bound fraction. The acetonitrile layer, containing the protein-bound fraction was mixed with 3 ml of scintillation fluid in a liquid scintillation vial. Plasma temperature and pH were maintained at 37°C and 7.4, respectively, to approximate the plasma binding environment in vivo. Four replicates (n=4) of ten concentrations for DLM, CIS and TRANS were tested ranging from 250 nM to 100 uM. The unbound, lipoprotein associated and protein bound fraction for DLM, CIS and TRANS was quantified by liquid scintillation counting using a Beckman Coulter LS 6500 (Brea, CA).

Validation Procedures:

Absolute recovery was calculated from total radioactive counts [unbound fraction + Lipoproteins associated fraction + protein (albumin) associated fraction] of DLM, CIS or TRANS in rat and human plasma compared with those of standard solutions. Validation for determination of inter-day (repeatability) and intraday precision (reproducibility) for unbound fraction, lipoproteins associated fraction and protein associated fraction for all three pyrethroids in human and rat plasma. For intra-day precision, the samples (n=4) spiked at concentrations of 250 nM, 10 µM and 100 µM were analyzed. Inter day precision was determined in four replicate of human and rat plasma spiked at three different concentrations (250 nM, 10 µM and 100 µM). The precision was expressed as the relative standard deviation (% R.S.D.) and calculated from standard deviation divided by the mean of unbound, lipoprotein associated and protein bound fraction. Since there are no reference standards for protein binding, it was not possible to test accuracy of the assay.

Validation Using Other Drugs:

The solvent extraction method was used to determine plasma protein binding of diazepam and cyclosporine. Stock solutions of radiolabeled diazepam (^{14}C) and cyclosporine (^{3}H) were prepared at a final concentration of 7023 nM and 8320 nM respectively. A volume of 80 µl of human or rat plasma was spiked with 10 µl of ^{14}C radiolabeled diazepam (702 nM or 0.2 mg/L) or 10 µl of ^{3}H radiolabeled cyclosporine (832 nM or 1mg/L) in silanized glass vials. Plasma samples were then treated with 10 µl of 0.64 M NaF to inhibit carboxylestrase. Samples were incubated in an orbital shaker (110 rpm) at 37°C for 3 hours. Samples were subjected to the three step solvent extraction procedure as described previously.

Results and Discussion:

Solvent Extraction Method:

The solvent extraction method proved to be rapid and accurate to determine plasma protein binding of three pyrethroids. The chemical structures of DLM, CIS and TRANS are shown in Figs. 2-2. It should be noted that the big advantage of the solvent extraction method is that it is more accurate for highly bound compounds. This method measures unbound, albumin and lipoprotein bound fractions in human and rat plasma. As pyrethroids are highly lipophilic, they might be anticipated to nonspecifically bind to polystyrene apparatus or equilibrium dialysis membrane. Wheelock et al. (2005) studied the influence of container adsorption upon observed pyrethroid toxicity to *Ceriodaphnia dubia* and *Hyalella azteca*. They reported up to 50% of the pyrethroid can adsorb to the container, with the highest adsorption to high-density polyethylene plastic containers. This resulted in time dependent decrease in the toxicity following pyrethroid adsorption to test containers for *C. dubia*. Previous published methods on plasma protein binding of pyrethroids (Abu-Qare and Abou-Donia 2002; Cui et al. 2006) have ignored the non-

specific binding of pyrethroids to apparatus. We observed significant non-specific adsorption of DLM (26.4 %), CIS (41.7%) and TRANS (59.4%) to plastic plates when pyrethroids were incubated in cell culture plates for 120 minutes (Fig 2-3). These results were similar to Lee et al. (2002) who reported losses of 28-42% in first 24 hours due to nonspecific bindings of pyrethroids. With the current method, glass vials were initially silanized to reduce nonspecific binding. The silanization of glassware has previously been reported to reduce the absorption of polar compounds such as carboxylic acid pesticides onto glassware surfaces (Hong et al. 2001). The absolute recoveries of DLM, CIS and TRANS from spiked plasma were in the range of 91-96 %. The percent unbound, lipoprotein associated and protein (albumin) bound values of DLM, CIS and TRANS in human plasma and rat plasma (spiked with 250 nM concentration) are listed in tables 2-1 and 2-2.

The unbound fraction was extracted using isooctane. Several studies have used isooctane to extract pyrethroids (López-Blanco et al. 2005; MO et al. 2009; Serodio and Nogueira 2005) from water, soil and tea samples. It has been reported that plasma proteins are not soluble in isooctane (Valdez et al. 2001) and conformational properties of proteins are not denatured during the solubilization process (Nicot and Waks 1995). Moreover, (Hušek et al. 2002) observed that isooctane alone cannot extract free fatty acids from serum. Therefore, it is reasonable to anticipate that isooctane is a suitable solvent to extract free pyrethroids (fraction) without changing the properties of the plasma.

Lipoprotein bound fraction was extracted using 2-octanol. (Waters 1964) demonstrated that extraction with 2-cotanol removes majority of the lipids from the human serum or plasma. Further, (Miller and Waters 1966) observed minimal alteration of serum proteins following extraction with 2-octanol. No changes on quantity or mobility of albumin were noted. Thus, 2-

octanol can remove significant amount of lipids from the plasma without altering the other protein fractions. The albumin bound fraction was extracted using acetonitrile. Acetonitrile acts by precipitating the plasma proteins and also serves as an excellent solvent for the highly lipophilic pyrethroids. Pyrethroids are highly lipophilic compounds with an octanol-water partition coefficient in the range of 5.5-7.5 (Laskowski 2002). High octanol-water partition values of pyrethroids make them much more soluble in the lipid-soluble solvent, acetonitrile, than a polar solvent.

Method Validation:

Intraday and interday precision were determined to evaluate the reliability of the current method. The inter-day and intraday precision for DLM, CIS and TRANS in rat and human plasma were evaluated using 250 nM, 10 µM and 100 µM. As shown in Tables 2-3 to 2-6 both inter- and intraday precision for all three pyrethroids in human and rat plasma were below 15 % R.S. D.

The suitability of this method was examined through measurement of plasma protein binding values for two well-studied drugs (diazepam and cyclosporine), and good correlation with literature values were obtained. Diazepam and cyclosporine were selected based on their binding properties to the plasma components. Although both diazepam and cyclosporine are highly bound to plasma proteins, diazepam primarily binds to albumin (Davis et al. 1985; Grandison and Boudinot 2000) whereas cyclosporine binds primarily to the lipoprotein fraction of plasma (Hughes et al. 1991; Wasan et al. 1997). In the current study, the total binding of diazepam to adult human plasma was found to be 95 ± 1% (Fig. 1-4). Our results are consistent with the previous studies, where diazepam was found bound up to 96.8 % to plasma proteins

(Klotz et al. 1976). Majority of the diazepam (92.36 ± 1.2 %) was recovered from the acetonitrile fraction suggesting that this layer contains the albumin bound fraction.

We observed high binding (95.46 ± 1.2 %) of cyclosporine to adult human plasma (Fig. 1-5). Yang and Elmquist (1996) used microdialysis to study plasma binding of cyclosporin and found the binding to be 98.5 ± 1.2 % at 37°C. It should be noted that in a three step extraction method, majority of the cyclosporine was present in the octanol fraction (85.98 ± 1.2 %). As stated earlier the octanol layer was expected to extract the lipoprotein fraction for the plasma. This is consistent with the earlier study by Sgoutas et al. (1986) where they demonstrated that up to 85 % of cyclosporine was associated with lipoproteins.

Conclusions:

Although the acute toxicity of DLM, CIS and TRANS is well characterized, there has been a lack of published simple and reliable methods to determine plasma protein binding of these compounds. Therefore it was necessary to develop a method and validate a method to determine the plasma protein binding of these compounds. A solvent extraction method using radiolabeled pyrethroids is an efficient method to quantify the binding of these highly hydrophobic compounds. Eliminating dialysis membrane, plastic apparatus enabled us to overcome the nonspecific binding that often serves as a hindrance to measure true plasma protein binding of these highly lipophilic compounds. The three step solvent extraction method not only measured the total bound and unbound fraction, but also enabled us to measure the lipoprotein bound fraction of pyrethroids in the plasma. This is a valuable advantage over the traditional methods used to determine plasma protein binding. This method is currently being used in our laboratory to investigate the age related changes in plasma protein binding of DLM, CIS and TRANS in human and rat plasma.

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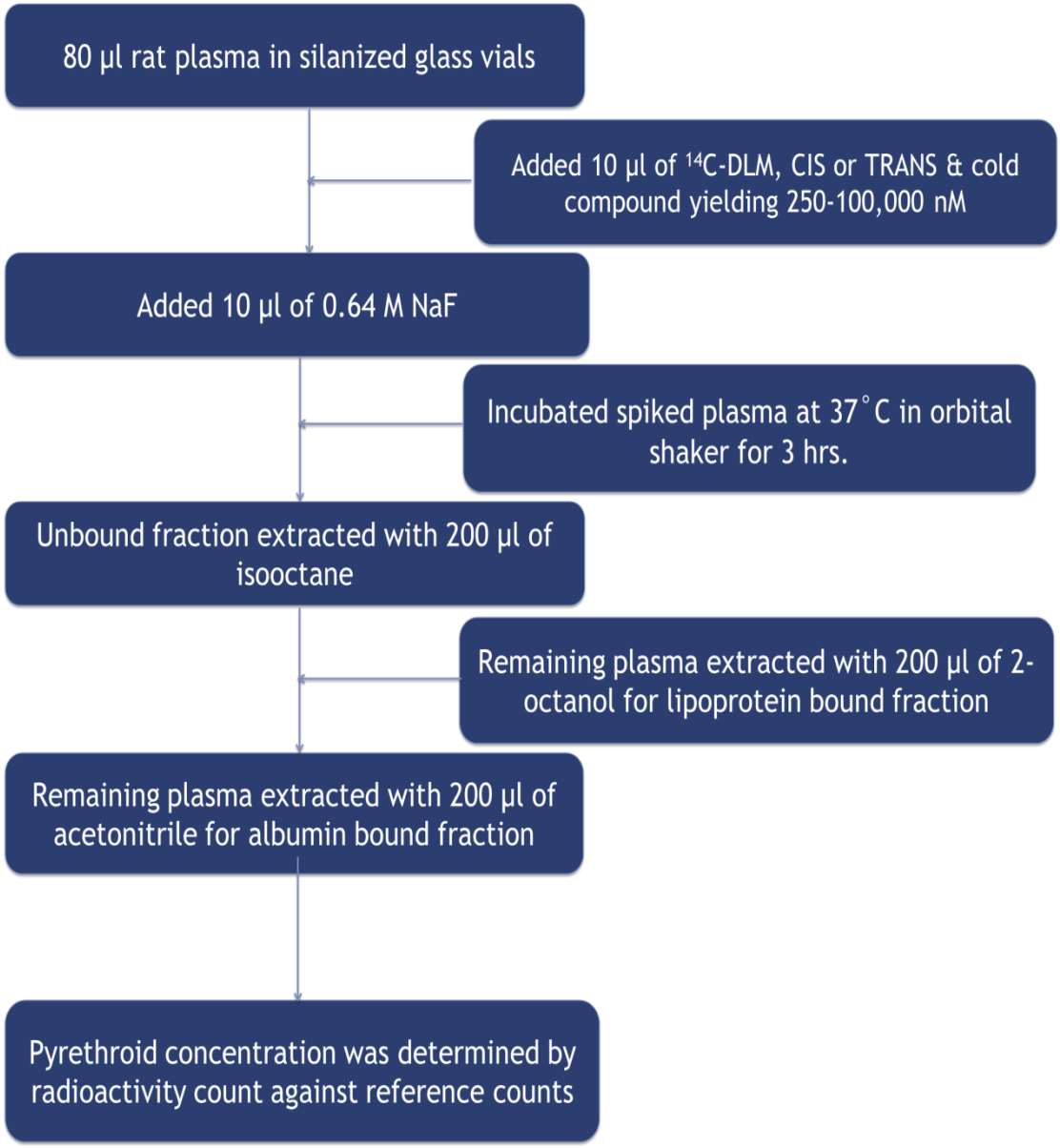

Figure 2-1. Scheme of solvent extraction method

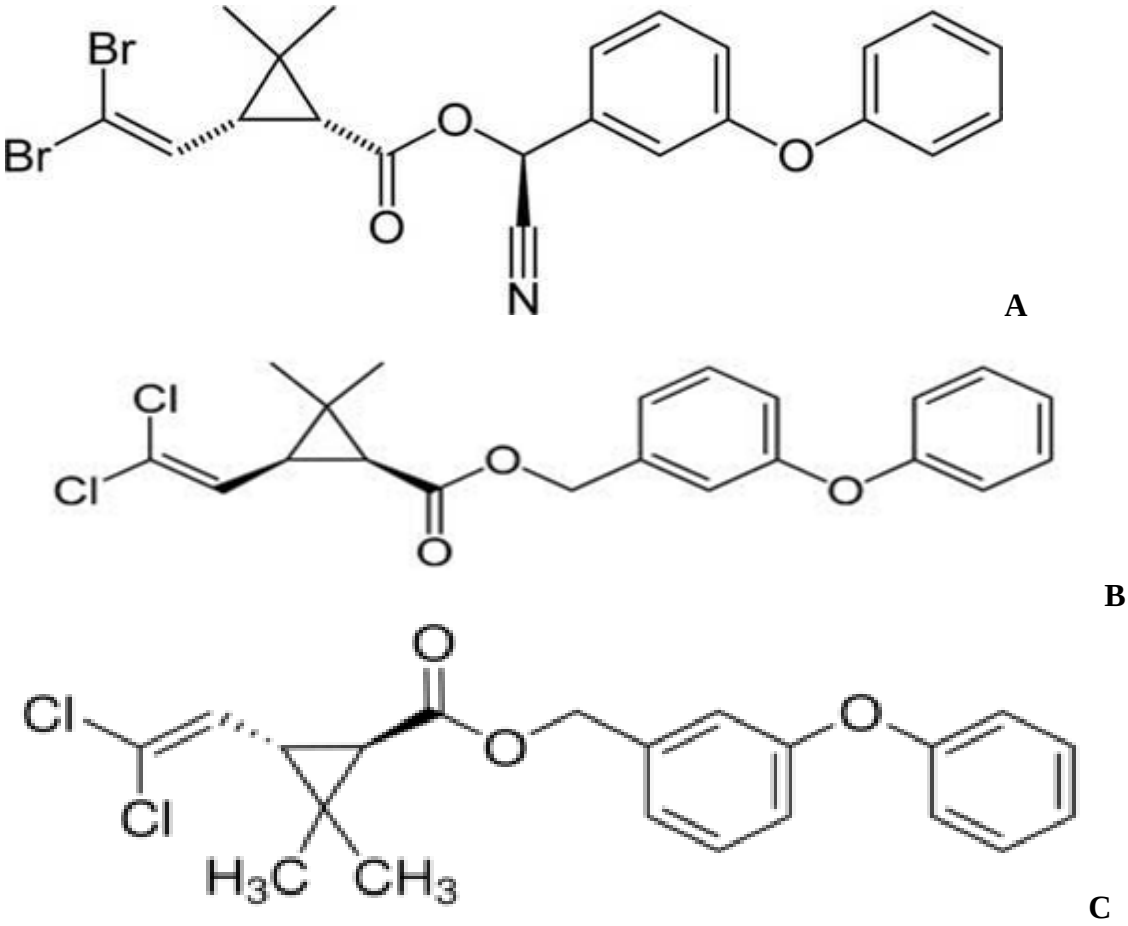

Figure 2-2. Chemical Structures of DLM (A), CIS (B) and TRANS (C)

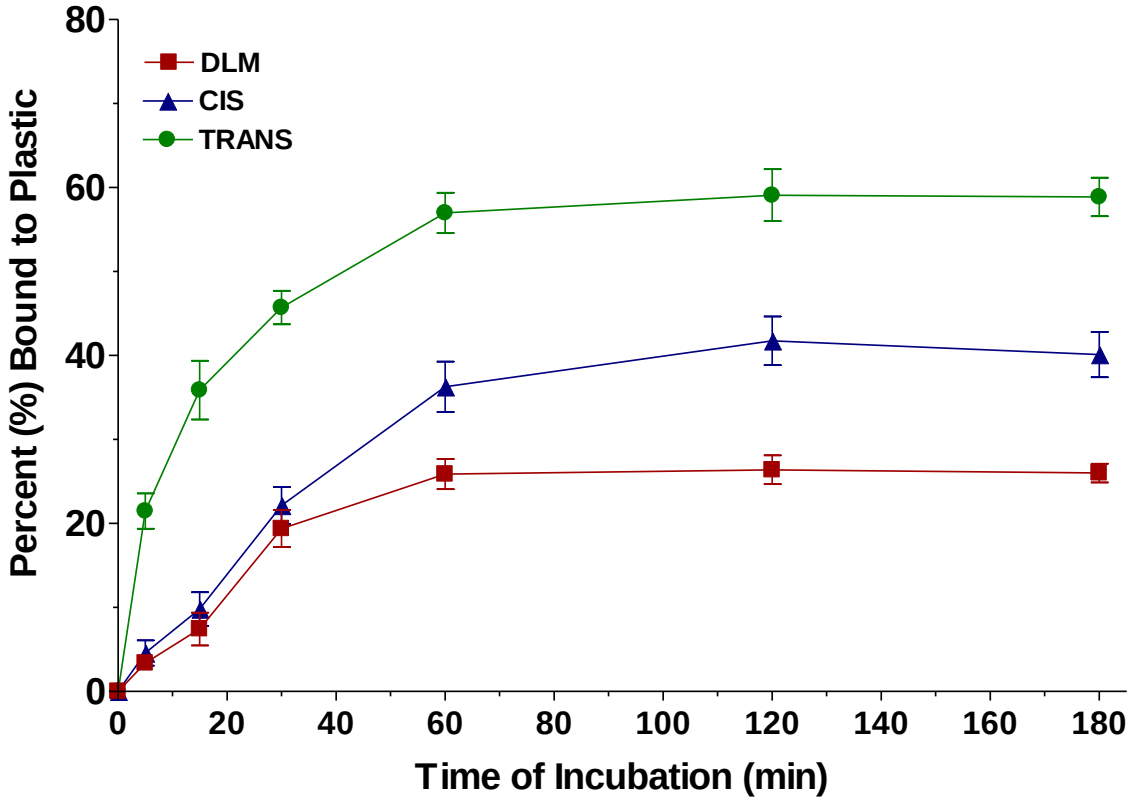

Figure 2-3. Binding of DLM, CIS and TRANS at a concentration of 250 nM to the cell culture plastic plates at 37°C for 120 minutes. Values represent mean ± SD, n=4

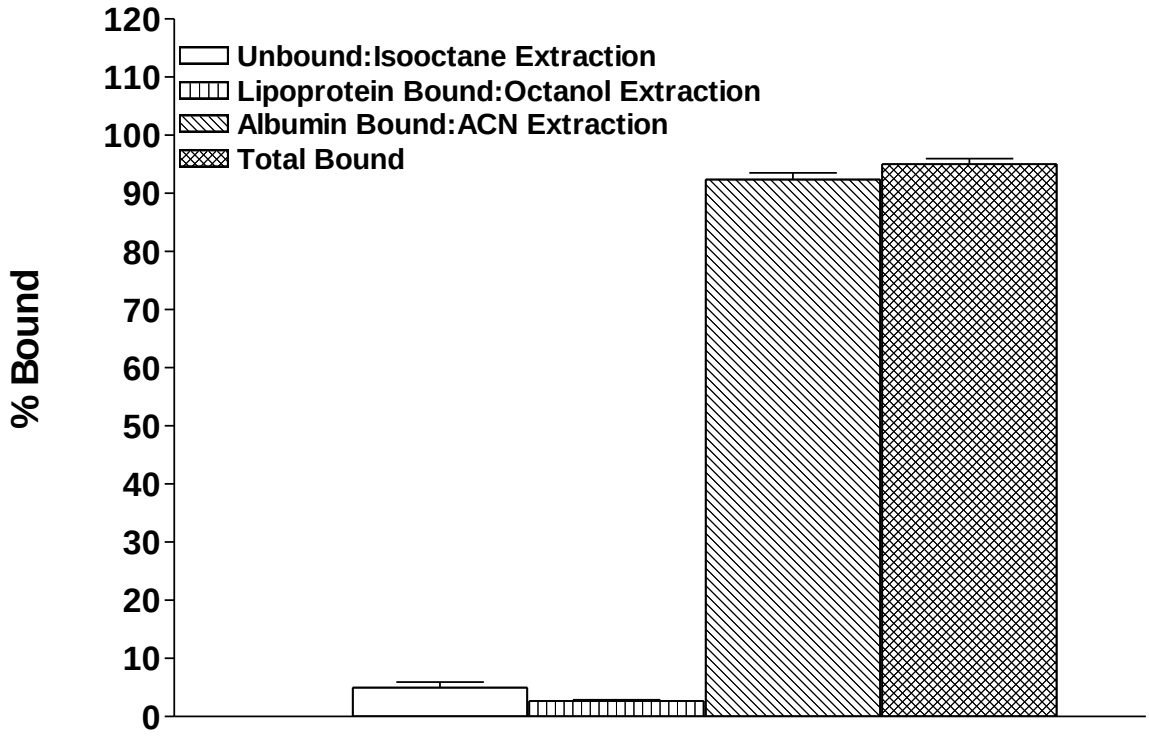

Figure 2-4. Plasma protein binding of diazepam (702 nM or 0.2 mg/L) to adult human plasma using solvent extraction method. Data represent mean ± SD, N=4.

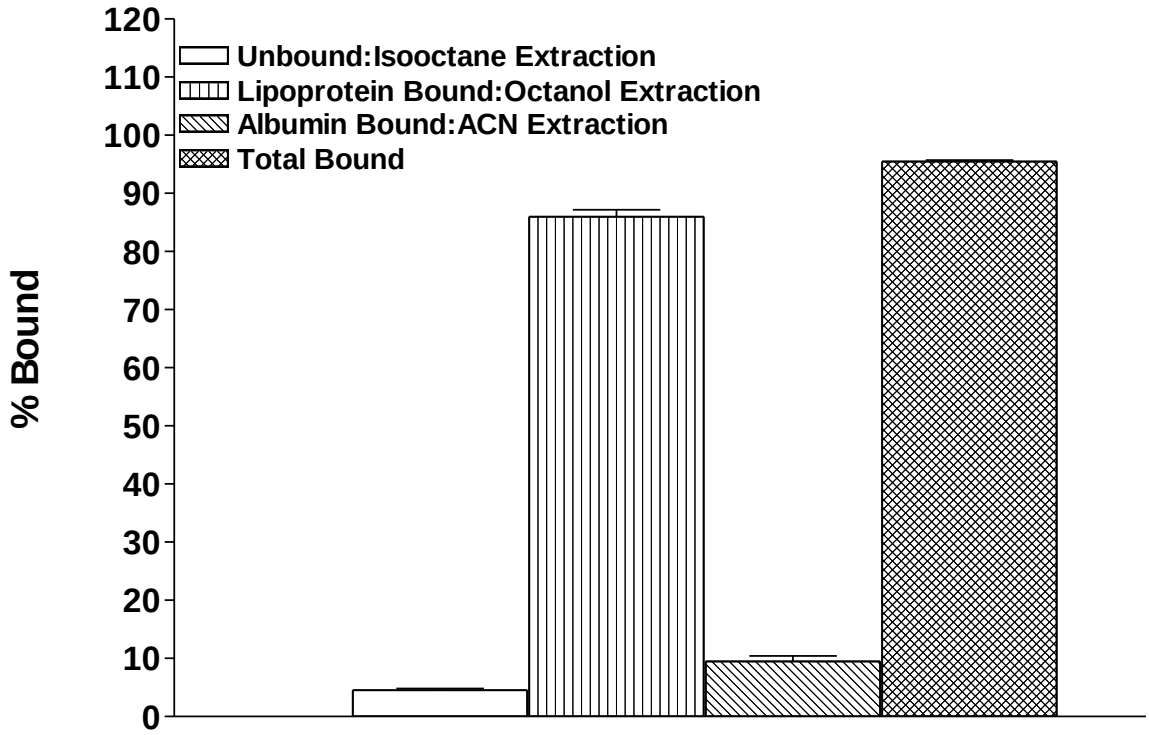

Figure 2-5. Plasma protein binding of cyclosporine (832 nM or 1mg/L) to adult human plasma using solvent extraction method. Data represent mean ± SD, N=4.

Table 2-1. The percent unbound, lipoprotein associated and protein (albumin) bound values of DLM, CIS and TRANS in human plasma

Compound	% unbound	% Associated to lipoproteins	% Protein Bound (Albumin)	% Total Bound
DLM	10.56 ± 1.0	31.30 ± 1.1	58.14 ± 2.0	89.44 ± 1.0
CIS	11.34 ± 1.7	29.40 ± 1.7	59.25 ± 2.4	88.66 ± 1.4
TRANS	11.57 ± 1.0	28.21 ± 3.4	60.21 ± 3.8	88.33 ± 1.0

Data represent Mean ± SD, N=4.

Table 2-2. The percent unbound, lipoprotein associated and protein (albumin) bound values of DLM, CIS and TRANS in rat plasma

Compound	% unbound	% Associated to lipoproteins	% Protein Bound (Albumin)	% Total Bound
DLM	19.61 ± 1.0	29.76 ± 2.5	50.63 ± 1.7	80.39 ± 1.0
CIS	22.54 ± 2.2	28.42 ± 3.6	49.04 ± 3.3	77.46 ± 2.2
TRANS	19.52 ± 1.0	30.17 ± 3.5	50.31 ± 3.7	80.48 ± 1.0

Data represent Mean ± SD, N=4.

Table 2-3. The intraday precision (% R.S.D.) for DLM, CIS and TRANS in human plasma

Compound	Concentration	Unbound Fraction (%)	Precision[a]	Lipoprotein Associated Fraction (%)	Precision	Protein Bound Fraction (%)	Precision
		(Mean ± SD)	(% R.S.D)	(Mean ± SD)	(% R.S.D)	(Mean ± SD)	(% R.S.D)
DLM	0.25	10.56 ± 0.9	8.2	31.31 ± 1.1	3.6	58.14 ± 2.0	3.4
	10	28.72 ± 1.2	5.2	26.31 ± 2.7	10.3	44.04 ± 2.7	6.0
	100	80.37 ± 1.5	1.9	9.10 ± 1.0	10.5	10.5 ± 1.0	8.1
CIS	0.25	11.34 ± 1.0	7.6	29.40 ± 1.7	5.6	59.25 ± 2.4	3.9
	10	39.19 ± 2.6	6.8	23.34 ± 1.3	5.3	37.66 ± 1.6	4.1
	100	78.78 ± 1.4	1.7	10.36 ± 1.0	9.7	10.86 ± 0.4	3.5
TRANS	0.25	11.57 ± 0.6	5.4	28.21 ± 3.4	11.9	60.21 ± 3.8	6.3
	10	29.27 ± 1.3	4.4	30.97 ± 2.8	9.1	39.75 ± .7	9.5
	100	76.35 ± 1.6	2.1	10.41 ± 1.0	8.5	13.24 ± 1.2	9.1

[a]The precision was evaluated as the % R.S.D. N=4.

Table 2-4. The inter-day precision (% R.S.D.) for DLM, CIS and TRANS in human plasma

Compound	Concentration	Unbound Fraction (%)	Precision[a]	Lipoprotein Associated Fraction (%)	Precision	Protein Bound Fraction (%)	Precision
		(Mean ± SD)	(% R.S.D)	(Mean ± SD)	(% R.S.D)	(Mean ± SD)	(% R.S.D)
DLM	0.25	10.27 ± 1.2	11.4	32.98 ± 1.3	3.8	56.75 ± 2.0	3.4
	10	29.19 ± 1.2	4.3	27.66 ± 0.4	1.3	43.15 ± 1.4	3.3
	100	80.18 ± 1.8	2.3	9.38 ± 1.0	10.0	10.44 ± 1.0	8.5
CIS	0.25	11.26 ± 1.2	10.6	28.75 ± 2.7	9.4	59.25 ± 2.6	4.4
	10	41.48 ± 3.5	8.5	21.28 ± 1.7	7.9	37.24 ± 2.0	5.5
	100	79.68 ± 1.0	1.2	10.04 ± 1.0	8.5	10.28 ± 0.5	4.4
TRANS	0.25	10.97 ± 0.7	6.1	29.63 ± 1.7	5.8	59.40 ± 1.5	2.5
	10	30.27 ± 2.6	8.5	30.97 ± 2.7	8.9	39.23 ± 1.8	4.6
	100	77.53 ± 1.3	1.6	10.28 ± 1.0	7.3	12.18 ± 1.2	8.3

[a]The precision was evaluated as the % R.S.D. N=8.

Table 2-5. The intraday precision (% R.S.D.) for DLM, CIS and TRANS in rat plasma

Compound	Concentration	Unbound Fraction (%)	Precision[a]	Lipoprotein Associated Fraction (%)	Precision	Protein Bound Fraction (%)	Precision
		(Mean ± SD)	(% R.S.D)	(Mean ± SD)	(% R.S.D)	(Mean ± SD)	(% R.S.D)
DLM	0.25	19.61 ± 0.8	4.2	29.76 ± 2.5	8.2	50.63 ± 1.7	3.4
	10	38.12 ± 4.7	12.3	21.19 ± 1.4	6.6	40.70 ± 4.5	11.2
	100	86.02 ± 1.0	1.9	6.93 ± 1.0	10.4	7.05 ± 13.1	8.1
CIS	0.25	22.00 ± 3.2	14.7	28.95 ± 4.3	14.6	49.05 ± 3.3	6.7
	10	50.45 ± 4.1	8.0	21.66 ± 2.5	11.3	27.89 ± 1.6	5.7
	100	87.32 ± 1.5	3.7	5.89 ± 1.0	12.7	6.79 ± 0.5	7.3
TRANS	0.25	22.54 ± 2.2	9.9	28.42 ± 3.6	12.6	49.04 ± 3.3	6.7
	10	41.19 ± 1.1	2.6	23.55 ± 1.0	4.3	35.26 ± 2.1	5.9
	100	87.04 ± 1.5	1.7	6.16 ± 1.0	13.2	6.80 ± 1.0	11.2

[a]The precision was evaluated as the % R.S.D. N=4.

Table 2-6. The inter-day precision (% R.S.D.) for DLM, CIS and TRANS in rat plasma

Compound	Concentration	Unbound Fraction (%)	Precision[a]	Lipoprotein Associated Fraction (%)	Precision	Protein Bound Fraction (%)	Precision
		(Mean ± SD)	(% R.S.D)	(Mean ± SD)	(% R.S.D)	(Mean ± SD)	(% R.S.D)
DLM	0.25	20.13 ± 1.7	8.5	29.32 ± 2.2	7.6	50.55 ± 1.0	1.7
	10	38.32 ± 1.2	3.1	21.52 ± 1.0	4.2	40.16 ± 1.0	2.1
	100	85.74 ± 1.2	2.3	6.89 ± 1.0	8.4	7.37 ± 1.0	11.6
CIS	0.25	23.33 ± 1.8	7.7	26.39 ± 1.9	7.2	50.28 ± 2.6	5.2
	10	48.52 ± 1.6	3.3	20.08 ± 1.1	5.5	31.40 ± 1.0	3.3
	100	86.35 ± 1.4	1.6	6.36 ± 1.0	10.4	7.29 ± 1.0	10.3
TRANS	0.25	19.17 ± 1.5	7.6	29.29 ± 1.7	5.7	51.54 ± 2.0	3.9
	10	39.69 ± 1.7	4.3	24.68 ± 1.7	7.1	35.63 ± 2.4	6.7
	100	86.53 ± 1.2	1.3	6.28 ± 1.0	11.2	6.67 ± 0.2	8.1

[a]The precision was evaluated as the % R.S.D. N=8.

CHAPTER 3

PLASMA PROTEIN BINDING OF PYRETHROIDS: ROLE OF ALBUMIN AND LIPOPROTEINS IN TRANSPORT OF PYRETHROIDS IN HUMAN AND RAT PLASMA

Pankaj Sethi, James V. Bruckner, Catherine White. To be submitted to Drug Metabolism and Disposition

Abstract:

Pyrethroids insecticides are widely used to control a wide variety of pests in and around homes, in food handing establishments, in mosquito control and in agricultural. Relatively little is known about the plasma protein binding of these compounds. The main aim of this study was to investigate the species differences in the binding of deltamethrin (DLM), cispermethrin (CIS) and transpermethrin (TRANS) to plasma proteins. We also wanted to determine the extent and relative contribution of plasma albumin and lipoproteins in the transport of DLM, CIS and TRANS in plasma. All three pyrethroids exhibited similar binding properties. The binding of DLM, CIS and TRANS to human and rat plasma was linear over the concentration of 250 nM to 750 µM and inversely proportional to concentration >750 nM. At concentrations below 750 nM, total binding of DLM, CIS and TRANS were in the range of 88-90 % and 76-83 % in human and rat plasma, respectively. All three pyrethroids had higher binding to albumin (approximately 54-60%) compared to lipoproteins (30-35%) in both human and rat plasma at concentrations below 750 nM. However, at higher concentrations there was an equal distribution of all three compounds to the albumin and lipoprotein fractions, indicating a greater role of lipoproteins in the transport of these compounds at higher concentrations. The higher free fraction observed in rat plasma might result in a greater toxicity of DLM, CIS and TRANS in rats. There were significant differences in Bmax for all three compounds between adult human and rat. Kd values were not significantly different between two species. These values will be used to generate a physiologically-based pharmacokinetic model

Introduction:

Pyrethroids, synthetic derivatives of pyrethrins, are used as insecticides throughout the world. Their use in the United States has increased dramatically during the past decade with the

phase-out of organophosphates (Power and Sudakin 2007). Pyrethroids are used widely to control insects in agriculture, horticulture, forestry, public health and residential settings (Heudorf and Angerer 2001). Pyrethroids are also used extensively in humans, small and large animals to control broad range of ectoparasites like mites, head lice and scabies (Anadón et al. 2009; Naeher et al. 2009).

Pyrethroids are used extensively to control pests due to their desirable properties like selective insecticide activity, low mammalian toxicity, and low environmental persistence (Casida et al. 1983; Soderlund and Knipple 2003). Although more than 1000 pyrethroids have been synthesized since 1973 (Todd et al. 2003), only about 20 pyrethroids have been approved by the U.S. Environment Protection Agency. Permethrin is the most widely used pyrethroid in the United States. A survey conducted by the U.S. Department of Housing and Urban Development in collaboration with the United States Environmental Protection Agency found permethrin as one of the most common insecticides detected in a randomly selected nationally representative sample of residential homes (Stout II et al. 2009). Thus, the widespread use of pyrethroids has resulted in low levels of contamination of soil, water and air (Zhang et al. 2011) as well as fruits, vegetables and grains (Pang et al. 1994).

Several recent studies have expressed concern about occupational and non-occupational exposure of human population to pyrethroids (Fortes et al. 2012; Wei et al. 2013; Wielgomas et al. 2012). Wei et al. (2012) studied pyrethroid exposure of flight attendants working on commercial aircrafts disinfected and non-disinfected with pyrethroids. Elevated levels of pyrethroid metabolites were observed in the urine of flight attendants working on commercial aircraft disinfected by pyrethroids. Fortes et al. (2012) found higher levels of 3-phenoxybenzoic acid, a common urinary metabolite in pyrethroid exposure, in subjects consuming both raw and

cooked vegetables five or more times weekly. Pyrethroids are generally regarded as safe insecticides as they are rapidly metabolized in mammals (Narahashi et al. 2007; Soderlund et al. 2002).

Pyrethroids are divided into two groups (type I or type II) based on their chemical structure and acute-high dose biological effects (Soderlund et al. 2002; Wolansky and Harrill 2008). Type II pyrethroids (Deltamethrin) contains α-cyano group, while type I (Cis- and Trans-permethrin) lacks it. Both type I and type II pyrethroids alter the normal function of the nervous system. Pyrethroids primarily act on the voltage sensitive sodium channels (VSSC), however the chloride, calcium, and other channels may also be targets (Ray et al. 2000; Shafer and Meyer 2004). Pyrethroids act by delaying the closure of VSSC (Soderlund, Clark et al. 2002). Insects are 2250-times more susceptible to pyrethroid toxicity as compared to mammals due to their increased sodium channel sensitivity, smaller body size and lower body temperature (Bradberry et al. 2005). The most common symptoms produced by acute intoxication of type I pyrethroids are whole-body tremors, also known as T-syndrome. Major symptoms produced by acute intoxication of type II pyrethroids are choreoathetosis and salivation, also known as CS-syndrome (Wolansky and Harrill 2008).

One of the important functions of the plasma is to serve as a transport medium for nutrients and drugs in the body. Plasma carrier proteins bind and transport a broad range of endogenous and exogenous hydrophobic substances (Baker 2002). Deschamps-Labat et al (1997) reported a linear relationship between lipophilicity and binding to human serum albumin of arylpropionic acid non-steroidal anti-inflammatory drugs. Lipoproteins are also responsible for binding and transport of lipophilic substances in the blood of humans and animals (Wasan et al. 2008). Brevetoxin (PbTx-3), a neurotoxin produced by *dinoflagellate Karenia brevis*, after in

vitro and in vivo exposures in mice (Woofter et al. 2005) and in vitro exposure in humans (Woofter and Ramsdell 2007), was found bound primarily to the high density lipoprotein (HDL) fraction in the plasma. In an early study, two insecticides, dieldrin and delodrin, were reported to partition, in part, to the lipoprotein fraction of rat and rabbit blood (Moss and Hathway 1964). Further investigation with the plasma revealed that these insecticides were primary associated with the HDL fraction. As pyrethroids are highly lipophilic compounds, it is reasonable to hypothesize that they will bind to both albumin and lipoprotein fractions in the plasma.

There are no studies that describe the distribution of pyrethroids in plasma. In one of the study, Gray and Rickard (1981) studied the tissue distribution of radiolabeled deltamethrin after intravenous administration to rats. They reported that the major portion of the radioactivity was derived from the alcohol and acid-labeled moieties of deltamethrin in the plasma.

Albumin differs in the amino acid sequence and protein structure between humans and rats (Mandula, Parepally et al. 2006). Further the distribution and composition of lipoproteins are different in rat and human plasma (Mills and Taylaur 1971). These changes might explain species difference in binding of these pyrethroids. Therefore, the overall objective of this study was to investigate the species differences in the binding of DLM, CIS and TRANS to plasma proteins. Previous studies have demonstrated that the interaction of hydrophobic compounds with plasma can modify their pharmacological activity, pharmacokinetics and tissue distribution (Lovich et al. 2001; Wasan and Lopez-Berestein 1994). We also wanted to determine the extent and relative contribution of plasma albumin and lipoproteins in the transport of DLM, CIS and TRANS in blood.

Materials and Methods:

Materials:

Radiolabeled [14C]-deltamethrin (54.1 mCi/mmol) was supplied by Bayer CropScience (Stilwell, KS), [14C]-Cis-permethrin (61 mCi/mmol) and [14C]-trans-permethrin (61 mCi/mmol) was provided by Symbiotic Research (Mount Olive, NJ). Standard DLM (purity, 98.8 %), CIS (purity, 99.0 %), and TRANS (purity, 99.3 %) were kindly provided by FMC Agricultural Product Group (Fresno, CA, USA). Acetonitrile (HPLC-grade), hexamethyldisilazane (Reagent-grade), sodium fluoride (NaF) (purity, 99.0 %) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Isooctane (purity, 99.0 %), 2-Octanol (Laboratory-grade) were purchased from Fisher Scientific (Pittsburgh, PA, USA). All clean glassware was silanized with 5% hexamethyldisilazane 24 hours prior to the start of experiments.

Plasma:

Frozen, heparinized, pooled adult human and rat plasma were obtained from Innovative Research (Novi, MI, USA). All plasma samples were stored at -80°C until use. All plasma samples were filtered through 0.45 µm Millipore® filters to remove precipitated fibrinogen.

Plasma Protein Binding Measurements:

Binding of DLM, CIS and TRANS were determined by a three step solvent extraction method. Stock solutions of ^{14}C radiolabelled DLM, CIS and TRANS were prepared at a final concentration of 2500 nM. Stock solutions of non-radiolabelled (cold) DLM, CIS and TRANS were prepared by dissolving the appropriate amount of DLM, CIS or TRANS in dimethyl sulfoxide. A volume of 9 µl of radiolabelled DLM, CIS or TRANS stock were mixed with 1 µl of non-radiolabelled stock to achieve a final concentrations of 250 nM to 100 uM. A volume of 80 µl of human or rat plasma was spiked with 10 µl of ^{14}C radiolabelled DLM, CIS or TRANS

(250 nM to 100 uM) in silanized glass vials. Plasma samples were then treated with 10 µl of 0.64 M NaF to inhibit carboxylestrase. Samples were incubated in an orbital shaker (110 rpm) at 37°C for 3 hours. Pilot experiments revealed that binding of all three pyrethroids had reached equilibrium by 3 hours (Sethi et al. 2013). Samples were mixed with 200 µl of isooctane and vortexed for 30 sec. The isooctane layer, containing the unbound fraction was removed and mixed with 3 ml of scintillation fluid in a liquid scintillation vial. The total amount of radiolabeled pyrethroid present in this 200 µl fraction was considered the unbound portion, or fraction. The remaining plasma was mixed with 200 µl of 2-octanol and vortexed for 30 sec. The total amount of radiolabeled pyrethroid present in these 200 µl was considered associated with lipoproteins. The 2-octanol layer, containing the lipoprotein-bound DLM, CIS or TRANS, was removed and mixed with 3 ml of scintillation fluid in a liquid scintillation vial. The remaining plasma was then mixed with 200 µl of acetonitrile and vortexed for 30 seconds. The total amount of radiolabeled pyrethroid present in these 200 µl was considered the protein (albumin) bound fraction. The acetonitrile layer, containing the protein-bound fraction was mixed with 3 ml of scintillation fluid in a liquid scintillation vial. Plasma temperature and pH were maintained at 37°C and 7.4, respectively, to approximate the plasma binding environment in vivo. Four replicates (n=4) of ten concentrations for DLM, CIS and TRANS were tested ranging from 250 nM to 100 uM. The unbound, lipoprotein associated and protein bound fraction for DLM, CIS and TRANS were quantified by liquid scintillation counting using a Beckman Coulter LS 6500 (Brea, CA).

Measurement of Albumin and Total Protein:

Albumin levels in human and rat plasma were measured by use of a kit (QuantiChrom™ BCG Albumin Assay Kit; BioAssay Systems, Hayward, CA, USA) according to the

manufacturer's instructions. Total protein estimations in human and rat plasma were performed using a BCA Protein assay kit (Pierce/Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions.

Data and Statistical Analysis:

All experiments were performed with a minimum of three independent experiments unless otherwise stated. Data were analyzed using nonlinear curve fitting (Graphpad Prism) using a one-site binding (hyperbola) to obtain KD (dissociation constant) and Bmax (maximum number of binding sites) values. Statistical significance was evaluated by two way ANOVA with a significance level of $p<0.05$ using Graphpad Prism 5.

Results:

Figure 3-1, shows the albumin and total protein levels in adult rat and human plasma. The concentrations of albumin and total proteins in human plasma were 4.01 ± 0.2 g/dl and 6.40 ± 0.7 g/dl, respectively. The concentrations of albumin and total proteins in rat plasma were 3.00 ± 0.2 g/dl and 5.37 ± 0.5 g/dl, respectively. This data shows that human plasma has 10-15 % higher albumin and total protein levels as compared to rat plasma.

The binding of DLM, CIS and TRANS to human plasma was linear over the concentration of 250 nM to 750 nM and inversely proportional to concentration >750 nM (Fig. 3-2). With concentrations < 750 nM, similar binding patterns were observed for all three compounds. Total binding of DLM, CIS and TRANS were in the range of 88-90 % (Fig 3-2). Approximately 10-12% of pyrethroids were unbound, 28-31% was associated with lipoproteins and 58-60% was bound to albumin (Fig. 3-3). However, as the concentrations of DLM, CIS and TRANS in the plasma increased, the ratio of compounds distributed to lipoproteins increased. At highest concentrations, there was an equal distribution of all three compounds between albumin

and the lipoprotein fraction of the plasma (Fig 3-4, 3-5 and 3-6). The saturation binding curves for DLM, CIS and TRANS in human plasma are shown in figure 3-7. Table 3-1 shows the binding parameters estimates for DLM, CIS and TRANS in human plasma.

In rat plasma binding was linear over the concentration of 0.25 µM to 0.75 µM and inversely proportional to concentrations >0.75 µM (Fig. 3-8). In adult rats, total binding of DLM, CIS and TRANS were in the range of 76-83 % in linear concentration range (Fig. 3-8). Approximately 17-24% of pyrethroids were unbound, 28-31% was bound/associated with lipoproteins and 47-50% was bound to albumin (Fig 3-9). However at higher concentrations there was an equal distribution of all three compounds between albumin and lipoprotein fraction of the plasma (Fig 3-10, 3-11, 3-12). Figure 3-13, shows the saturation binding curves for DLM, CIS and TRANS in rat plasma. Binding parameter estimates for DLM, CIS and TRANS in rat plasma are listed in table 3-1.

Figures 3-14, 3-15 and 3-16, shows the comparative binding of DLM, CIS and TRANS respectively, in human and rat plasma in the linear concentration range. Between rat and human, the percent unbound and percent albumin bound fractions were significantly different ($p<0.05$) for all three pyrethroids. In rat, the percent unbound fraction observed was approximately twice that of human. The distribution of DLM, CIS and TRANS to the lipoprotein fraction was similar in both rat and human plasma. These values were not significantly different from each other.

Discussion:

Despite frequent exposure of the populace to relatively low levels of pyrethroids, relatively little is known about the plasma protein binding, and transport of DLM, CIS, TRANS or other pyrethroids in the systemic circulation of humans or other mammals. The determination of plasma protein binding of these highly lipophilic pyrethroids is difficult due to their poor

solubility in the polar solvents and nonspecific adsorption to the equipment used to measure plasma protein binding. A solvent extraction method was developed to determine the binding of these compounds. A key contribution of the work was to determine the relative distribution of these pyrethroids into albumin and lipoprotein fraction of the plasma.

Findings in the present study demonstrated relatively high binding of DLM, CIS and TRANS in adult human (88-90%) and rat (78-80 %) plasma at concentrations where linear binding was observed. We found that DLM, CIS and TRANS were bound to both albumin and lipoproteins in the plasma. Hence, it is reasonable to assume a role of both albumin and lipoproteins in the transport of DLM, CIS and TRAN in blood. Skalsky and Guthrie (1978) described the binding of DDT, dieldrin, carbaryl, and parathion to human serum proteins. All four insecticides were bound significantly to both albumin and lipoproteins. The binding was demonstrated to be low affinity, reversible and hydrophobic in nature. Vomachka et al. (1983) reported that 2, 4, 5, 2', 4', 5'-hexachlorobiphenyl, the predominant polychlorinated biphenyl congener in humans, binds to both albumin and lipoprotein fractions in the plasma.

In the current study, DLM, CIS and TRANS associated (approximately 30-35%) with the total lipoprotein fraction in both human and rat plasma. Pyrethroids are highly lipophilic (log Kow in the range of 5.7–7.6) compounds. Therefore, they were anticipated to associate with the lipoprotein fraction in the plasma. The inner hydrophobic core of lipoproteins facilitates the incorporation of poorly water soluble drugs, while the outer apolipoproteins monolayer helps to solubilize and stabilize the insoluble lipids of the lipoprotein particle (Wasan and Cassidy 1998). Wasan et al. (1999) reported the distribution profile of a novel endotoxin antagonist, [^{14}C] E5531, in human plasma. They found the majority of E5531 bound with HDLs in the plasma. Mathews et al. (1984) described the in vivo transport of halogenated biphenyl in rat blood.

During the initial phase, these compounds were associated with all plasma proteins. With the passage of time, higher levels of halogenated biphenyl were observed in lipoproteins. The distribution of halogenated biphenyl to the lipoproteins was proportional to the lipid solubility of the compound involved. This is consistent with our current study where we observed more of these compounds in lipoprotein fraction of the plasma at higher concentrations. Polyakov et al (1996) observed the highest association of benzo[a]pyrene with the HDL fraction following in vitro incubation in rat plasma. Vomachka et al. (1983) reported that after the intravenous administration of 2, 4, 5, 2', 4', 5'-hexachlorobiphenyl to the rat, for a short time following injection, the LDL fraction served as the primary transport vehicle. However, after 24 hours there was a shift in the distribution of the hexachlorobiphenyl from LDL to HDL and the remaining protein-rich fraction. It was suggested that lipoproteins compete with albumin for binding to hexachlorobiphenyl. Similarly, Maliwal and Guthrie (1981a) found that the binding of carbaryl and carbofuran was greater for HDL than LDL even though LDL has about 1.5 times the lipid content. The results of these studies indicated a complex interaction between lipoproteins and hexachlorobiphenyl rather than a simple dissolution into the lipoprotein core (Hjelmborg et al. 2008). In our study in the linear concentration range, all three compounds were only ~30-35% bound to lipoproteins. This indicates that at lower concentrations, lipoproteins have to compete with albumin for binding to pyrethroids. However, at higher concentrations, we observed an equal distribution of DLM, CIS and TRANS in lipoprotein and albumin fractions. This indicates that higher concentrations of lipoproteins might play a more important role in the transport of these compounds in vivo.

Data from our preliminary invivo study suggested that DLM binds predominantly to the HDL class of lipoproteins in rats given an oral dose of DLM (data not shown). We also found

low levels of DLM in LDL fraction. However, barely detectable levels were found in the VLDL fraction of the rat plasma. Vost and Maclean (1984) describe finding HDL as the major acceptor of benzo(α)pyrene, hexadecane, octadecane and DDT when rats were given these compounds intravenously with chylomicrons. Sonie et al (1982) measured the distribution of chlordecone (organochlorine pesticide) in rat plasma after in vivo administration and in vitro incubation. In both situations, chlordecone was approximately 80% bound to HDL, 11% to LDL, and 9% to VLDL. The reason for the higher distribution of chlordecone with HDL is its several-fold higher affinity for HDL than for LDL or VLDL (Maliwal and Guthrie 1982). Polyakov et al (1996) examined the distribution of [^{3}H]benzo[a]pyrene in rat plasma 30 minutes post iv injection of benzo-[a]pyrene-lipoproteins complexes. Benzo[a]pyrene was found distributed to HDL (40%), LDL (14%), VLDL (23%) and other plasma proteins (23%).

We observed that all three pyrethroids were bound to a greater extent to albumin (approximately 54-60%) as compared to lipoproteins in the linear concentration range. Hence, albumin might play a major role in the transport of these three pyrethroids at low concentrations. The lipophilic nature of pyrethroids can be the key for binding to albumin. Cszerhárti and Forgács (1995) reported that the hydrophobicity of pesticides exert a significant influence on their albumin binding capacity. They suggested that the pesticide-albumin complex can be less hydrophobic than the uncomplexed pesticide molecule. Mourik and Jong (1978) described high affinity binding of parathion (organophosphate) to bovine and human serum albumin. Maliwal and Guthrie (1981b) reported the binding of organophosphates, carbamates and chlorinated hydrocarbon insecticides to human serum albumin. One site of higher affinity and 4 to 6 binding sites of moderate affinity, inversely related to aqueous solubility of the compounds were observed. The interaction was primarily hydrophobic in nature. In an in vitro study, diazinon, an

organophosphate, was found distributed to albumin (53.6 %), LDL (29.9 %) and HDL (13.0 %) in human plasma (Maliwal and Guthrie 1981). Mohammed et al. (1990) described the distribution of DDT, PCB and toxaphene in vitro and in vivo. They found up to 52% of these compounds in albumin and 37% in the HDL_2 fraction in rat plasma. Our results were similar to their study for all three pyrethroids. Interestingly, we also observed DLM, CIS and TRANS bound approximately 55% to purified human serum albumin. These findings suggest that although albumin is the major transport protein of DLM, CIS and TRANS in plasma, it has limited capacity.

The binding of DLM, CIS and TRANS to human and rat plasma was quantitatively different. We observed a significant difference in the unbound and total bound fraction between rat and human plasma. Rat plasma exhibited approximately twice the unbound fraction when compared to human plasma. The total bound fraction was significantly higher in human plasma. Although the levels of DLM, CIS and TRANS distributed to lipoproteins were higher in human than rat plasma, however, they were not significantly different from each other. Although the amino acid sequence on human serum albumin are highly conserved (~80 %) and homologous to rat serum albumin, interspecies differences in binding site activity has been observed (Lavan 2008; Martinez 2011). Kosa et al. (1997) reported that human albumin binding site I primarily binds to heterocyclic (warfarin and phenylbutazone) molecules via hydrophobic interactions. Site II preferably binds to aromatic carboxylic acids (diazepam and ibuprofen) (Hideto and Hirayama 2013). Binding parameters of site I was found similar between rats and human. In contrast, marked differences between rat and human albumin were observed at site II due to changes in the microenvironment resulting from changes of size and/or hydrophobicity of binding pocket (Kosa, Maruyama et al. 1997). In our study we observed significant difference in Bmax for all

three compounds between adult human and rat at PND 90. Kd values were similar in both adult human and PND 90 rat plasma. Pistolozzi and Bertucci (2008) reported a tenfold lower binding affinity of diazepam in rat than that observed with human albumin. Lumpkin et al. (2003) found lower binding of trichloroacetic acid in rat plasma as compared to human plasma. Mandhula et al. (2006) pointed out that rat has lower albumin and total protein content as compared to human plasma. The modestly higher total protein levels, along with higher binding capacity of human plasma might be responsible for finding higher binding of DLM, CIS and TRANS in human plasma.

Conclusions:

It is apparent from our current study that both albumin and lipoprotein plays a role in the transport of DLM, CIS and TRANS in blood. Albumin has a major role, while lipoproteins play a minor role in transport. All three pyrethroids exhibited similar binding properties in human and rat plasma. The binding of DLM, CIS and TRANS were higher in human than in rat. The unbound fraction was significantly higher (approximately twice) of all three pyrethroids in rat when compared to human plasma. The higher unbound fraction in rat plasma might result in greater distribution of DLM, CIS and TRANS to different and increased toxicity.

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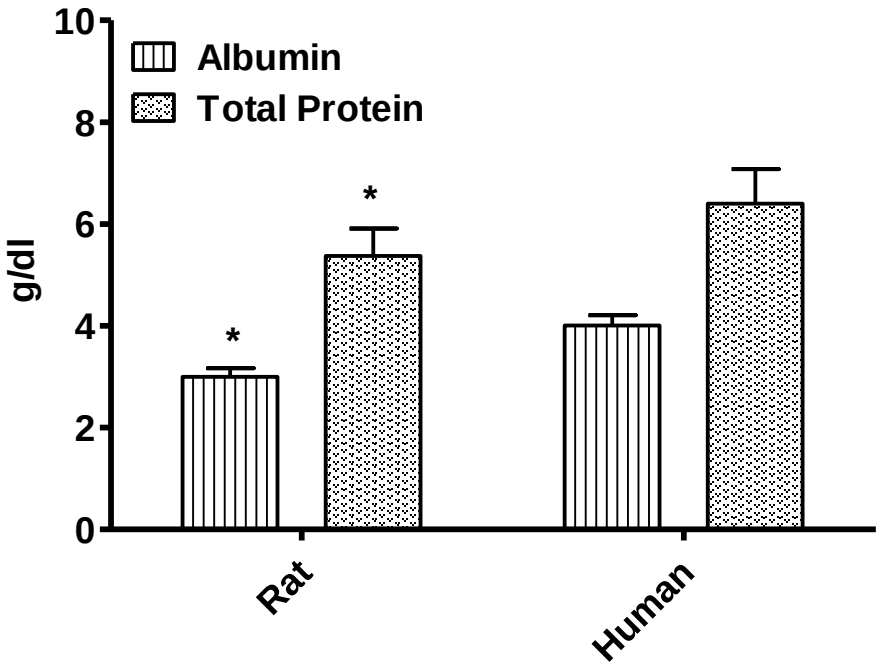

Figure 3-1. Albumin and total protein levels in rat and human plasma. Data represent mean ± SD, N=3. (*) Statistical significance was evaluated between groups using an unpaired student t-test with a significance level of $p<0.05$ using Graphpad Prism.

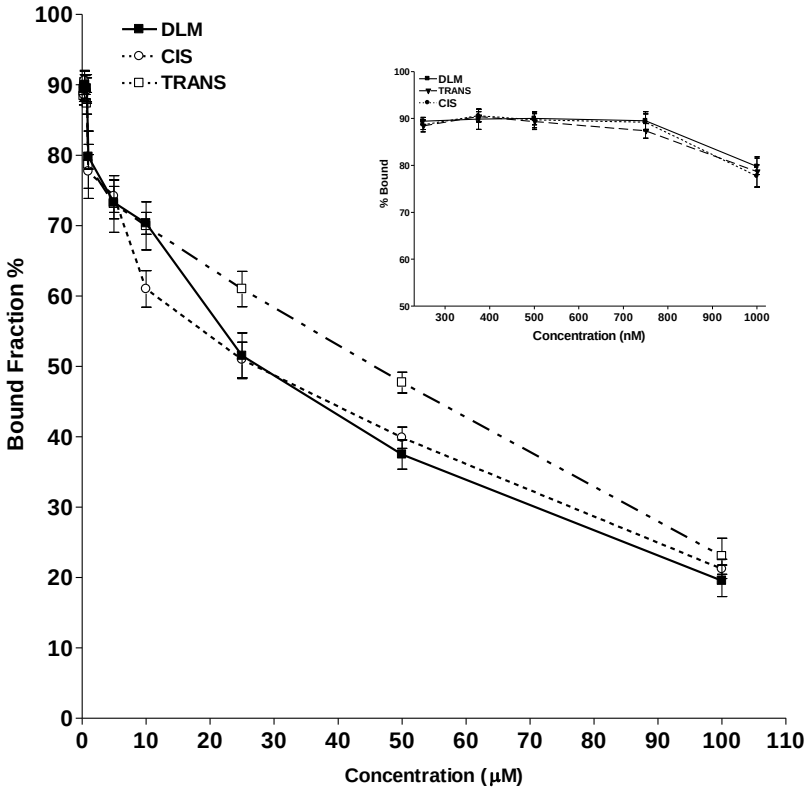

Figure 3-2. Insert shows linear binding of DLM, CIS and TRANS in human plasma (at concentrations below 750 nM). Binding was non-linear at concentrations >750 nM. A Similar pattern of binding was observed for all three compounds. Data represent mean ± SD, N=4.

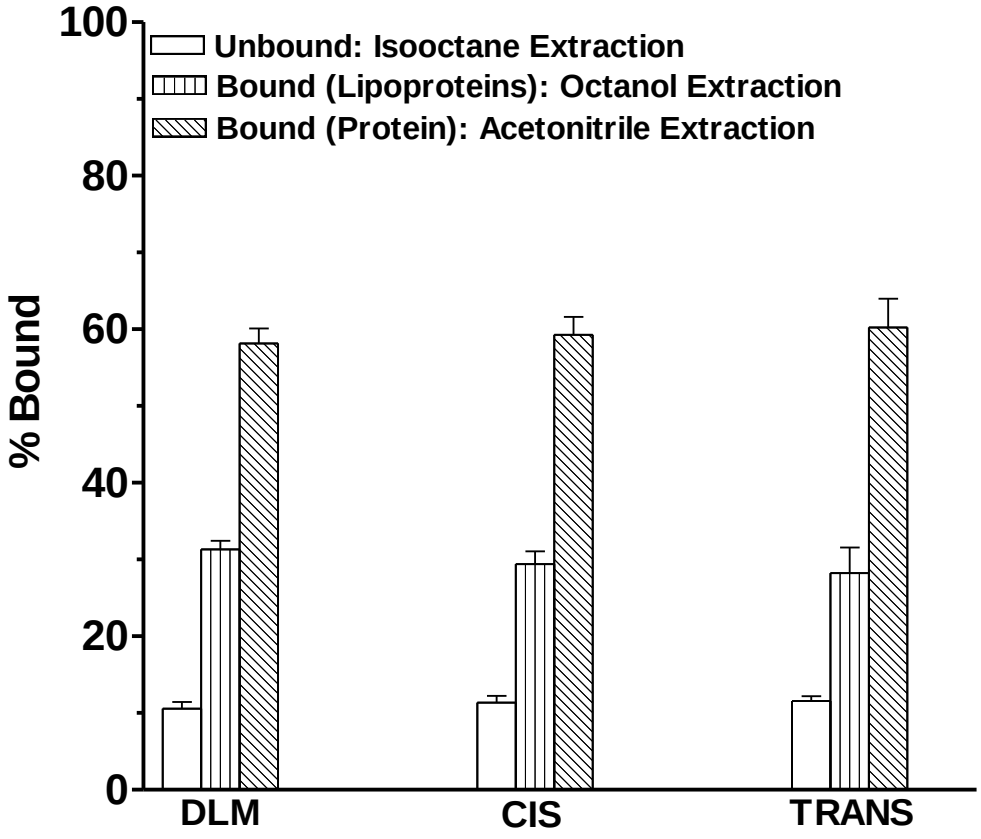

Figure 3-3. Relative distribution of DLM, CIS and TRANS in human plasma in linear range (250 nM concentration). A Similar pattern of distribution in lipoprotein and albumin was observed for all three compounds. Bar heights and brackets represent mean ± SD, N=4.

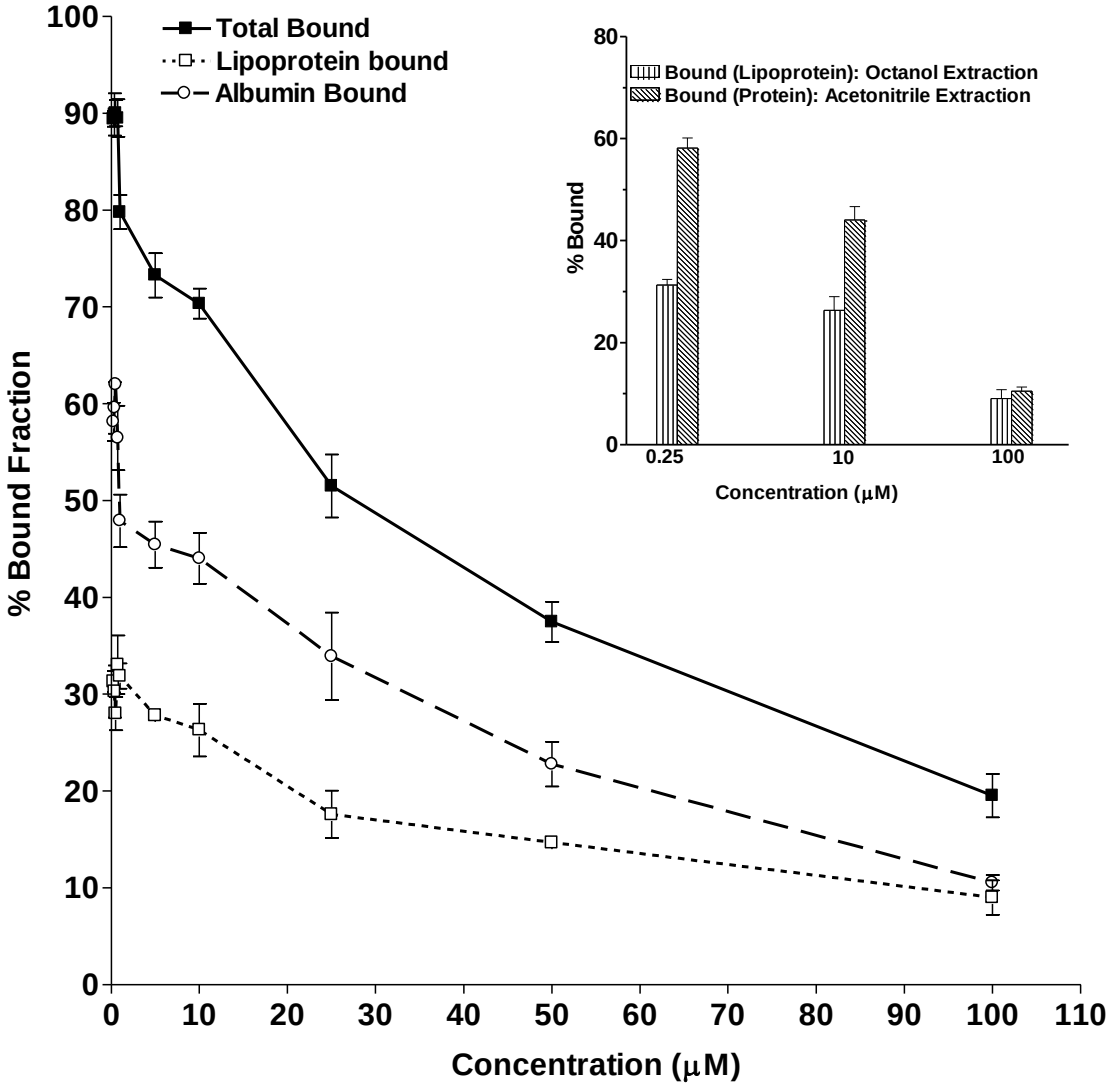

Figure 3-4. Non-linear binding of DLM in human plasma (at concentrations above 750 nM). Insert shows changes in the distribution of DLM to the lipoprotein and albumin fractions at higher concentrations. Data represent mean ± SD, N=4.

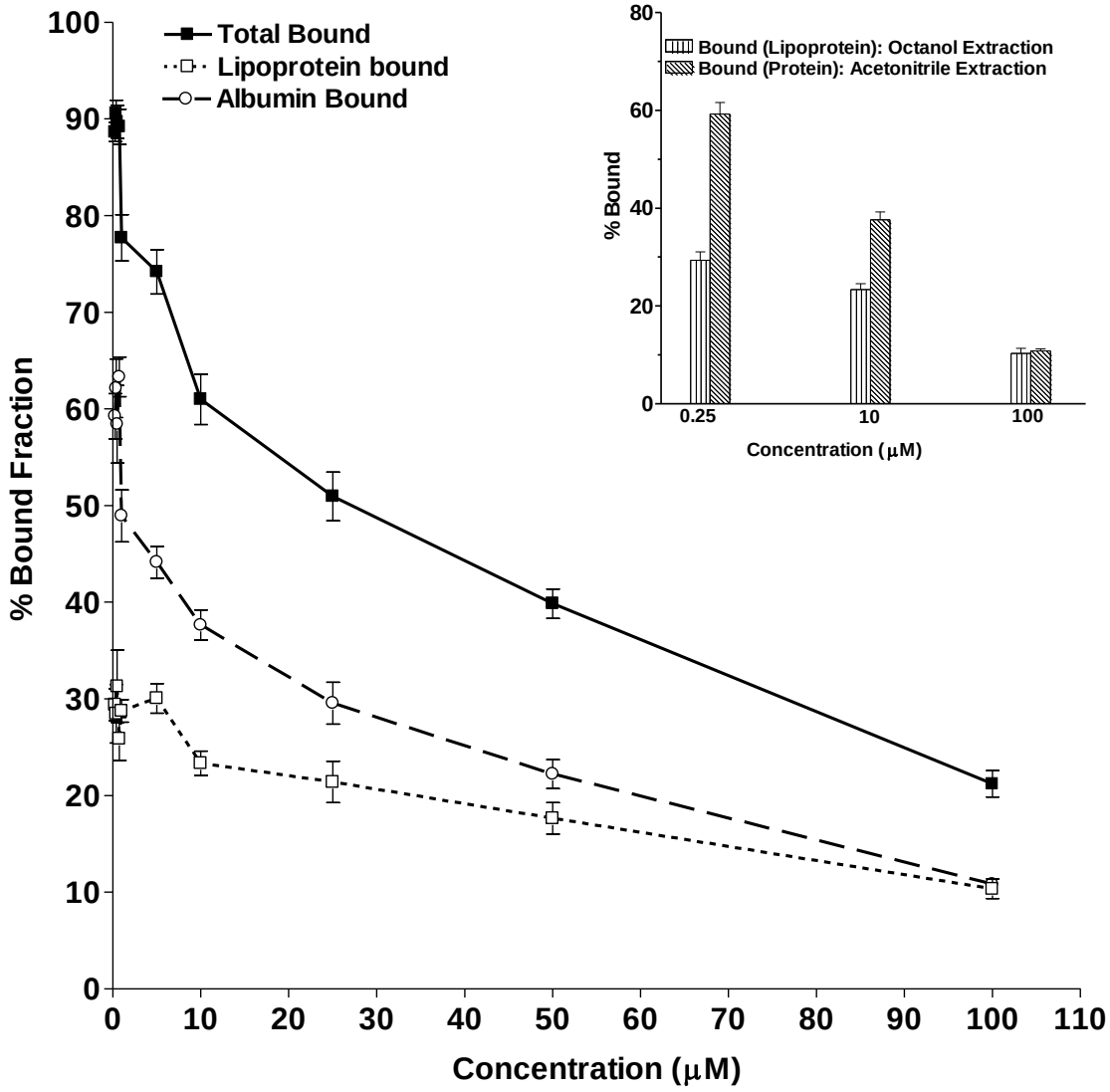

Figure 3-5. Non-linear binding of CIS in human plasma (at concentrations above 750 nM). Insert shows changes in the distribution of DLM to the lipoprotein and albumin fractions at higher concentrations. Data represent mean ± SD, N=4.

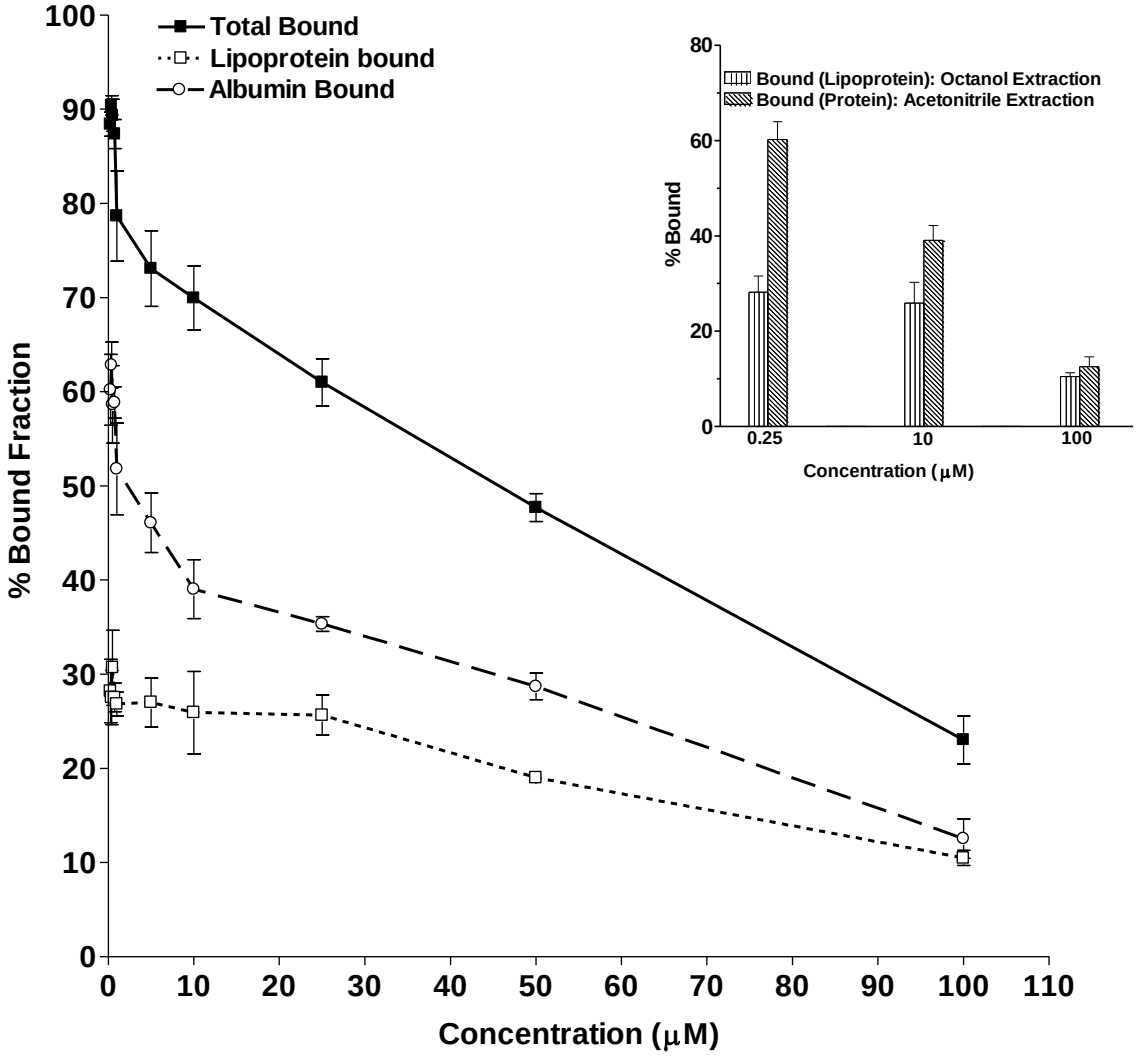

Figure 3-6. Non-linear binding of TRANS in human plasma (at concentrations above 750 nM). Insert shows changes in the distribution of DLM to the lipoprotein and albumin fractions at higher concentrations. Data represent mean ± SD, N=4.

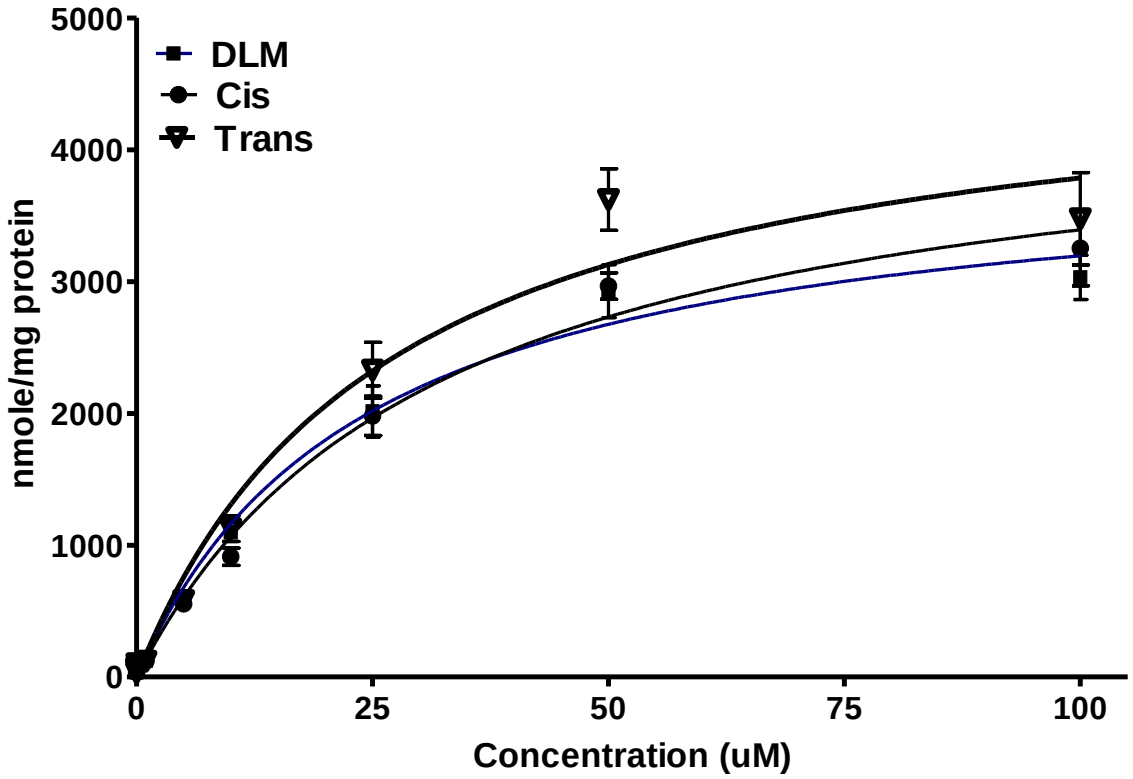

Figure 3-7. Saturation binding curves of DLM, CIS and TRANS in human plasma (250 nM to 100 µM concentration). Solid lines represent the fitted curves. Data represent mean ± SD, N=4.

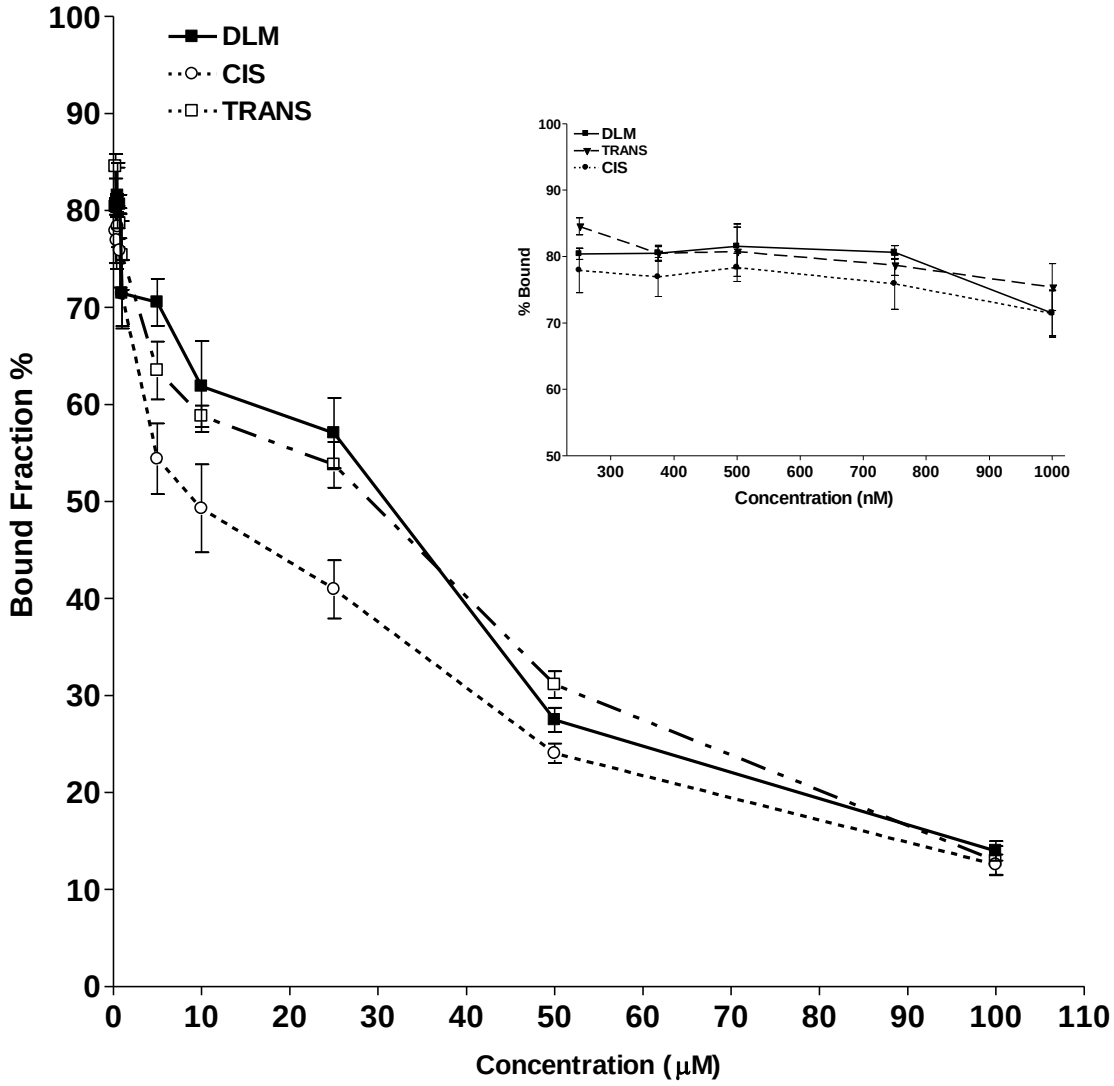

Figure 3-8. Insert shows linear binding of DLM, CIS and TRANS in rat plasma (at concentrations below 750 nM). Binding was non-linear at concentrations >750 nM. A Similar pattern of binding was observed for all three compounds. Data represent mean ± SD, N=4.

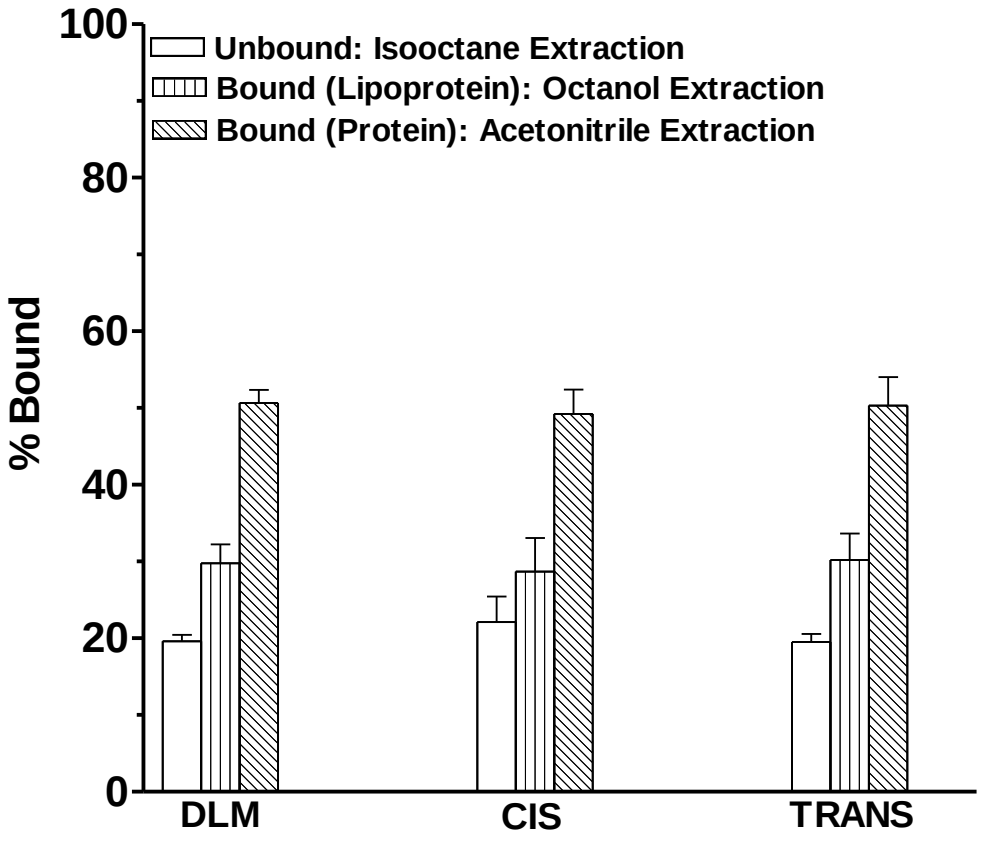

Figure 3-9. Relative distribution of DLM, CIS and TRANS in rat plasma in the linear range (250 nM concentration). A Similar pattern of distribution in lipoprotein and albumin for all three compounds. Bar heights and brackets represent mean ± SD, N=4.

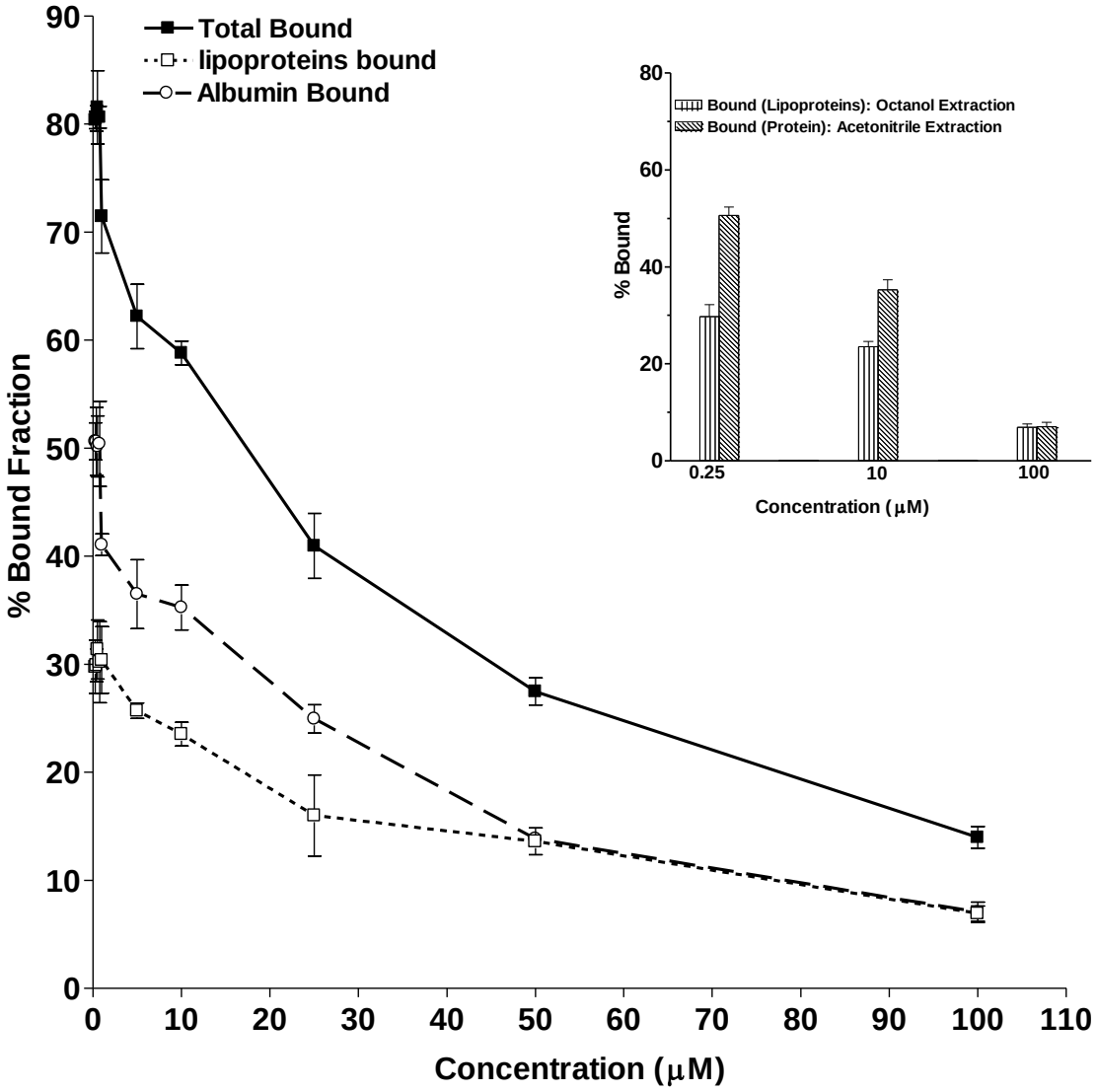

Figure 3-10. Non-linear binding of DLM in rat plasma (at concentrations above 750 nM). Insert shows changes in the distribution of DLM to the lipoprotein and albumin fractions at higher concentrations. Data represent mean ± SD, N=4.

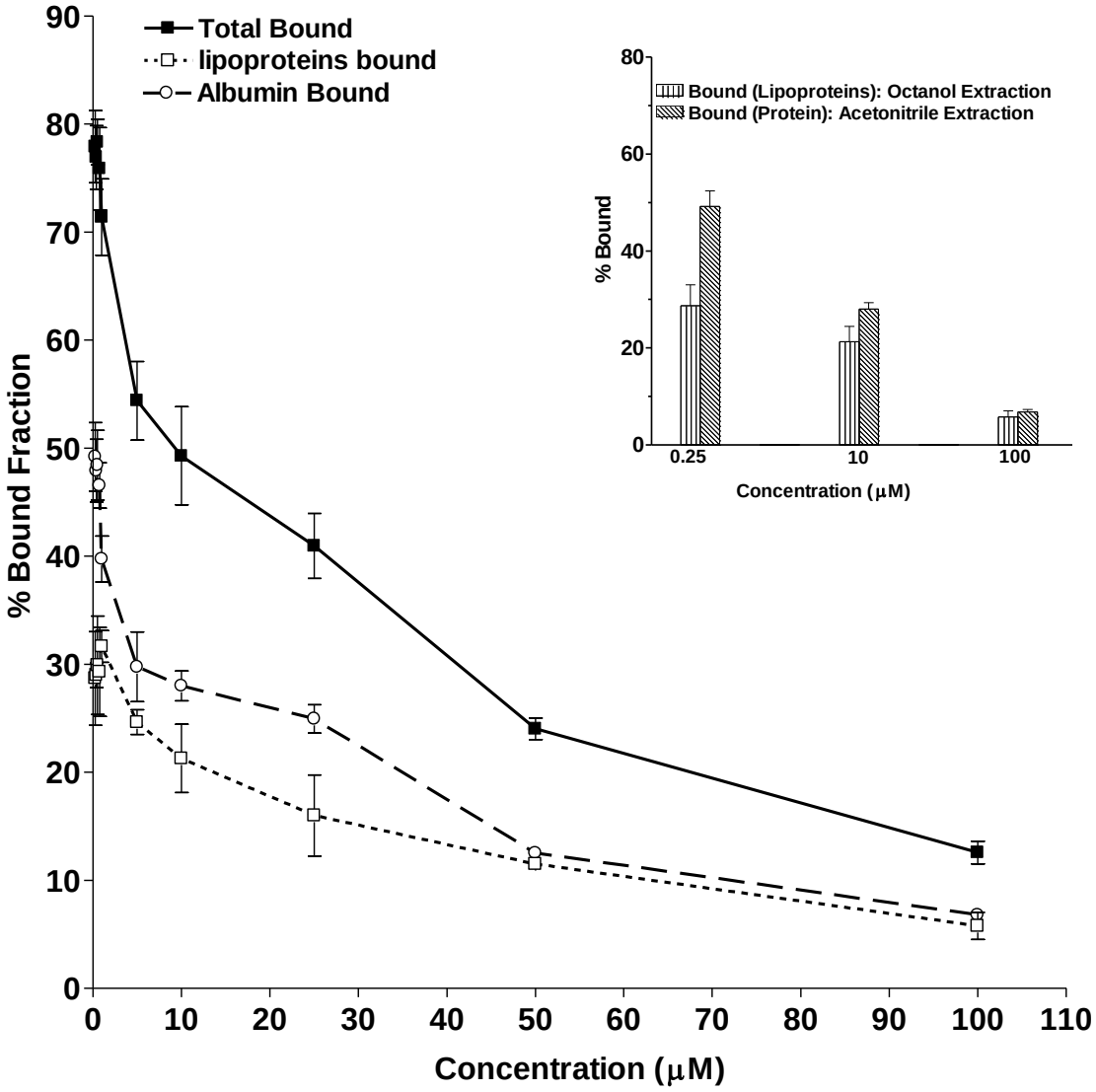

Figure 3-11. Non-linear binding of CIS in rat plasma (at concentrations above 750 nM). Insert shows changes in the distribution of DLM to the lipoprotein and albumin fractions at higher concentrations. Data represent mean ± SD, N=4.

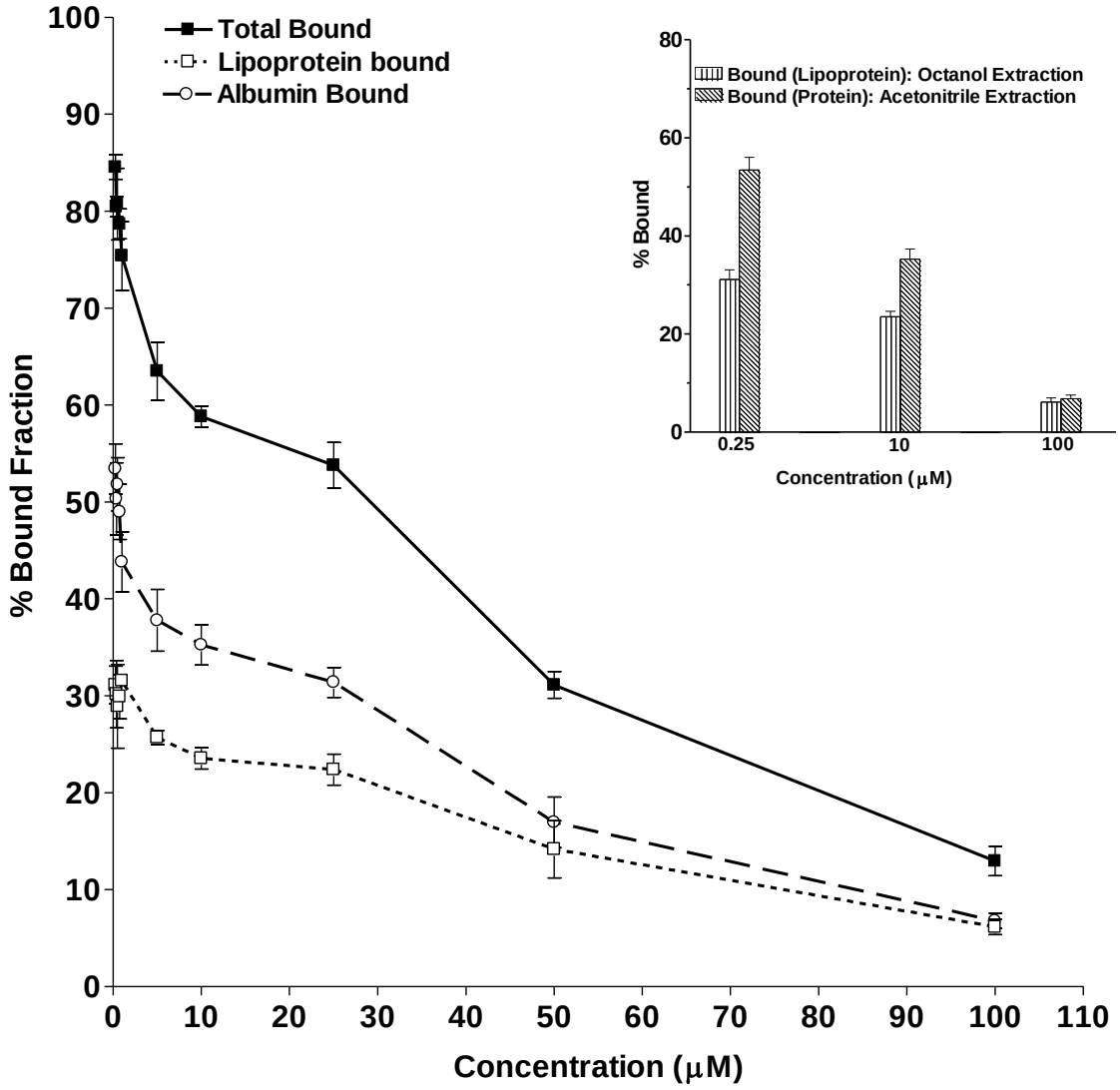

Figure 3-12. Non-linear binding of TRANS in rat plasma (at concentrations above 750 nM). Insert shows changes in the distribution of DLM to the lipoprotein and albumin fractions at higher concentrations. Data represent mean ± SD, N=4.

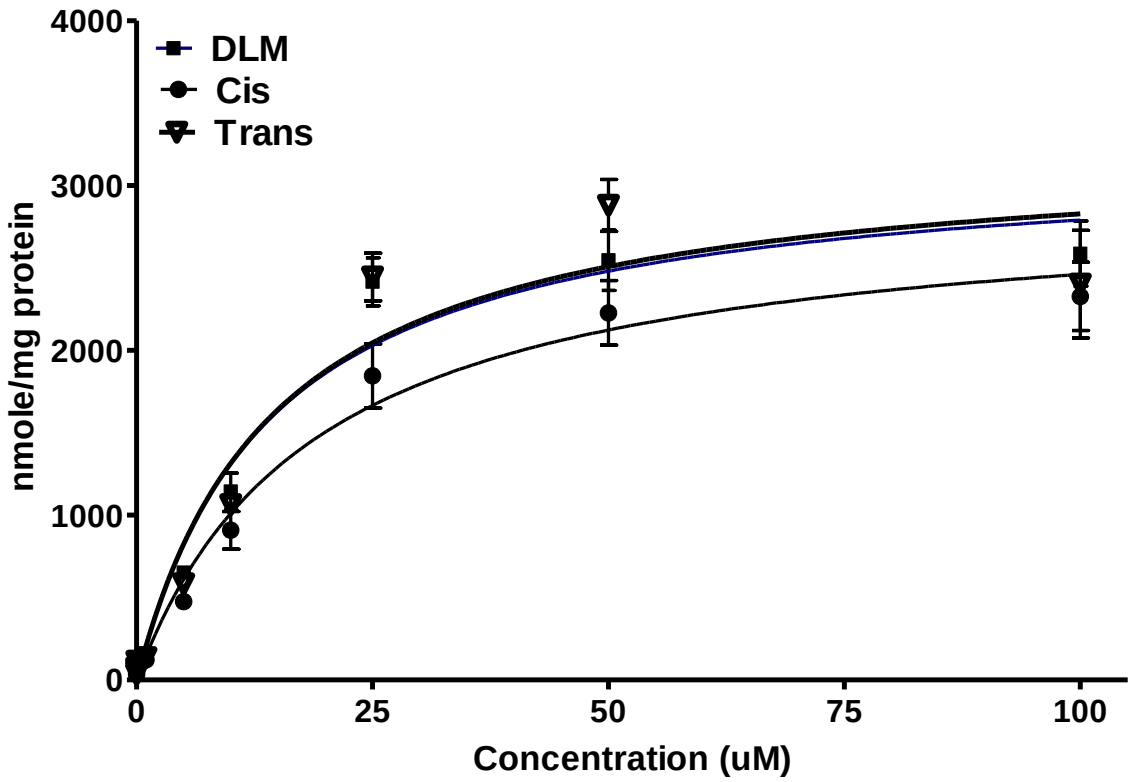

Figure 3-13. Saturation binding curves of DLM, CIS and TRANS in rat plasma (250 nM to 100 µM concentration). Solid lines represent the fitted curves. Data represent mean ± SD, N=4.

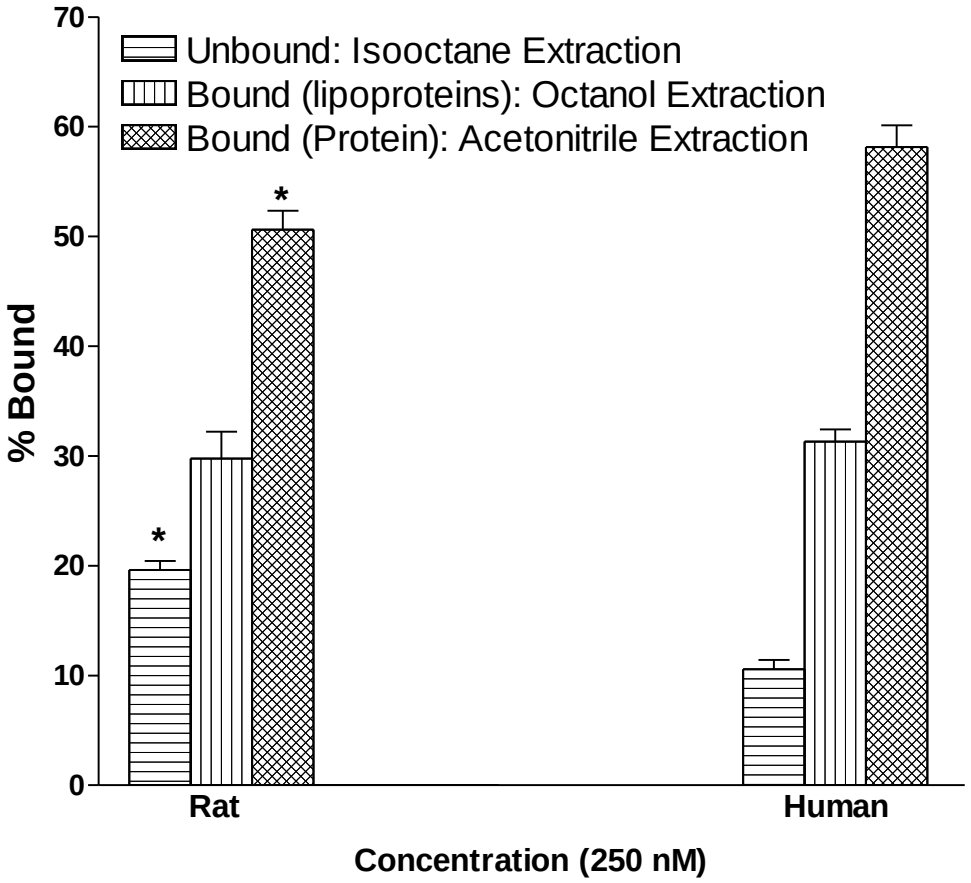

Figure 3-14. Comparative binding of DLM in human and rat plasma (at 250 nM concentration). (*) Statistical significance ($p<0.05$) was evaluated by one-way ANOVA with post-hoc Dunnett's test comparisons between unbound, lipoprotein bound and protein bound in rat and human plasma. Data represent mean ± SD, N=4.

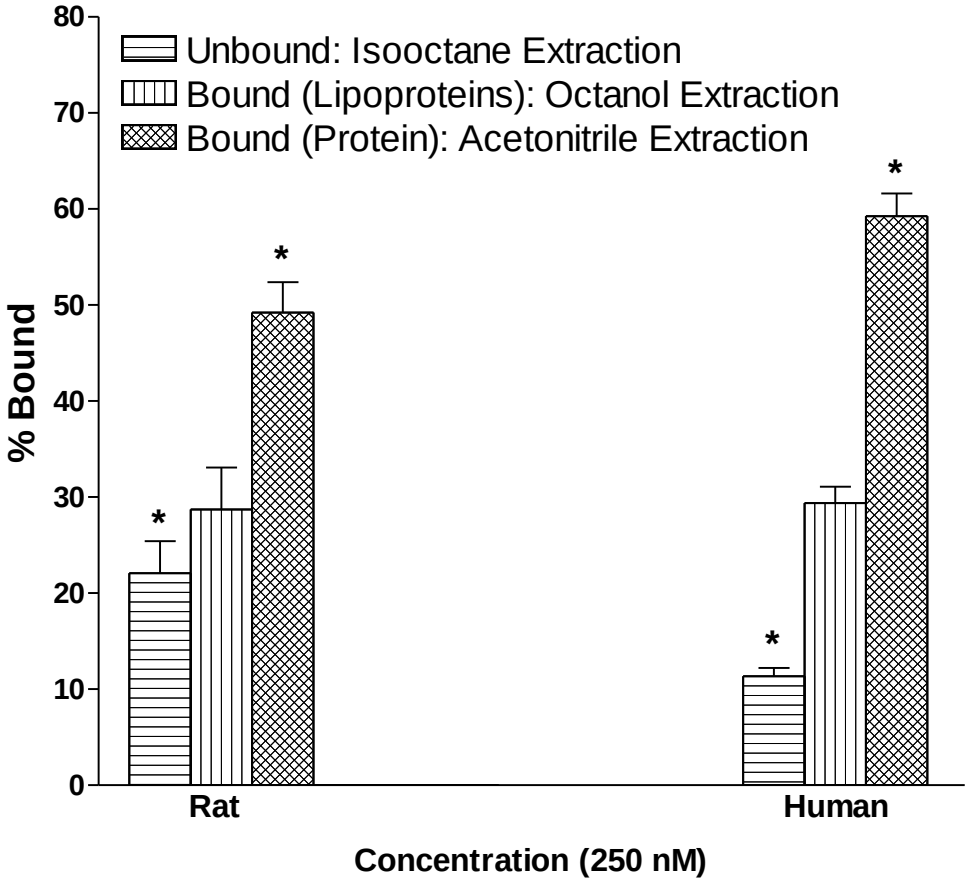

Figure 3-15. Comparative binding of CIS in human and rat plasma (at 250 nM concentration). (*) Statistical significance ($p<0.05$) was evaluated by one-way ANOVA with post-hoc Dunnett's test comparisons between unbound, lipoprotein bound and protein bound in rat and human plasma. Data represent mean ± SD, N=4.

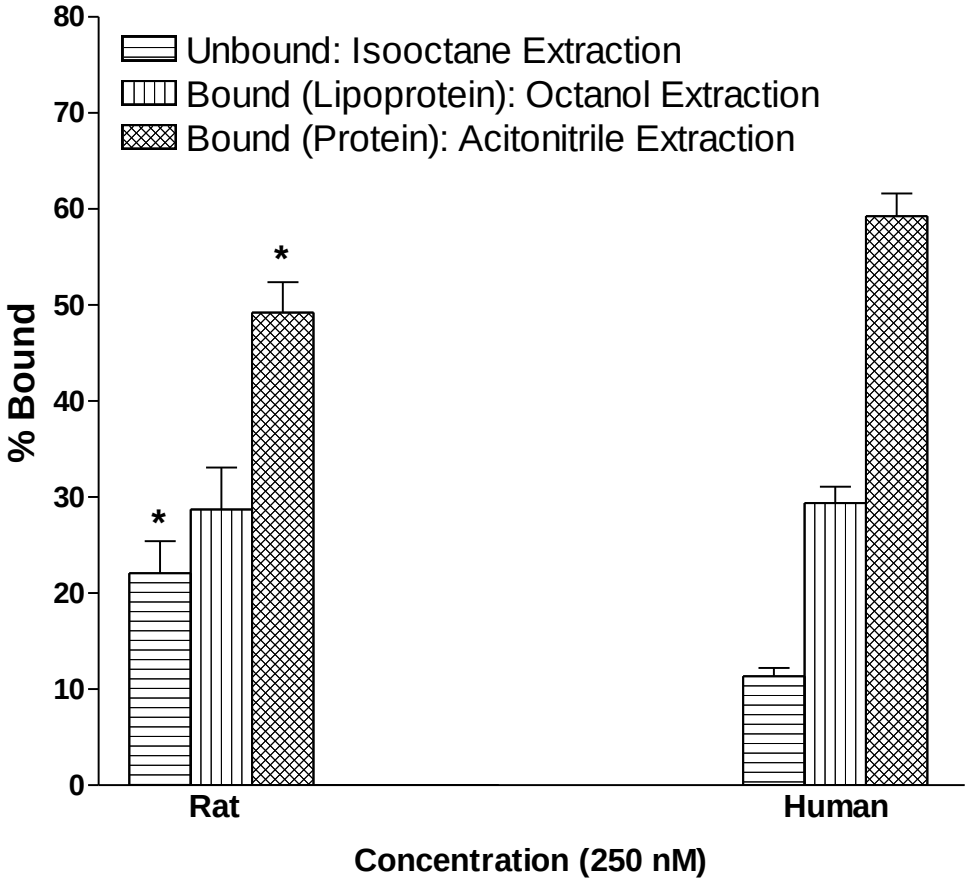

Figure 3-16. Comparative binding of TRANS in human and rat plasma (at 250 nM concentration). (*) Statistical significance ($p<0.05$) was evaluated by one-way ANOVA with post-hoc Dunnett's test comparisons between unbound, lipoprotein bound and protein bound in rat and human plasma. Data represent mean ± SD, N=4.

Table 3-1 Binding Parameters Estimates for Human and Rat Plasma Obtained by Using One-site Binding (Hyperbola) by Nonlinear Regression Using the Prism Program.

Compound	Bmax (µM/mg protein)		Kd (M)	
	Human	Rat	Human	Rat
DLM	3.2 ± 0.1	2.8 ± 0.1*	5.9 ± 0.7 × 10-6	$5.6 \pm 1.0 \times 10^{-6}$
CIS	3.5 ± 0.2	2.6 ± 0.1*	$5.7 \pm 0.1 \times 10^{-6}$	$6.2 \pm 0.9 \times 10^{-6}$
TRANS	4.0 ± 0.2	2.9 ± 0.2*	$6.1 \pm 0.1 \times 10^{-6}$	$5.9 \pm 1.0 \times 10^{-6}$

Data represent Mean ± SD, N=4. Statistical significance ($p<0.05$) was evaluated by two-way ANOVA. (*) indicate significantly different from human.

CHAPTER 4

EFFECT OF MATURITY ON PLASMA PROTEIN BINDING OF DELTAMETHRIN, CIS-PERMETHRIN AND TRANS-PERMETHRIN IN HUMAN AND RAT PLASMA

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Abstract:

Plasma protein binding can influence the toxicokinetics of highly-bound chemicals by limiting the amount of compound free to reach target organs and sites of elimination. Because the influence of age on plasma protein binding of common pyrethroids is unclear, this study was undertaken to determine if binding of DLM, CIS or TRANS changes during maturation. The plasma protein binding of DLM, CIS and TRANS was studied in plasma of 10-, 15-, 21- and 90-day-old (adult) rats and birth -1 week, > 1 week – 4 week, > 4 week – 1 year, > 1 year- 3 years, > 3 years- 6 years, > 6 years – 18 years and adult human plasma. In all age groups, rat and human binding was linear over the concentration range of 0.25 µM to 0.75 µM and inversely proportional to concentrations >0.75µM. In rat plasma, we observed a significant decrease in the total binding of all three compounds in PND 10 (70-73%) and PND 15 (70-75 %) as compared to PND 90 (77-83 %) at concentrations lower than 750 nM. However, no significant difference in the binding of all three compounds was observed between PND 21 (78-80 %) and PND 90 plasma. Binding of all three compounds to albumin was significantly different at PND 10 (35-36 %) and PND 15 (39-42 %) as compared to PND 90 (47-50%). Lipoprotein binding of DLM, CIS and TRANS were significantly higher in PND 10 (35-36 %) as compared to PND 90 (28-31%). In human plasma, the total binding of all three compounds in birth -1 week (74-77%) and > 1 week – 4 weeks (75-77%) was significantly different as compared to the adult at concentration below 750 nM. However, after 4 weeks no significant difference in binding of all three compounds was observed in other age groups as compared to adult plasma. Higher free fractions of DLM, CIS and TRANS at PND 10, PND 15 in rats and birth -1 week, >1 week – 4 weeks in humans may result in greater toxicity in these age groups.

Introduction:

Pyrethroids are the synthetic analogs of pyrethrins, used as an insecticide in household sprays, insect repellants, pet shampoos, and lice treatments. Pyrethroids are highly hydrophobic (log Kow in the range 5.7–7.6) and have low water solubility (of a few μg L−1) (Laskowski 2002). They have low volatility, high affinity for soil and sediment particulate matter and are rapidly and completely adsorbed to sediment particles. Pyrethroids are biodegraded in the soil and water and can also undergo hydrolysis under alkaline conditions. In the last decades, with the phase out of organophosphate, they are the pesticides of choice in the USA due to their selectively insecticidal activity, relatively lower mammalian toxicity, high activity and lower environmental persistence (Feo et al. 2010). Pyrethroids are often applied to agricultural fields, commercial nurseries, residential structures and lawns. Permethrin is the most widely used pyrethroid insecticide.

Since these compounds are extensively used, pesticides are present in a small amount in the food consumed by humans (Lu et al. 2010). Thus, the primary route of exposure is through ingestion of food. Becker et al. (2006) observed a significant correlation between permethrin in house dust and the metabolite concentrations in urine of German children. Lu et al. (2010) assessed the exposure of 23 elementary school–age children to pyrethroid pesticides in the USA. They observed an association between the pyrethroid use in the residential environment and elevated pyrethroid metabolite levels found in the children's urine. Occupationally, dermal exposure is considered the most important route in exposure in agricultural workers (Bradberry et al. 2005). Wang et al (2007) (Wang et al. 2007) reported increased levels of 3-PBA (3-phenoxybenzoic acid), urinary biomarker for pyrethroids exposure, in Japanese pest control operators.

Pyrethroids like many other insecticides are neurotoxins and have been classified into two major categories: Type I or type II, based on distinct toxicological mechanisms (Breckenridge et al. 2009). The major signs of acute poisoning of type I pyrethroids in mammals are aggressive sparring, sensitivity to external stimuli, fine tremor progressing to whole body tremor and prostration (Casida et al. 1983). These signs are often referred as T-syndrome (tremor syndrome). Major signs of acute poisoning with type II pyrethroids include salivation, hyperexcitability, choreoathetosis, and seizures (Casida et al. 1983). These signs are referred as CS-syndrome (choreoathetosis and salivation). The primary mechanism of toxicity of both type I and type II pyrethroids is interaction with neuronal voltage-sensitive sodium channels (Soderlund et al. 2002). Neonatal rats are more susceptible to acute toxicity of pyrethroids as compared to adults (Cantalamessa 1993; Shafer et al. 2005; Sheets 2000) Cantalamessa, (1993) observed increased sensitivity of neonates to both type I (cypermethrin) and type II (deltamethrin) than adults. Sheets et al. (2000) also observed that the young Sprauge-Dawley rats were much more susceptible to acute toxicity of type II pyrethroids than were adults.

The overall pharmacokinetics and pharmacodynamics of a drug can be influenced by plasma protein binding (Yasuhara et al. 1985; Zeitlinger et al. 2011). The therapeutic and toxic effects of a drug are often linked to the amount of free drug in the blood. An increase in the amount of free drugs results in more drug being available for binding to the receptor site. Thus, the degree of plasma or tissue protein binding is a major determinant of drug action on the target site. Plasma protein binding also influences drug disposition. Higher unbound fraction results in enhanced distribution into tissues and a larger volume of distribution. Plasma protein binding also affects clearance of a drug from the body (Zeitlinger et al. 2011).

The plasma protein binding of drugs is altered by several factors like age, gender, and disease conditions (Crooks et al. 1976;Ronfeld et al. 1997). Age-dependent changes in the plasma proteins influence the distribution of a chemical throughout the body. This factor is more important during early developmental stages when plasma protein shows both quantitative and qualitative changes (Ehrnebo et al. 1971) to endogenous substrates in the plasma may be present in higher concentrations. In neonates, plasma protein binding of drugs is generally lower than in adults. Ehrnebo et al (1971) found low binding of ampicillin, α-azidobenzylpencillin, benzylpenecillin, phenobarbital and diphenyldantoin to human foetal and neonatal plasma as compared to adult plasma. Similarly, Herngren et al (1987) found a lower mean protein binding (88.6%) of flucloxacillin in newborn infants as compared to (95.7%) healthy adults (Espersen 1995). The high unbound fraction at the receptor site leads to more intense pharmacological effect and may in part be responsible for the increase sensitivity of newborns to certain drugs (Simons et al. 1981).

There have been several studies to determine pharmacokinetics of pyrethroids, but relatively little information is available about their plasma protein binding. One study (Cui et al. 2006) observed significant binding of cypermethrin to bovine serum albumin and bovine hemoglobin. However, other study (Abu-Qare and Abou-Donia 2002) noted no significant interaction of permethrin with human serum albumin after 1 hour of incubation. Further there is no information available on the effect of maturity on plasma protein binding of pyrethroids.

The primary objective of the current investigation was to determine the effect of maturity (age) on the plasma protein binding of Deltamethrin (DLM), Cis-Permethrin (CIS) and Trans-permethrin (TRANS) to rat and human plasma. A related objective was to determine relative distribution of pyrethroids in lipoprotein and albumin fractions of the plasma. Another aim of the

study was to calculate maximum binding capacity and dissociation constant in various age groups.

Materials and Methods:

Materials:

Radiolabeled [14C]-deltamethrin (54.1 mCi/mmol) was supplied by Bayer CropScience (Stilwell, KS), [14C]-Cis-permethrin (61 mCi/mmol) and [14C]-trans-permethrin (61 mCi/mmol) was provided by Symbiotic Research (Mount Olive, NJ). Radiolabeled [14C]-diazepam (55 mCi/mmol) and [3H]-Cyclosporine (20 Ci/mmol) were purchased from American Radiolabeled Chemicals (St.Lous, MO). Standard DLM (purity, 98.8 %), CIS (purity, 99.0 %), and TRANS (purity, 99.3 %) were kindly provided by FMC Agricultural Product Group (Fresno, CA, USA). Purified human serum albumin, Acetonitrile (HPLC-grade), hexamethyldisilazane (Reagent-grade), sodium fluoride (NaF) (purity, 99.0 %) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Isooctane (purity, 99.0 %) and 2-octanol (Laboratory-grade) were purchased from Fisher Scientific (Pittsburgh, PA, USA). All clean glassware was silanized with 5% hexamethyldisilazane 24 hours prior to the start of experiments.

Plasma:

Adult Human and Rat Plasma:

Frozen, heparinized, pooled adult human and rat plasma were obtained from Innovative Research (Novi, MI, USA). All plasma samples were stored at -80°C until use. All plasma samples were filtered through 0.45 µm Millipore® filters to remove precipitated fibrinogen.

Rat Plasma (PND 10, 15 and 21):

Animals for plasma: Pregnant Sprague-Dawley rats were obtained from Charles River Breeding Laboratories (Raleigh, NC). Pups were delivered and grown until the assigned

postnatal day (PND 10, 15 and 21). The day of birth was defined as PND 0. The protocol for this study was approved by the University of Georgia Animal Use and Care Committee and rats were housed in polycarbonate cages on a 12-hr light/dark cycle at ambient temperature (22°C) and relative humidity (55±5%). Food (5001 Rodent Diet, PMI Nutrition International LLC, Brentwood, MO) and tap water were provided ad libitum. All the immature rats were delivered in animal facilities located in College of Pharmacy of the University of Georgia. Weanling pups were separated from their dam only after 21 days.

Each rat was sacrificed by cervical dislocation (at PND 10, 15 and 21) and blood was withdrawn by closed-chest cardiac puncture into a heparinized syringe. The maximum amount of blood possible was withdrawn and transferred to heparinized tubes. Tubes were centrifuged (4500 rpm, 15 min) and plasma transferred to a clean tube. Plasma from different animals at each age group was pooled and stored at -80°C until use. All plasma samples were filtered through 0.45 μm Millipore® filters to remove precipitated fibrinogen.

Pediatric Plasma:

Plasma samples were obtained from the Children's Hospital of Wisconsin Clinical Laboratory. Plasma samples were obtained from discarded blood procured as part of an IRB-approved research study with an informed consent exemption. A sufficient number of samples (at least 30) were retrieved such that pooling provided a total volume of at least 5 mL for each of the following age brackets. Birth to 1week, >1 week to 4 weeks, >4 weeks to 1 year, >1 year to 3 years, >3 years to 6 years, and >1 years to 18 years. Plasma samples were stored at -80°C until use. All plasma samples were filtered through 0.45 μm Millipore® filters to remove precipitated fibrinogen.

Plasma Protein Binding Measurements:

Binding to selected pyrethroids was determined by three step solvent extraction method (Sethi et al. 2013). Stock solutions of ^{14}C radiolabelled DLM, CIS and TRANS were prepared at final concentration of 2500 nM. A volume of 80 µl of human or rat plasma was spiked with 10 µl of 14C radiolabelled DLM, CIS or TRANS (250 nM to 100 uM) in silanized glass vials. Plasma samples were then treated with 10 µl of 0.64 M NaF to inhibit carboxylestrase. Samples were incubated in an orbital shaker (110 rpm) at 37°C for 3 hours. Pilot experiments revealed that all three pyrethroids binding reached equilibrium by 3 hours (data not shown). Samples were mixed with 200 µl of isooctane and vortexed for 30 sec. The isooctane layer, containing the unbound fraction was removed and mixed with 3 ml of scintillation fluid in a liquid scintillation vial. The total amount of radiolabeled pyrethroid present in these 200 µl was considered the unbound portion, or fraction. The remaining plasma was mixed with 200 µl of 2-octanol and vortexed for 30 sec. The total amount of radiolabeled pyrethroid present in this 200 µl fraction was considered associated with lipoproteins. The 2-octanol layer, containing the lipoprotein-bound DLM, CIS or TRANS, was removed and mixed with 3 ml of scintillation fluid in a liquid scintillation vial. The remaining plasma was then mixed with 200 µl of acetonitrile and vortexed for 30 seconds. The total amount of radiolabeled pyrethroid present in these 200 µl was considered the protein (albumin) bound fraction. The acetonitrile layer, containing the protein-bound fraction was mixed with 3 ml of scintillation fluid in a liquid scintillation vial. Plasma temperature and pH were maintained at 37°C and 7.4, respectively, to approximate the plasma binding environment in vivo. Four replicates (n=4) of ten concentrations for DLM, CIS and TRANS were tested ranging from 250 nM to 100 uM. The unbound, lipoprotein associated and

protein bound fraction for DLM, CIS and TRANS was quantified by liquid scintillation counting using a Beckman Coulter LS 6500 (Brea, CA).

Determination of Protein Binding to Purified Human Serum Albumin:

A stock solution of human serum albumin was prepared in PBS buffer. A volume of 90 µl of human serum albumin (40 mg/ml) was spiked with 10 µl of ^{14}C radiolabelled DLM, CIS or TRANS (250 nM) in silanized glass vials. Solvent extraction method was used to determine binding of DLM, CIS and TRANS to human serum albumin. The temperature and pH were maintained at 37°C and 7.4, respectively, to approximate the plasma binding environment in vivo. The unbound, lipoprotein associated and protein bound fraction for DLM, CIS and TRANS was quantified by liquid scintillation counting using a Beckman Coulter LS 6500 (Brea, CA).

Measurement of Albumin and Total Protein:

Albumin levels in human and rat plasma were measured by use of a kit (QuantiChrom™ BCG Albumin Assay Kit; BioAssay Systems, Hayward, CA, USA) according to the manufacturer's instructions. Total protein estimations in human and rat plasma were performed using a BCA Protein assay kit (Pierce/Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions.

Statistical Analysis:

All experiments were performed with a minimum of three independent experiments unless otherwise stated. All values are represented as mean ± SD unless otherwise stated. Data were analyzed using nonlinear curve fitting (Graphpad Prism) using a one-site binding (hyperbola) to obtain KD and Bmax values. Statistical significance was evaluated by one-way ANOVA with post-hoc Dunnett's test, with a significance level of $p<0.05$ using Graphpad Prism 5.

Results:

Figure 4-1 shows albumin and total protein levels at different ages in rat plasma. Significantly higher albumin levels were recorded in adult (PND 90) plasma as compared to PND 10 and PND 15. However, no statistically significant differences were observed between day 21 and adult plasma. Total proteins levels were significantly higher in adult compared to PND 10, PND 15 and PND 21.

In all age groups binding were linear over the concentration of 0.25 µM to 0.75 µM and inversely proportional to concentrations >0.75 µM. Figure 4-2 shows the linear and non-linear pattern of DLM binding in different age groups. CIS and TRANS followed the similar pattern (data not shown). Relative distributions of DLM, CIS and TRANS in different age groups of rats are shown in Figures 4-3. In the linear concentration range the highest distribution of all three compounds was to albumin. However, at higher concentration DLM was equally distributed between albumin and lipoproteins (Figure 4-4). CIS and TRANS followed the similar pattern (data not shown). Saturation binding curves for DLM, CIS and TRANS in rat plasma in all age groups are shown in Figures 4-5. Binding parameters estimates for DLM, CIS and TRANS for all groups in rat plasma are listed in table 4-1, 4-2, and 4-3 respectively. No significant differences were noted in binding affinities as a function of age. Bmax was significantly reduced in PND 10 and PND 15 pups.

Figure 4-6 shows total biding of DLM, CIS and TRANS in all groups of rats. In adult rat, total binding of DLM, CIS and TRANS were in the range of 77-83 % at concentration < 750 nM. Total binding of all three compounds (78-80 %) at PND 21 were not significantly different from the adult. However, total binding for all three compounds in PND 15 (70-75 %) and PND 10 (70-73%) were significantly different from PND 90 (77-83 %).

Albumin binding of DLM, CIS and TRANS (at concentration < 750 nM) in all age groups of rats is shown in figure 4-7. In adult rat plasma DLM, CIS and TRANS were 47-50% was bound to albumin. We observed significant differences in albumin binding for all three compounds at PND 10 (35-36 %) and PND 15 (39-42 %) from PND 90. Binding to albumin for all three compounds at PND 21 (49-50%) was not significantly different from PND 90.

Figure 4-8 shows lipoproteins biding of DLM, CIS and TRANS in all groups of rats. In adult rat plasma DLM, CIS and TRANS were 28-31% was bound to lipoproteins. We observed significant differences in lipoproteins binding for all three compounds at PND 10 (35-36 %) from PND 90. There was an equal distribution of all three compounds at concentrations below 750 nM. Binding to lipoprotein for all three compounds in PND 15 (30-32 %) and PND 21 (28-30%) were not significantly different from PND 90.

Effect of Maturity on Plasma Protein Binding of Pyrethroids in Human Plasma:

Figure 3-8 shows albumin and total protein levels at different ages in human plasma. We observed statistically significant difference in albumin levels at birth-1week and > 1week-4 weeks from adult albumin levels. Albumin levels in all other age groups were not significantly different from adult levels. Total proteins levels in birth-1week, > 1week-4 weeks, and > 4weeks-1year were significantly different from adult levels. However, no statistically significant differences in total protein levels were observed for all age groups after 4 weeks of age.

In human plasma binding was linear over the concentration of 0.25 µM to 0.75 µM and inversely proportional to concentrations >0.75 µM. Figure 3-10 shows the linear and non-linear patter of DLM in different age groups. CIS and TRANS followed a similar pattern (data not shown). Relative distributions of DLM, CIS and TRANS in different age groups in human plasma are shown in Figures 4-11. At low concentrations, the highest distribution of all three

compounds at all groups was to albumin. However, at higher concentrations DLM was equally distributed between albumin and lipoproteins (Figure 4-12). CIS and TRANS followed the similar pattern (data not shown). Saturation binding curves for DLM, CIS and TRANS for all age groups in human are shown in Figures 4-13. Binding parameters estimates for DLM, CIS and TRANS for all groups in human plasma are listed in table 4-5, 4-6, and 4-7 respectively. In human plasma Bmax values were significantly lower in birth -1 week and > 1 week - 4weeks as compared to adults. Kd values were similar in all age groups of rat. However, Kd values for all three pyrethroids in birth – 1 week was significantly different than the adult.

Figure 4-14 shows total biding of DLM, CIS and TRANS in all groups of humans. In adult human plasma total binding of DLM, CIS and TRANS were in the range of 88-90 % in linear concentration range. Total binding of all three compounds in birth-1week (74-77%) and > 1week – 4 weeks (75-77%) were significantly different from the adult. However, total binding for all three compounds were not significantly different in other age groups from adults after 4 weeks of age.

Albumin binding of DLM, CIS and TRANS (in concentrations < 750 nM) in all age groups of humans is shown in figure 4-15. In adult human plasma DLM, CIS and TRANS were 58-60% was bound to albumin. We observed significant difference in albumin binding for all three compounds at birth – 1 week (27-30%) and > 1 week – 4 week (45-48%) from adults. Binding to albumin for all three compounds were not significantly different in other age groups from adults after 4 weeks of age.

Figure 4-16 shows lipoproteins biding of DLM, CIS and TRANS in all groups of human plasma. In adult human plasma DLM, CIS and TRANS were 28-31% was bound to lipoproteins.

Binding to lipoproteins for all three compounds were not significantly different in age groups from adults

Binding of Pyrethroids to Purified Human Serum Albumin:

Binding of DLM, CIS and TRANS to purified adult human serum albumin was 50.40 ± 2.6 %, 52.32 ± 3.0 %, and 57.25 ± 1.5 % respectively (figure 4-17).

Discussion:

Results from our study show that greater free fraction of DLM, CIS and TRANS were observed up to 4 weeks of age compared to adult human plasma. Similarly in rat, free fractions for all three compounds were greater in PND 10 and PND 15 as compared to adults. In newborn humans several factors like quantity and quality of albumin, plasma lipoproteins, plasma globulins, α-fetal protein, PH and endogenous substances (bilirubin and free fatty acids) may result in reduced plasma protein binding (Notarianni 1990). The distribution and clearance of a drug in the body is influenced by the amount and character of plasma proteins. The results of this study provide important insights into the effect of maturity on plasma protein binding of pyrethroids. This is a first comprehensive study to demonstrate age related changes in the plasma protein binding of representative pyrethroids in human and rat. All the three pyrethroids were bound to both albumin and lipoproteins; however albumin played a more important role in binding at low concentrations.

In adult human and rat plasma all three pyrethroids were 50-60 % bound to albumin. Interestingly, we also found all the three pyrethroids bound approximately 55% to purified adult human serum albumin. Hence, it is reasonable to assume that these pyrethroids primarily bind to albumin. Maliwal and Guthrie (1981) reported highly bound (96.6%) organophosphate insecticide (diazinon) distribution to albumin (~55 %), LDL (~30 %) and HDL (~13%) in human

plasma. Since albumin is the primary plasma protein, it appears that changes in plasma pyrethroids binding during maturation are largely the result of differences in albumin levels. Findings in this study indicated reduced plasma protein binding of DLM, CIS and TRANS in newborn (birth – 1 week and > 1 week – 4 weeks) as compared to adults. This is consistent with reduced plasma protein binding of several other drugs in newborns. Ehrnebo et al. (1971) observed very low binding of ampicillin, azidobenzylpencillin, benzylpencillin, phenobarbital and diphenylhydantoin to fetal and neonatal plasma as compared to the plasma of adult humans. Similarly, reduced levels of protein binding of furosemide (Viani and Pacifici 1992), salicylate (Stewart and Hampton 1987), sufentanil (Meistelman et al. 1990) and micafungin (Yanni et al. 2011) in newborns have been reported.

As determined in our study birth – 1 week, > 1 week – 4 weeks have reduced levels of plasma albumin and total proteins. These results are consistent with the previously reported studies (Ehrnebo et al. 1971;Kanakoudi et al. 1995). Lower albumin and total proteins may in part explain the observed decrease in protein binding of newborns. Kanakoudi et al. (1995) reported significant lower levels of albumin in healthy infants (birth to 6 months) as compared to adult levels. We also observed lower levels of plasma albumin and total protein in rats at PND 10 and PND 15. Oliver at al. (1962) reported that rat plasma proteins are ~30% that of the adult levels by day ten (PND 10), after which they increases rapidly to adult levels. McNamara and Alcorn (2002) suggested that a strong correlation exists between the fraction bound and postnatal increase in plasma albumin concentrations. It should be noted that not only the concentration, but also the neonatal albumin also differs in terms of quality from the adult plasma. Wallace (1977) reported variations in amino acid content of neonatal and adult albumin. This study suggested that observed alterations in the composition of neonatal and adult rat albumin may result in

differences in drug binding capacity. Notarianni et al. (1990) pointed out that albumin concentration and binding affinity increases with gestational and postnatal age. Further, they suggested that plasma volume and therefore absolute number of drug binding sites increase during gestational and postnatal growth. Miyoshi et al. (1961) and O'Connor et al. (1964) reported that reduced binding of acidic drugs in newborn may be due to alteration in the albumin, which attains adult properties at 10 to 12 months.

Pyrethroids being highly lipophilic compounds are anticipated to partition into triglycerides, cholesterol and other lipoproteins in the plasma. Lipoproteins are macromolecular complexes of lipids and proteins and are the primary vehicles responsible for the transport of hydrophobic material through the aqueous circulatory system (Wasan et al. 2008). All three pyrethroids partitioned to lipoproteins (27-30 %) in adult human and rat plasma. Maliwal and Guthrie (1981) studied binding of chlorinated hydrocarbon, carbamates and organophosphate insecticides to human low density lipoproteins and high density plasma proteins. They suggested the interaction to be due to partitioning rather than saturable binding. Further, they found distribution was related to lipid content and composition of lipoproteins. Mohammed et al. (1990) studied the distribution of toxaphene, DDT and PCB among lipoprotein and protein fractions in rat plasma. They observed ~ 55% of the each compound distributed to plasma proteins and ~ 35 % to lipoprotein fraction.

The distribution of all three pyrethroids to lipoproteins at PND 10 was significantly higher from PND 90 rat plasma at concentration below 750 nM. However distribution of all three compounds to lipoprotein was similar in all age groups in human plasma. This might be due to the differences in the plasma lipoprotein profile of human and rat (Mills and Taylaur 1971; Haa and Barter 1982). Despite little differences in the structure, substantially higher HDL/LDL ratios

were reported in rats than in humans (Mills and Taylaur 1971). These differences were due to the difference in metabolism of lipoproteins in rats and humans in several respects (Oschry and Eisenberg 1982). Human plasma is reported to have elevated plasma cholesteryl ester transfer protein (van Tol, Zock et al. 1995). This activity of transfer protein results in a greater rate of transfer of cholesteryl esters from HDL to lower density lipoproteins. In contrast, rats lack this transfer protein, which results in transport of the majority of endogenous cholesterol via HDL (Gatto, Lyons et al. 2002). Further, in rat the clearance of chylomicron and VLDL remnants from the circulation is due to interactions of remnants with specific hepatic receptors (Windler, Chao et al. 1980). This results in high HDL levels in rats. Data from our preliminary in-vivo study suggested that DLM binds predominantly to the HDL class of lipoproteins in rats. Therefore, higher HDL levels along with immature and lower albumin levels might be responsible for greater association of DLM, CIS and TRANS to lipoproteins in PND 10 and PND 15. However these differences were not observed in adult human and rat plasma. This might be due to greater role of albumin in binding of these compounds. When pyrethroids were incubated with isolated lipoproteins, about 90% of the pyrethroids were associated with the lipoproteins (data not shown). This implies that pyrethroids have a higher binding affinity for albumin than the lipoproteins. Data from our study at concentrations where non-linear binding occurred also support this hypothesis. With linear binding however, we observed greater distribution of DLM, CIS and TRANS to lipoproteins at higher concentrations.

Binding of DLM, CIS and TRANS to human plasma after 4 weeks of age was not significantly different from adults. This was not unexpected. Meistelman et al. (1990) reported no difference in plasma protein binding of sufentanil between children and adults. Similarly Roure et al. (1987) and Meistelman et al. (1987) observed comparable binding of alfentanil

between children and adults. This might in part be due to non-significant differences in albumin levels of children (3.31 g/dl) and adults (3.38 g/dl) as reported in previous studies (Weeke and Krasilnikoff 1972; Meistelman et al. 1990). In the current study, in rats although levels of albumin and total proteins at PND 21 were lower by ~ 23% and ~ 32% respectively from the adults, the binding had reached the adult levels. This suggests that by PND 21 the rat plasma attains the adult properties. Thus higher unbound fraction of pyrethroids at PND 10, PND 15 in rats and birth-1week, >1week-4weeks in humans may alter the tissue distribution in these age groups.

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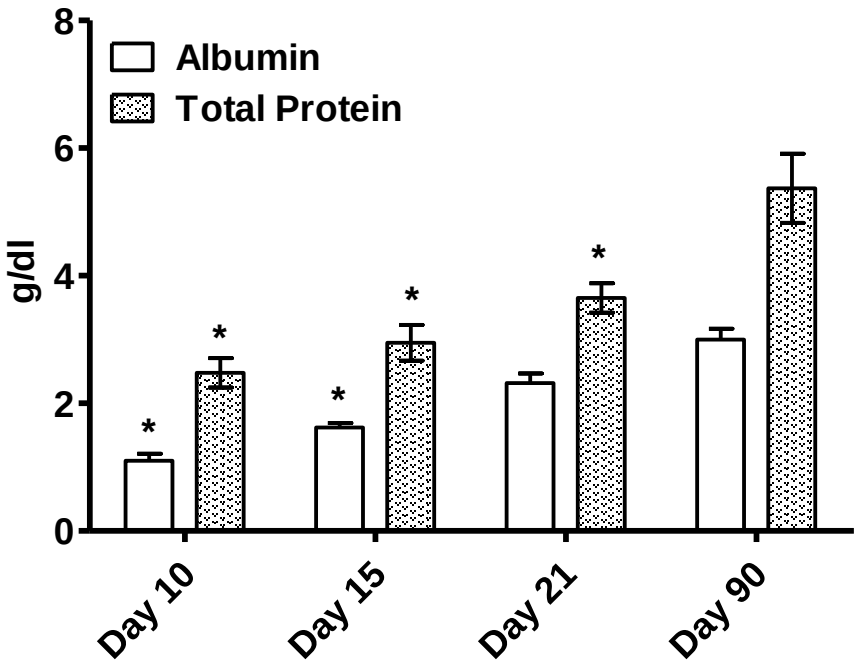

Figure 4-1. Albumin and total protein levels (g/dl) in different age groups in rat plasma. Data represent mean ± SD, N=3. Statistical significance was evaluated by one-way ANOVA with post-hoc Dunnett's test using Graphpad Prism. (*) Indicates statistically significant difference from adult ($P \leq 0.05$).

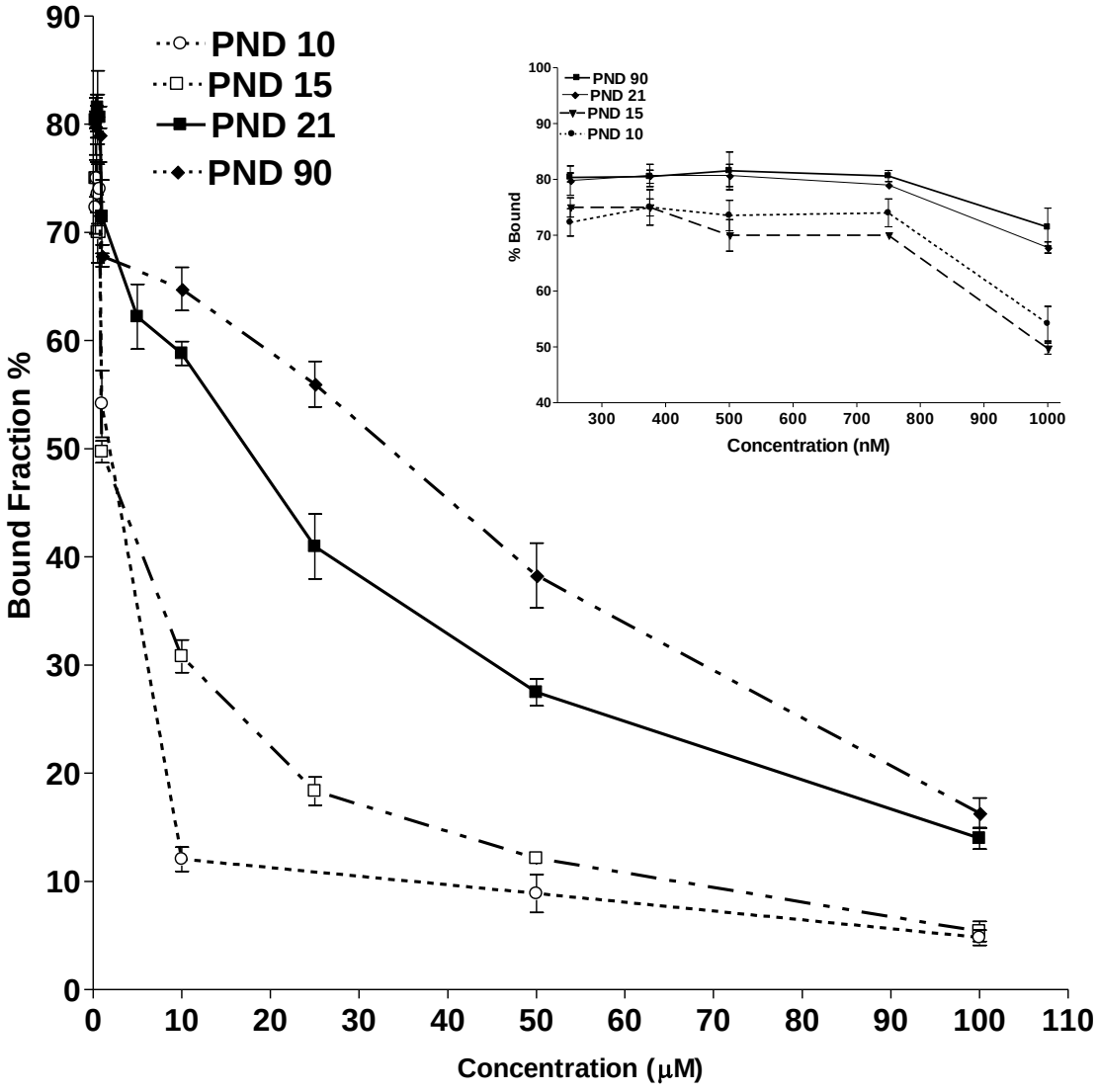

Figure 4-2. Non-linear binding at concentrations >750 nM in different age groups in rats. Insert shows linear binding of DLM in different age groups in rat plasma (at concentrations below 750 nM). Data represent mean ± SD, N=4.

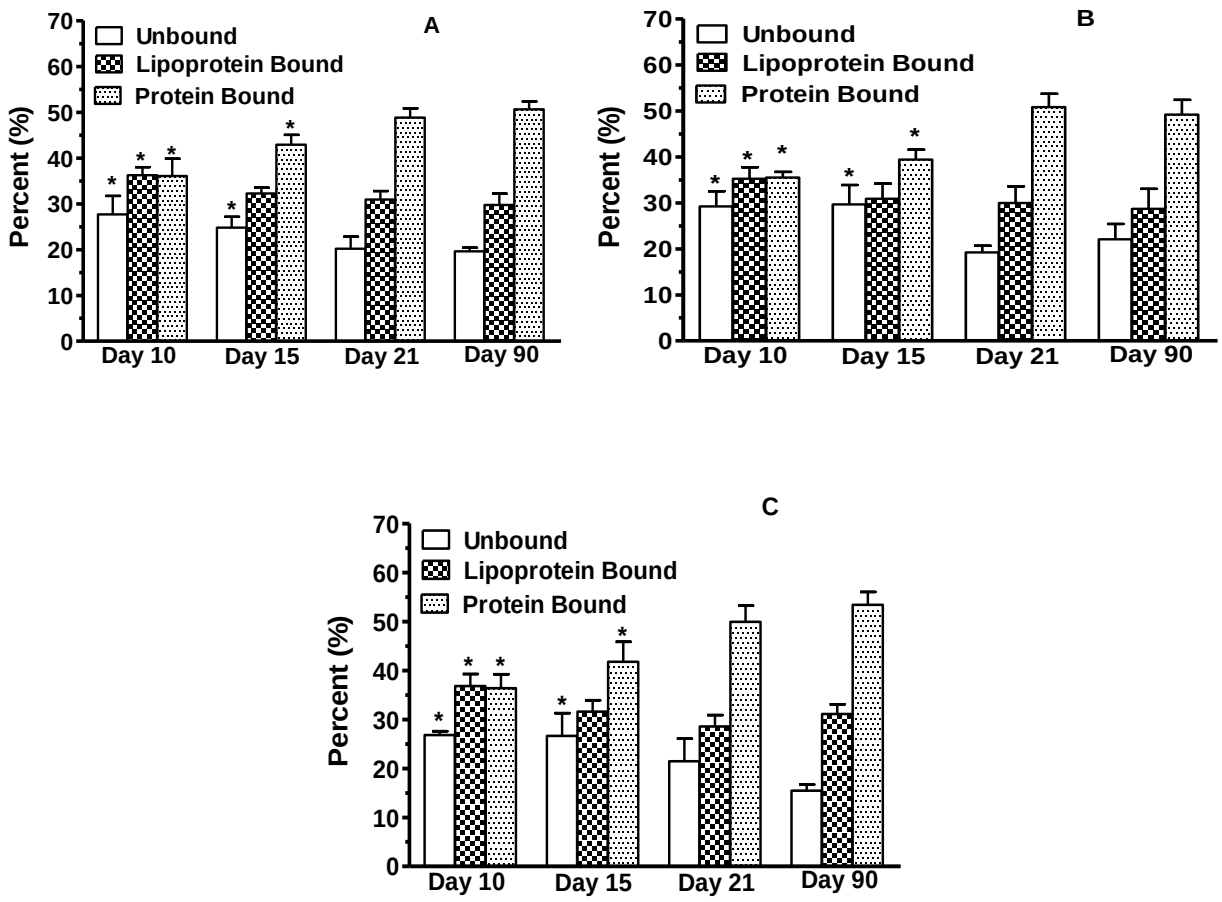

Figure 4-3. Effect of age on plasma protein binding DLM (A), CIS (B) and TRANS (C) in rat plasma (250 nM concentration). (*) Statistical significance ($p<0.05$) was evaluated by one-way ANOVA with post-hoc Dunnett's test comparisons between unbound, lipoprotein bound and protein bound in rat and human plasma. Data represent mean ± SD, N=4.

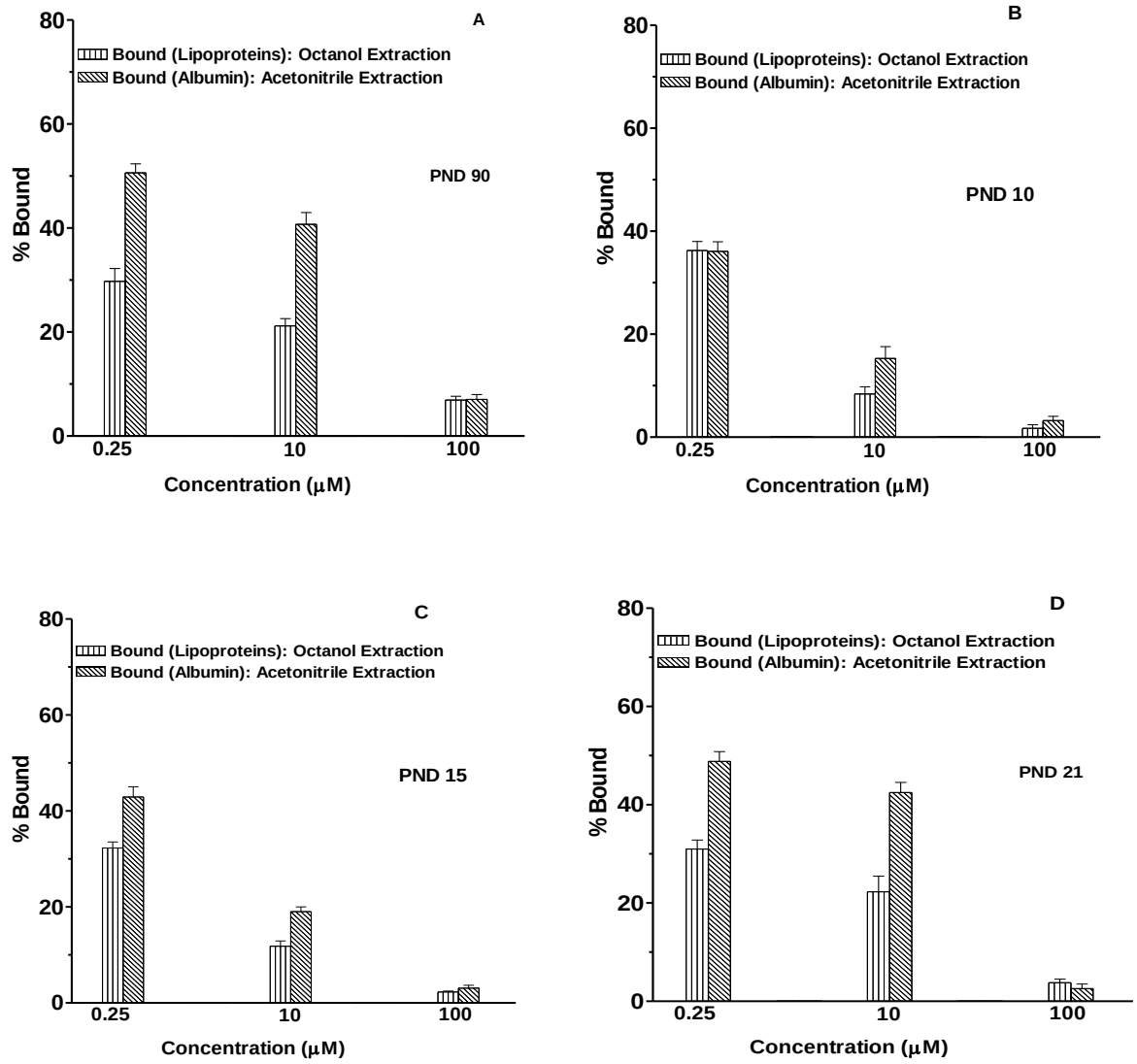

Figure 4-4. Distribution of DLM to albumin and lipoproteins at different concentrations in rat plasma (0.25 µM, 10 µM and 100 µM). Panel (A) represent PND 90, Panel (B) PND 10, Panel (C) PND 15, Panel (D) PND 21. Data represent mean ± SEM, N=4.

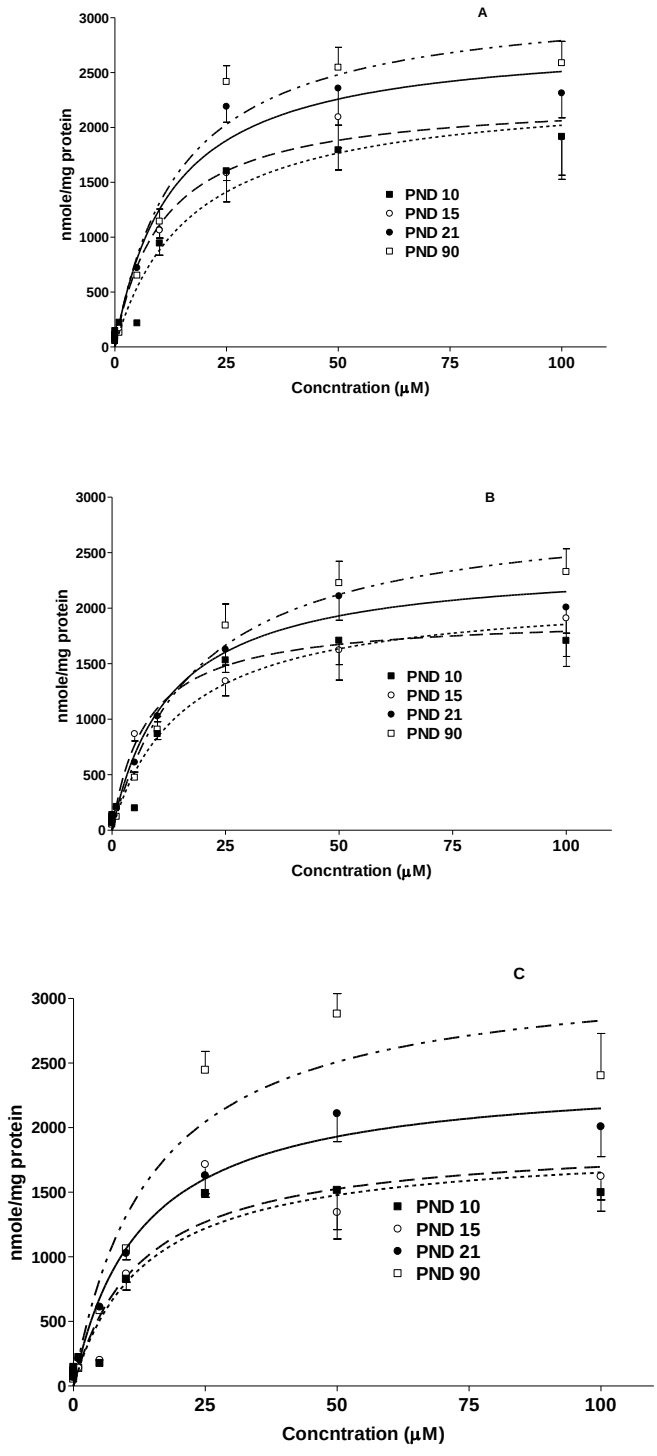

Figure 4-5. Saturation binding curves of DLM (A), CIS (B) and TRANS (C) in rat plasma in PND 10, PND 15, PND 21 and PND 90. (250 nM to 100 µM concentration). Lines are fitted curves. Data represent mean ± SD, N=4.

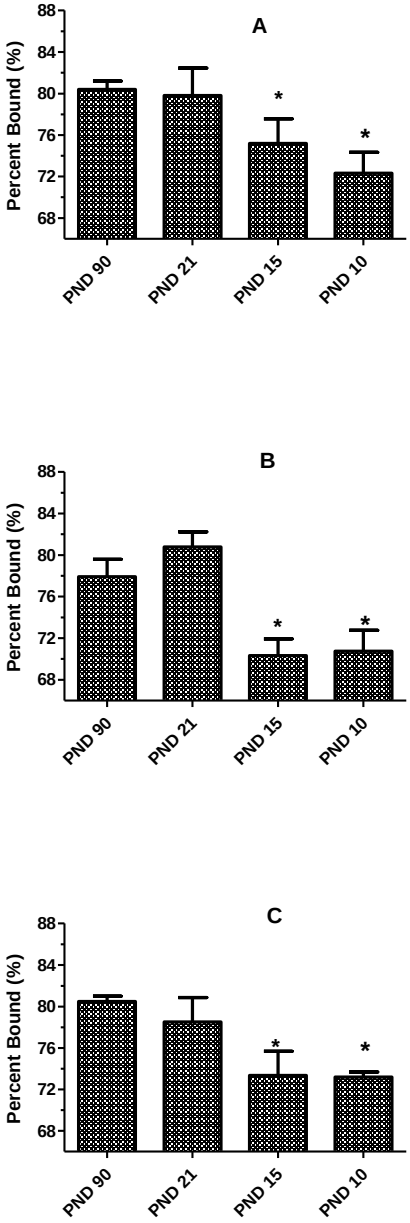

Figure 4-6. Total binding of DLM (A), CIS (B) and TRANS (C) in different age groups in rat. (*) Statistical significance ($p<0.05$) was evaluated by one-way ANOVA with post-hoc Dunnett's test comparisons between unbound, lipoprotein bound and protein bound in rat and human plasma. Data represent mean ± SD, N=4.

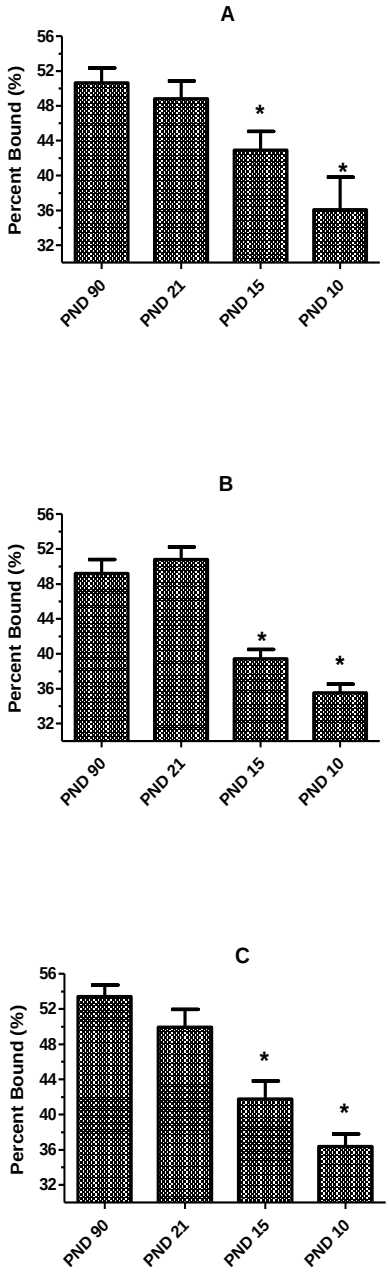

Figure 4-7. Albumin binding of DLM (A), CIS (B) and TRANS (C) in different age groups in rat. (*) Statistical significance ($p<0.05$) was evaluated by one-way ANOVA with post-hoc Dunnett's test comparisons between unbound, lipoprotein bound and protein bound in rat and human plasma. Data represent mean ± SD, N=4.

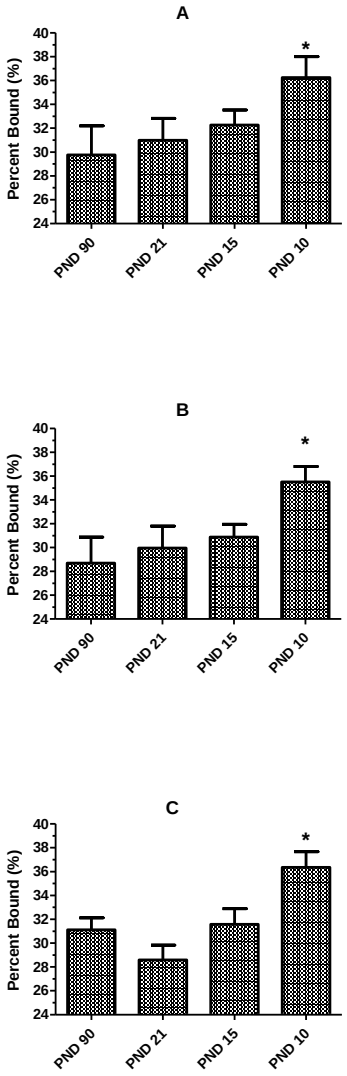

Figure 4-8. Lipoprotein binding of DLM (A), CIS (B) and TRANS (C) in different age groups in rat. (*) Statistical significance ($p<0.05$) was evaluated by one-way ANOVA with post-hoc Dunnett's test comparisons between unbound, lipoprotein bound and protein bound in rat and human plasma. Data represent mean ± SD, N=4.

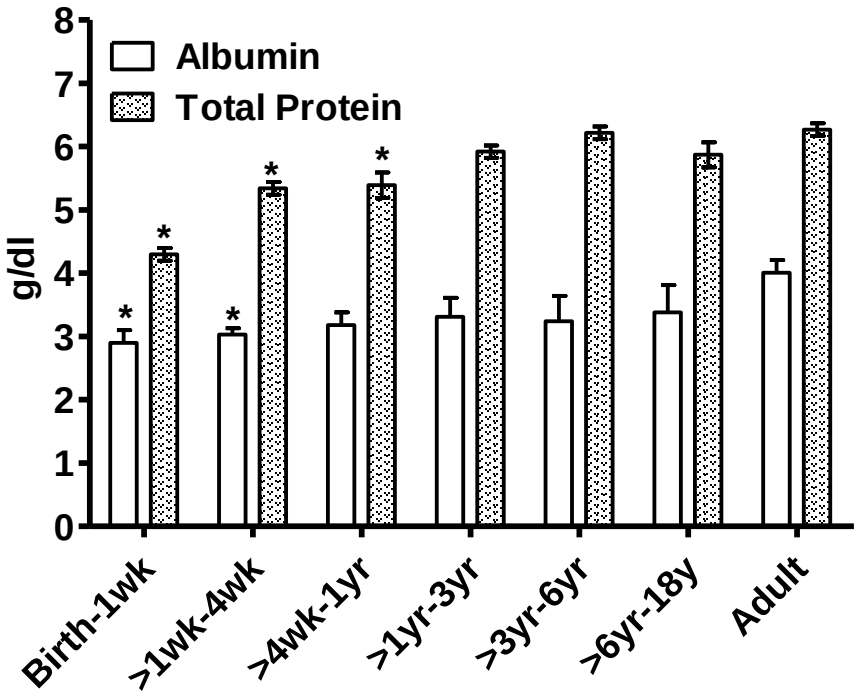

Figure 4-9. Albumin and total protein levels (g/dl) in different age groups in human plasma. Data represent mean ± SD, N=3. Statistical significance was evaluated by one-way ANOVA with post-hoc Dunnett's test, with a significance level of $p<0.05$ using Graphpad Prism. (*) Indicates statistically significant difference from adult ($P \leq 0.05$).

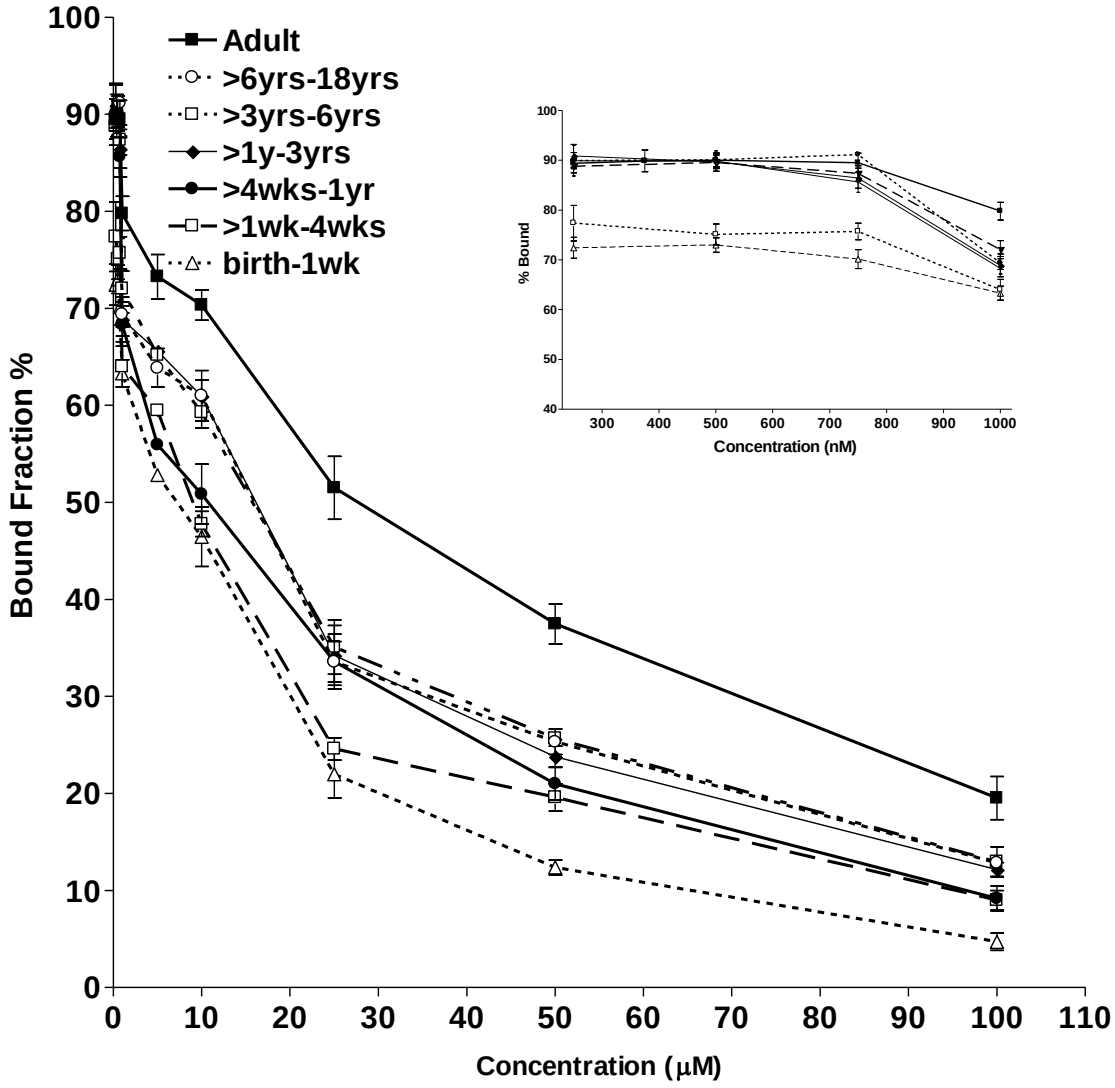

Figure 4-10. Non-linear binding at concentrations >750 nM in different age groups in humans. Insert shows linear binding of DLM in different age groups in human plasma (at concentrations below 750 nM). Data represent mean ± SD, N=4.

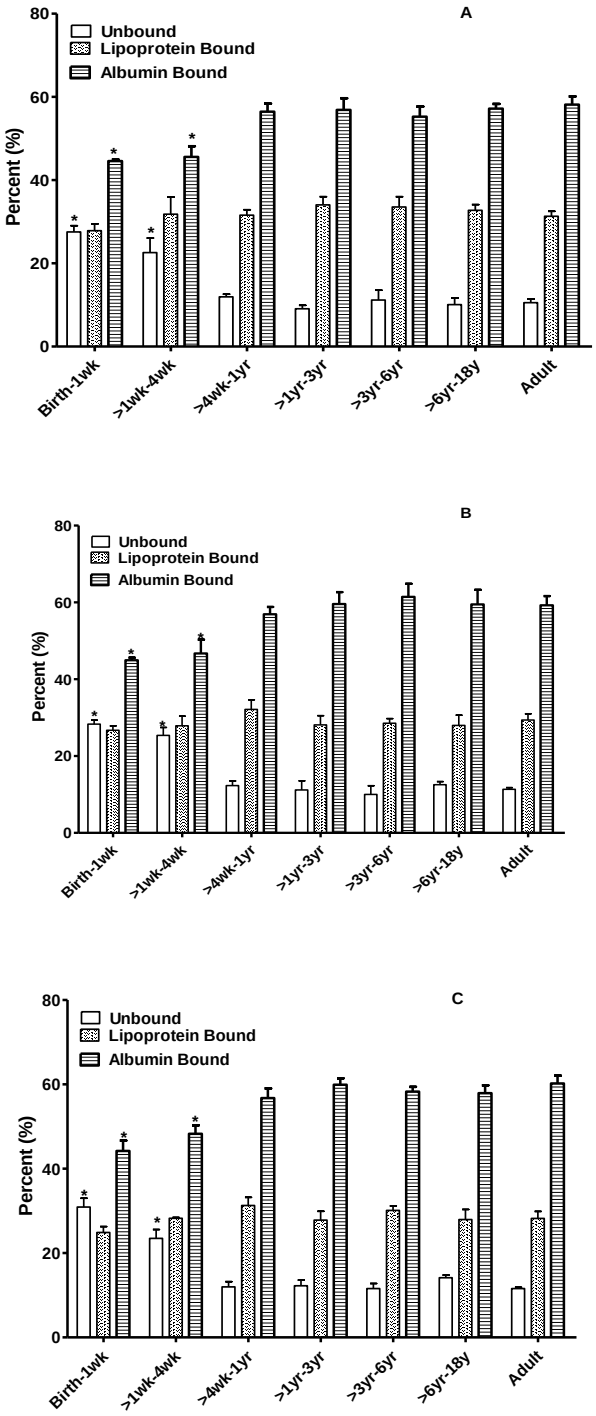

Figure 4-11. Relative distributions of DLM (A), CIS (B) and TRANS (C) in different age groups in human plasma (250 nM concentration). (*) Statistical significance ($p<0.05$) was evaluated by one-way ANOVA with post-hoc Dunnett's test comparisons between unbound, lipoprotein bound and protein bound in rat and human plasma. Data represent mean ± SD, N=4.

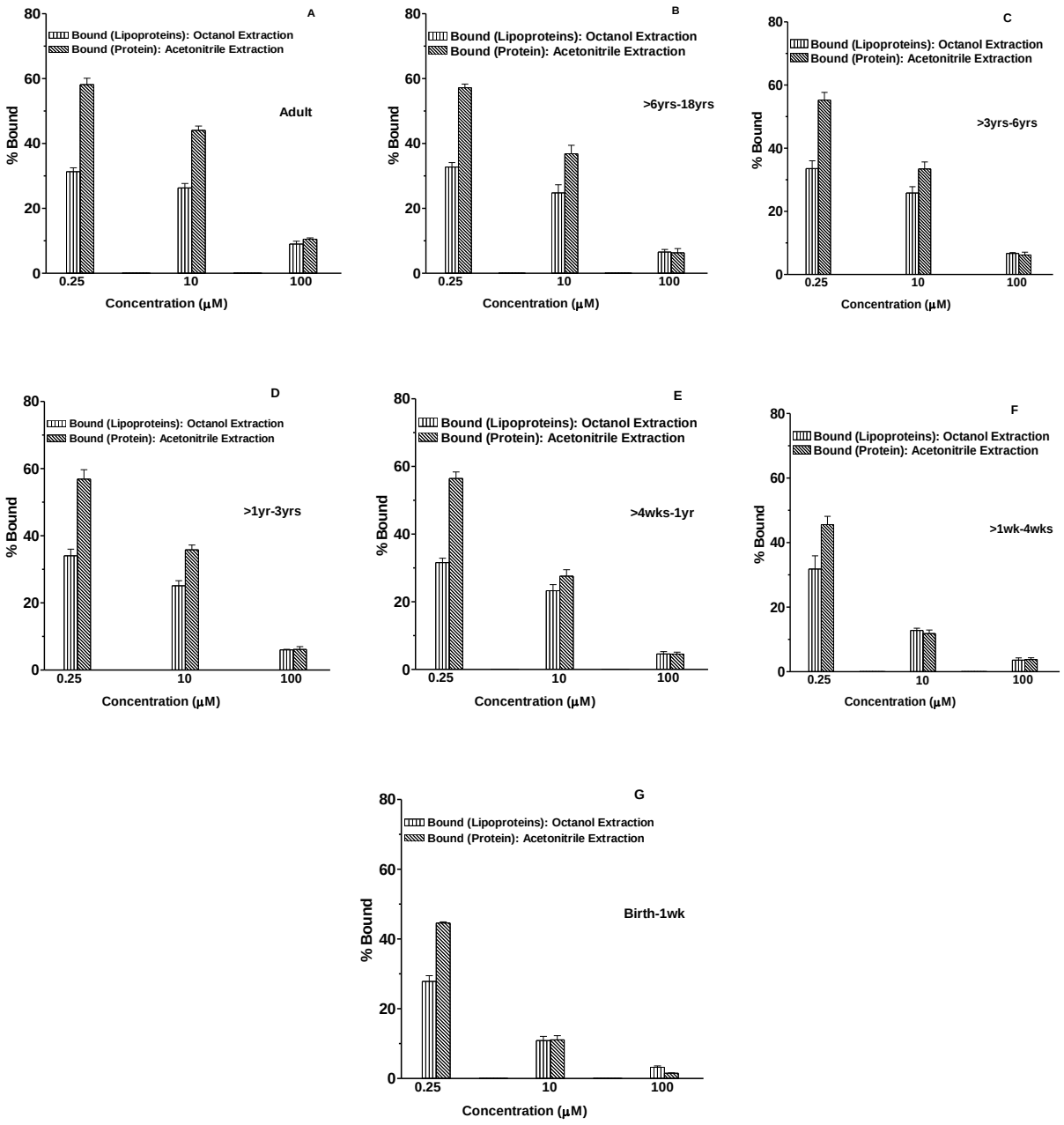

Figure 4-12. Distribution of DLM to albumin and lipoproteins at different concentrations (0.25 µM, 10 µM and 100 µM) in human plasma. Panel (A) represent Adult, Panel (B) >6yrs-18yrs, Panel (C) >3yrs-6yrs, Panel (D) >1yr-3yrs, Panel (E) >4wks-1yr, Panel (F) >1wk-4wks, and Panel (G) Bith-1wk. Data represent mean ± SD, N=4.

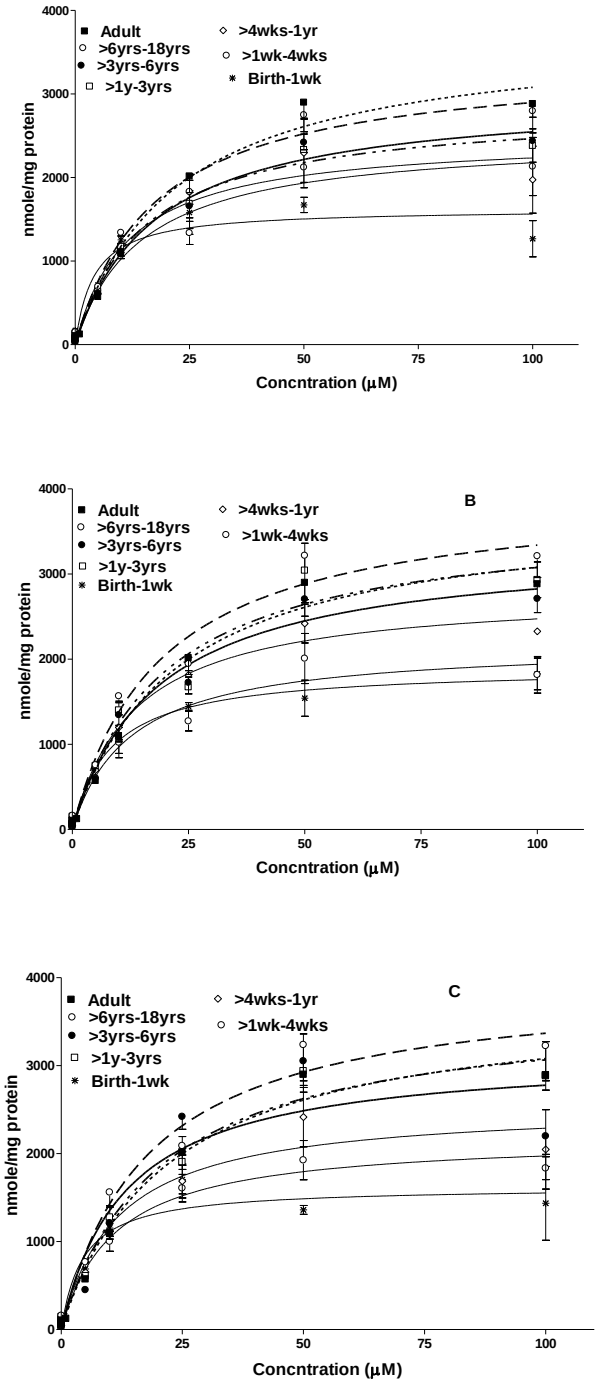

Figure 4-13. Saturation binding curves of DLM (A), CIS (B) and TRANS (C) in human plasma in different age groups. (250 nM to 100 µM concentration). Lines are fitted curves. Data represent mean ± SD, N=4.

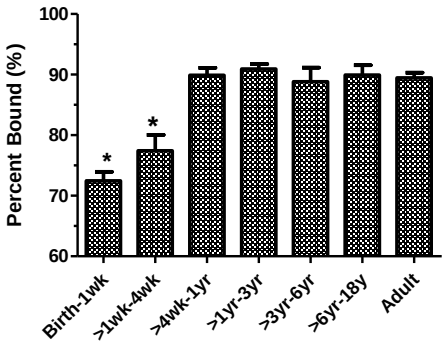

Figure 4-14. Total binding of DLM in different age groups in human plasma. (*) Statistical significance ($p<0.05$) was evaluated by one-way ANOVA with post-hoc Dunnett's test comparisons between unbound, lipoprotein bound and protein bound in human plasma. Data represent mean ± SD, N=4.

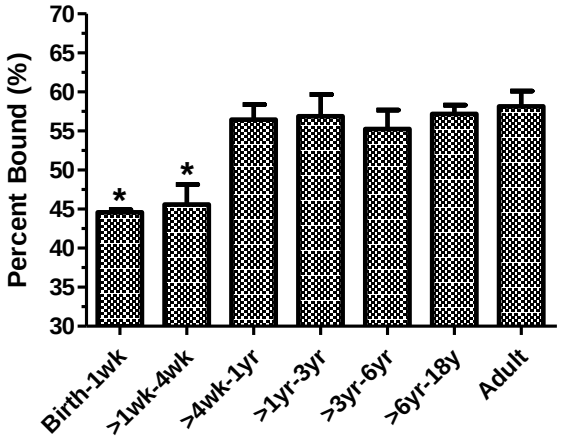

Figure 4-15. Albumin binding of DLM in different age groups in human plasma. (*) Statistical significance ($p<0.05$) was evaluated by one-way ANOVA with post-hoc Dunnett's test comparisons between unbound, lipoprotein bound and protein bound in human plasma. Data represent mean ± SD, N=4.

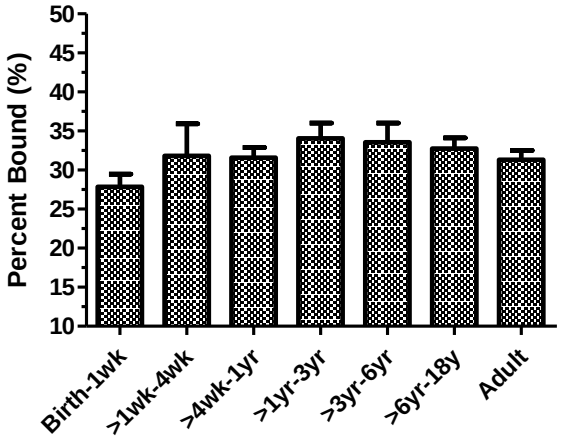

Figure 4-16. Lipoprotein binding of DLM in different age groups in human plasma. (*) Statistical significance ($p<0.05$) was evaluated by one-way ANOVA with post-hoc Dunnett's test comparisons between unbound, lipoprotein bound and protein bound in human plasma. Data represent mean ± SD, N=4.

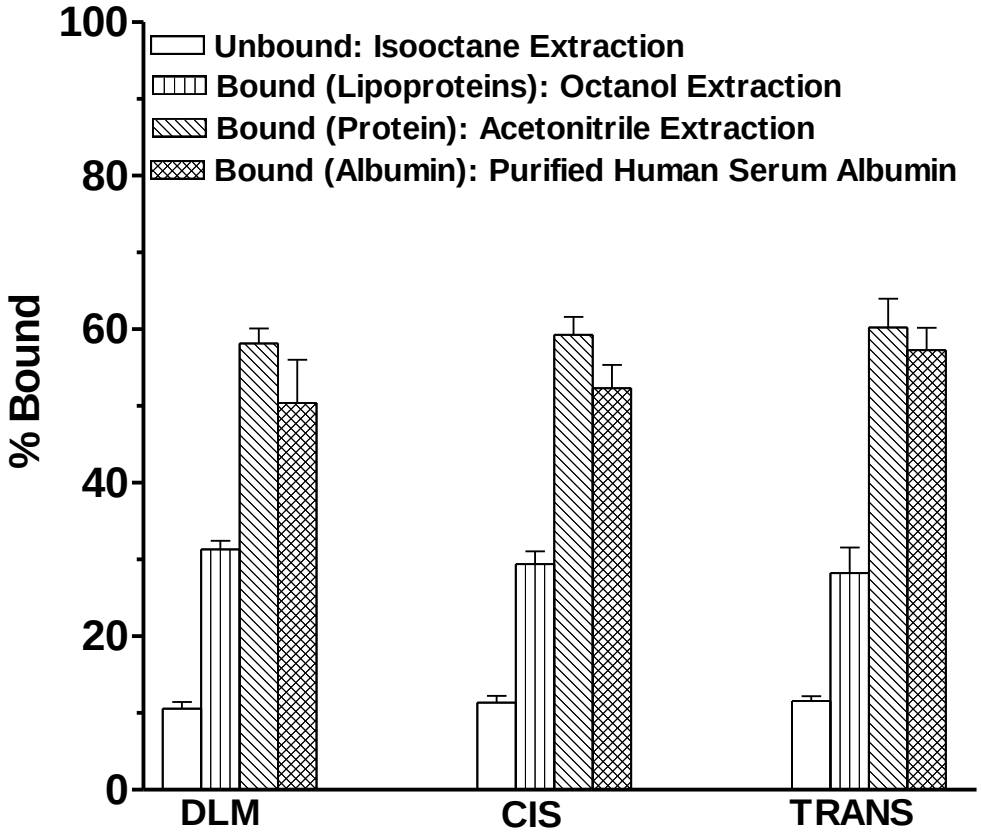

Figure 4-17. Relative distribution of Deltamethrin (DLM), Cis-Permethrin (CIS) and Trans-Permethrin (TRANS) in human plasma (250 nM concentration). Binding of DLM, CIS and TRANS to purified human serum albumin (4.0 g/dl). Unbound fraction was obtained in the isooctane extraction, lipoprotein bound/associated fraction was obtained in octanol extraction and protein bound fraction was obtained in the acetonitrile extraction. Bar heights and brackets represent mean ± SD, N=4.

Table 4-1 Binding Parameters Estimates for Rat Plasma Obtained by Using One-site Binding (Hyperbola) by Nonlinear Regression Using the Prism Program.

Compound **DLM**	Bmax (µM/mg protein)	Kd (M)
PND 10	2.0 ± 0.1*	$6.1 \pm 0.2 \times 10^{-6}$
PND 15	2.2 ± 0.1*	$6.2 \pm 0.1 \times 10^{-6}$
PND 21	2.6 ± 0.1	$6.2 \pm 0.3 \times 10^{-6}$
PND 90	2.8 ± 0.1	$5.6 \pm 0.7 \times 10^{-6}$

Data represent Mean ± SD, N=4. * Indicates statistically significant difference from adult ($P \leq 0.05$).

Table 4-2 Binding Parameters Estimates for Rat Plasma Obtained by Using One-site Binding (Hyperbola) by Nonlinear Regression Using the Prism Program.

Compound **CIS**	Bmax (µM/mg protein)	Kd (M)
PND 10	1.9 ± 0.1*	$5.8 \pm 0.2 \times 10^{-6}$
PND 15	1.7 ± 0.1*	$5.1 \pm 0.3 \times 10^{-6}$
PND 21	2.2± 0.1	$6.0 \pm 0.3 \times 10^{-6}$
PND 90	2.6 ± 0.1	$6.2 \pm 0.7 \times 10^{-6}$

Data represent Mean ± SD, N=4. * Indicates statistically significant difference from adult ($P \leq 0.05$).

Table 4-3 Binding Parameters Estimates for Rat Plasma Obtained by Using One-site Binding (Hyperbola) by Nonlinear Regression Using the Prism Program.

Compound **TRANS**	Bmax (µM/mg protein)	Kd (M)
PND 10	1.6 ± 0.1*	$5.5 \pm 0.2 \times 10^{-6}$
PND 15	1.8 ± 0.1*	$5.1 \pm 0.1 \times 10^{-6}$
PND 21	2.3 ± 0.1	$5.8 \pm 0.3 \times 10^{-6}$
PND 90	2.9 ± 0.9	$5.9 \pm 0.7 \times 10^{-6}$

Data represent Mean ± SD, N=4. * Indicates statistically significant difference from adult ($P \leq 0.05$).

Table 4-4. Binding Parameters Estimates for Human Plasma Obtained by Using One-site Binding (Hyperbola) by Nonlinear Regression Using the Prism Program.

Compound **DLM**	Bmax (µM/mg protein)	Kd (M)
Birth-1week	1.6 ± 0.1*	$2.1 \pm 0.7 \times 10^{-6}$*
>1week-4weeks	2.3 ± 0.1*	$6.3 \pm 0.1 \times 10^{-6}$
>4weeks-1year	2.4 ± 0.3	$5.2 \pm 0.9 \times 10^{-6}$
>1years-3years	2.5 ± 0.6	$5.0 \pm 0.8 \times 10^{-6}$
>3years-6years	2.6 ± 0.7	$6.2 \pm 0.6 \times 10^{-6}$
>6years-18years	2.9 ± 0.2	$5.9 \pm 0.5 \times 10^{-6}$
Adult	3.2 ± 0.1	$5.9 \pm 0.7 \times 10^{-6}$

Data represent Mean ± SD, N=4. * Indicates statistically significant difference from adult ($P \leq 0.05$).

Table 4-5. Binding Parameters Estimates for Human Plasma Obtained by Using One-site Binding (Hyperbola) by Nonlinear Regression Using the Prism Program.

Compound **CIS**	Bmax (µM/mg protein)	Kd (M)
Birth-1week	1.7 ± 0.1*	$3.9 \pm 0.6 \times 10^{-6}$*
>1week-4weeks	1.9 ± 0.3*	$5.5 \pm 0.8 \times 10^{-6}$
>4weeks-1year	2.7 ± 0.3	$5.0 \pm 0.5 \times 10^{-6}$
>1year-3years	2.9 ± 0.4	$4.8 \pm 0.8 \times 10^{-6}$
>3years-6years	2.8 ± 0.4	$5.2 \pm 0.4 \times 10^{-6}$
>6years-18years	3.3 ± 0.1	$4.9 \pm 0.9 \times 10^{-6}$
Adult	3.5 ± 0.7	$5.7 \pm 0.5 \times 10^{-6}$

Data represent Mean ± SD, N=4. * Indicates statistically significant difference from adult ($P \leq 0.05$).

Table 4-6. Binding Parameters Estimates for Human Plasma Obtained by Using One-site Binding (Hyperbola) by Nonlinear Regression Using the Prism Program.

Compound **TRANS**	Bmax (µM/mg protein)	Kd (M)
Birth-1week	1.5 ± 0.1*	$2.5 \pm 0.8 \times 10^{-6}$*
>1week-4weeks	2.0 ± 0.1*	$5.1 \pm 0.7 \times 10^{-6}$
>4weeks-1year	2.5 ± 0.4	$4.7 \pm 0.4 \times 10^{-6}$
>1year-3years	2.8 ± 0.1	$5.9 \pm 0.7 \times 10^{-6}$
>3years-6years	2.9 ± 0.3	$4.9 \pm 0.6 \times 10^{-6}$
>6years-18years	3.3 ± 0.2	$4.8 \pm 0.9 \times 10^{-6}$
Adult	4.0 ± 0.1	$6.1 \pm 0.4 \times 10^{-6}$

Data represent Mean ± SD, N=4. * Indicates statistically significant difference from adult ($P \leq 0.05$).

CHAPTER 5

EFFECT OF MATURITY ON PLASMA PROTEIN BINDING OF DIAZEPAM AND CYCLOSPORINE IN HUMAN PLASMA

Pankaj Sethi, James V. Bruckner, Catherine White. To be submitted to Clinical Pharmacokinetics

Abstract:

Plasma protein binding is often a significant determinant of drug disposition in the body. Among the various factors capable of altering plasma protein binding of a drug, age is very important one. This study was conducted to determine plasma protein binding of diazepam and cyclosporine in human plasma. These drugs are chosen for their ability to bind extensively and reversibly to albumin and lipoproteins respectively. The percent free fraction of diazepam was significantly higher in birth-1week, >1 week – 4 week and > 4week – 1 year than in adults. The percent free fraction of diazepam in > 1 year – 3 years, > 3 years - 6years and > 6years – 18 years were not significantly different from adult. The percent free fraction of cyclosporine was significantly higher in birth – 1 week, > 1 week - 4 weeks, > 4 weeks - 1 year and > 1 year - 3 years than in adults. The percent free fraction of diazepam > 3 years – 6 years and > 6 years - 18 years were not significantly different from adult. Albumin, lipoproteins and total protein levels as well as the presence of endogenous substances like bilirubin and free fatty acids might be responsible for the higher free fractions of diazepam and cyclosporine. Thus, the clinical effect of highly bound drugs may be significantly increased in children less than 3 years of age and care should be taken with the dosing of these drugs.

Introduction:

Plasma serves as a transport medium to carry wide variety of compounds from the site of absorption to the site of action and elimination. Some compounds are transported as free (unbound) while many others are reversibly associated with plasma constituents such as albumin, α1-acid glycoprotein, and lipoproteins. Albumin is one of the most abundant proteins in the plasma, accounting for 50-60% of total protein (Nicholson, Wolmarans et al. 2000) and plays an important drug carrier role in the plasma. Many commonly used acidic drugs (e.g. diazepam,

warfarin, and ibuprofen) binds to site I or II of albumin (Ghuman, Zunszain et al. 2005). Viani et al. (1991) demonstrated that diazepam primarily binds to albumin isolated from fetal and adult serum. Sudlow et al (1976) pointed out that warfarin binds to site I of albumin with high affinity.

Several studies have demonstrated the role of lipoproteins in the transport of lipophilic drugs. Lipoproteins are complex macromolecules made of lipids and proteins that are responsible for the transport of hydrophobic drugs through the aqueous circulatory system (Wasan and Cassidy 1998). Groen (1988) suggested that cyclosporine binds to low-density lipoprotein, which serves as an important carrier of cyclosporine in plasma. Brajtburg et al. (1984) reported that Amphotericin B binds to lipoproteins in the human plasma. Further, they observed that Amphotericin B interacts more rapidly with low- and very-low-density lipoproteins than with high-density lipoproteins.

Plasma protein binding is often a significant determinant of drug disposition (distribution and clearance) in the body. It is widely accepted that the pharmacological action of a drug is dependent on the unbound concentration of drug at the site of action (Muller and Milton 2012). It is the unbound drug that diffuses across the biological membrane to reach the site of action and produce a pharmacological effect. Therefore, important pharmacokinetics characteristic such as clearance, volume of distribution, and efficacy of a drug is dependent on the extent of plasma protein binding (Zeitlinger, Derendorf et al. 2011). Plasma protein binding data along with other parameters such as apparent volume of distribution and clearance are used to determine the dosing regimen of a drug (Roberts, Pea et al. 2013).

Among the various factors capable of altering plasma protein binding of a drug, age is very important one. Determination of plasma protein binding is critical in neonates and children as they undergo rapid anatomical and physiological changes. Qualitative and quantitative

changes in the circulating protein (albumin, α1-acid glycoprotein, and lipoproteins) can influence the distribution of highly bound drugs (Rakhmanina and van den Anker 2006). In general, neonates exhibit lower plasma protein binding as compared to adults. Aranda et al. (1997) described the protein binding of intravenous ibuprofen in the premature newborn infant. Plasma protein binding was significantly lower in in the cord plasma compared to the adult human plasma. Meuldermans et al (1986) reported lower plasma protein binding of alfentanil and sufentanil in neonates compared to maternal plasma. However, similar plasma protein binding of alfentanil was observed for children (aged 10 months 6.5 yrs.) and adult patients (Roure, Jean et al. 1987).

Although it is widely accepted fact that "newborn/infants are not small adults", the qualitative differences in the physiological functions among infants, children and adults are often ignored (Morselli 1976). Limitations of methods for drug analysis with small volume samples and ethical considerations are responsible of lack of detailed studies in newborn, infants and small children (Koren 1997). There have been numerous studies to determine the plasma protein binding of drugs in adults, but little information is available about plasma protein binding in neonates, infants and children.

The overall objective of this study is to determine the effect of age on the plasma protein binding of diazepam and cyclosporine in human plasma. These drugs are chosen for their ability to bind extensively and reversibly to different plasma components. Diazepam binds mainly to albumin (Nau, Luck et al. 1984), whereas cyclosporine primarily binds to lipoproteins (Wasan and Cassidy 1998). Both diazepam and cyclosporine are prescribed at various stages in the life to treat different conditions. Neonates are exposed to diazepam during breastfeeding (Kelly, Poon et al. 2012). Diazepam is also used in children, adolescents, and young adults to treat status

epilepticus (Mitchell 1996). Cyclosporine is used in transplant recipients during pregnancy and in children as young as 1 year of age (Ryan, Amor et al. 2010).

Materials:

Radiolabeled [^{14}C]-diazepam (55 mCi/mmol) and [^{3}H]-Cyclosporine (20 Ci/mmol) were purchased from American Radiolabeled Chemicals (St.Lous, MO, USA). Dispo Equilibrium Dialyzer was purchased from Harvard Apparatus (Holliston, MA, USA).

Human Plasma:

Frozen, heparinized, pooled adult human and rat plasma were obtained from Innovative Research (Novi, MI, USA). Pediatric plasma samples were obtained from the Children's Hospital of Wisconsin Clinical Laboratory. Plasma samples were obtained from discard blood procured as part of an IRB-approved research study with an informed consent exemption. A sufficient number of samples (at least 30) were retrieved such that pooling provided a total volume of at least 5 mL for each of the following age brackets. Birth to 1week, >1 week to 4 weeks, >4 weeks to 1 year, >1 year to 3 years, >3 years to 6 years, and >1 years to 18 years. Plasma samples were stored at -80°C until use. All plasma samples were filtered through 0.45 µm Millipore® filters to remove precipitated fibrinogen

Plasma Binding Measurements:

Plasma (100 µl) spiked with [^{3}H] cyclosporine (1mg/L), was dialyzed against isotonic phosphate buffer at 37°C for 18 hours to establish equilibrium. For diazepam, plasma (200 µl) spiked with [^{14}C] diazepam (0.2 mg/L), was dialyzed against isotonic phosphate buffer at 37°C for 4 hours to establish equilibrium. No fluid shifts were noted during the 4 hour incubations. After equilibration, 100-µl volumes of plasma and buffer were removed simultaneously and mixed with 3 ml of scintillation fluid in a liquid scintillation vial. The radioactivity of the sample

was measured using a Beckman Coulter LS 6500 (Brea, CA, USA). The extent of plasma binding was then calculated from:

$$\%\ bound\ fraction = \frac{plasma\ dpm - buffer\ dpm}{plasma\ dpm} X\ 100$$

$$\%\ free\ fraction = 1 - \%\ bound\ fraction$$

Measurement of Albumin and Total Protein:

Albumin levels in human and rat plasma were measured by use of a kit (QuantiChrom™ BCG Albumin Assay Kit; BioAssay Systems, Hayward, CA, USA) according to the manufacturer's instructions. Total protein estimations in human and rat plasma were performed using a BCA Protein assay kit (Pierce/Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions.

Data and Statistical Analysis:

All experiments were performed with a minimum of three independent experiments unless otherwise stated. All values are represented as mean ± SD unless otherwise stated. Statistical significance was evaluated by one-way ANOVA with post-hoc Dunnett's test, with a significance level of $p<0.05$ using Graphpad Prism.

Results:

Figure 5-1 shows albumin and total protein levels at different ages in human plasma. Albumin and total protein levels for different age groups are summarized in table 4-1. Albumin levels were statistically significantly lower in infants less than 4 weeks of age as compared to adults. Total proteins levels were significantly lower in infants less than 1 year as compared to adult protein levels.

Figure 5-2 shows the unbound fraction of diazepam in different age groups. The results are summarized in table 5-2. The free fraction of diazepam was significantly higher in infants up

to 1 year of age as compared to adults. Figure 5-3 shows the unbound fraction of cyclosporine in different age groups. Results are summarized in table 5-2. The percent free fraction of cyclosporine was significantly higher the younger age brackets. The binding of cyclosporine in children older than 3 was not significantly different from binding in adult plasma.

Discussion:

The determination of free (unbound) drug concentration is important in understanding the pharmacodynamics action of a drug. Although numerous studies are conducted to determine plasma protein binding of drugs in adult human plasma, but very few such studies are conducted to study effect of maturity in plasma protein binding of neonates, infants and children (Pruitt and Dayton 1971). The binding of drugs to plasma proteins is dependent on several factors that may not be fully mature in neonate infants (Milsap and Jusko 1994). During this period factors like decreased protein quality and quantity, changing lipoproteins levels, fluctuating pH and presence of endogenous substances (bilirubin and free fatty acids) may result in reduced plasma protein binding of drugs (Notarianni 1990). The demonstration of age dependent changes in plasma protein binding of diazepam and cyclosporine is interesting from the point of developmental pharmacokinetics.

Results from this study show that the degree of plasma protein binding of diazepam in neonates and infants plasma is significantly lower than in adult plasma. These results were consistent with the previously reported results by Morselii et al. (1980). Similarly, Nau et al. (1984) reported significantly higher unbound fraction of diazepam in newborn plasma as compared to adult human plasma. It is well known that diazepam primarily binds to albumin in the plasma (Ghuman, Zunszain et al. 2005). The differences in binding between neonates and infants as compared to adult appear to result, in part, from the reduced albumin concentration in

neonates and infants. Dean et al. (1980) reported a positive correlation between the serum albumin concentration and diazepam binding capacity. We observed significantly lower albumin in newborns (birth-1 week and >1 week-4 weeks), plasma as compared to adult human plasma. Total proteins levels were also significantly lower in newborn (birth-1 week, >1 week-4 weeks), infants (>4 weeks-1year) from adult human plasma. It has previously been reported that plasma albumin and total protein concentration approaches the adult levels at about 1 year of age (Herngren, Ehrnebo et al. 1983; Milsap and Jusko 1994). However, the difference in the concentration of albumin and total protein cannot alone account for the 3-4-fold increases in free fraction observed in neonates (less than 1 year of age) in this study.

Along with the quantity, infant albumin may also different from adult human albumin in terms of quality. Wallace (1977) pointed out the albumin of neonatal plasma is different to that of the adult. This might lead to difference in binding capacity of neonate and adult plasma. Another reason for lower plasma binding of diazepam in early phases of life might be due to the presence of increased concentrations of endogenous substances like bilirubin and free fatty acids in neonate and infant plasma. Krasner et al. (1973) reported that neonatal albumin, although having a reduced affinity for many drugs, has a greater ability to bind bilirubin than has adult albumin. Bilirubin binds to albumin strongly and can displace drugs from the binding site on albumin molecule (Kurz, Mauser-Ganshorn et al. 1977). Nau et al. (1984) reported a positive correlation between increasing fatty acids and unbound fraction of diazepam during first week of life and suggested that the greatly increased free fatty acids levels shortly after birth result in increased free fractions of diazepam.

We observed similar binding of diazepam in children > 3 years of age as compared to adults. Meistelman et al. (1990) reported no difference in plasma protein binding of sufentanil

between children and adults. Similarly Roure et al. (1987) and Meistelman et al. (1987) observed comparable binding of alfentanil between children and adults. This might in part be due to non-significant differences in albumin levels of children (3.31 g/dl) and adults (3.38 g/dl) as reported in previous studies (Weeke and Krasilnikoff 1972; Meistelman, Benhamou et al. 1990).

In our study total binding of cyclosporine was more than 95% in adult human plasma. This result was consistent with previously reported results by Yatscoff et al. (1993) and Yang and Elmquist (1996). Cyclosporine predominantly binds to cholesterol-rich lipoproteins including low-density lipoproteins (LDL) and high density lipoproteins (HDL) (Niederberger, Lemaire et al. 1983). Hence, changes in LDH and HDL levels can have a significant impact on the distribution of cyclosporine in plasma. Wasan et al. (1997) reported that increases in LDL, VLDL and triglyceride concentrations resulted in greater recovery of cyclosporine in these fractions. The ontogeny of lipoproteins in human plasma is complex and is dependent on age, sex, diet, environmental effects and genetic factors (Lusis 1988).

We observed a significant 2- to 5-fold increase in the unbound fraction of cyclosporine in infants and children less than 3 years of age as compared to adults. This might be due, in part, to lower levels of total cholesterol and triglyceride in these age groups as compared to adults. Several studies have reported dramatic changes in the concentration of serum lipids and lipoproteins during first few years of life. Farris et al. (1982) reported that total cholesterol increased by 100 % and triglycerides by 130 % above cord levels in infants fed a polyunsaturated, fatty acid rich, cholesterol free diet by 6 months of age. Berson et al. pointed out that serum lipids and lipoprotein increased dramatically during first year of life. Serum total cholesterol, β-lipoproteins, α-lipoproteins increase rapidly after birth and reach maximum levels by two years of age. Lehtimaki et al. (1994) reported that total cholesterol increased from 55.6

mg/dl in newborns to 164.5 mg/dl in children 3 years of age. LDL cholesterol levels increased from 22.25 mg/dl in newborns to 98.99 mg/dl in 3 years old. Lindholm and Henricsson (1989) examined the plasma protein binding of cyclosporine A in the plasma of renal transplant recipient. They reported that increased free fraction of cyclosporine in the plasma was negatively correlated with serum HDL and postitively correlated with bilirubin concentration. Thus, these changes might in part, explain the higher unbound fraction of cyclosporine we observed in younger age groups.

Plasma protein binding to albumin and lipoproteins is certainly reduced in infants and young children. This effect is magnified when compounds are extensively bound as observed with diazepam and cyclosporine. Other reports examining the plasma binding of pyrethroids (50% bound to albumin, 35% bound to lipoproteins) in neonates and children only found significant differences in binding from birth to 4 weeks. Thus the clinical effect of highly bound drugs may be significantly increased in children less than 3 years of age and care should be taken with the dosing of these drugs.

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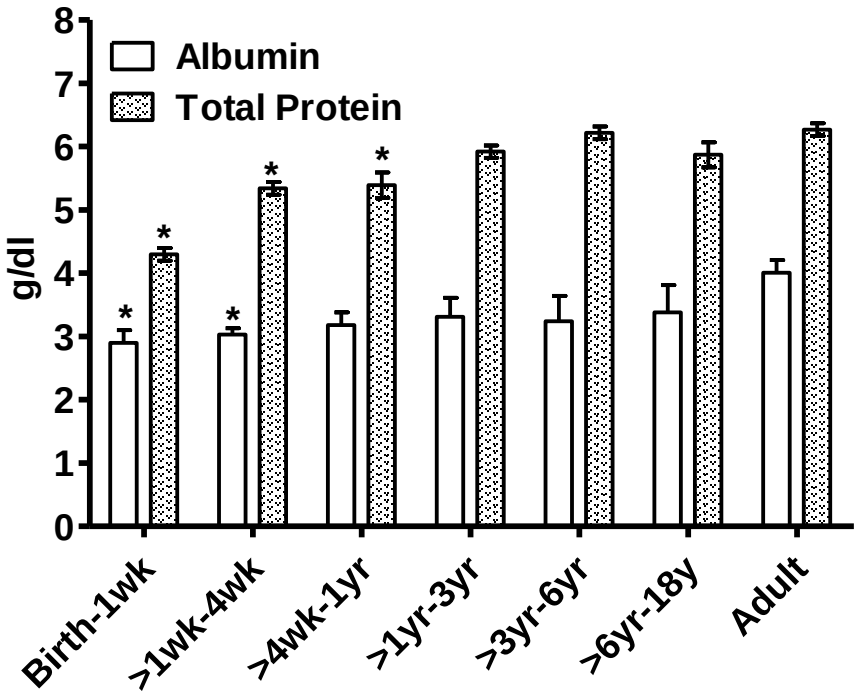

Figure 5-1. Albumin and total protein levels (g/dl) in different age groups (birth-1wk, >1wk-4wk, >4wks-1yr, >1yr-3yr,>3yr-6year, >6yr-18yr and adults) in human plasma. Data represent mean ± SD, N=3. Statistical significance was evaluated by one-way ANOVA with post-hoc Dunnett's test, with a significance level of $p<0.05$ using Graphpad Prism. (*) Indicates statistically significant difference from adult.

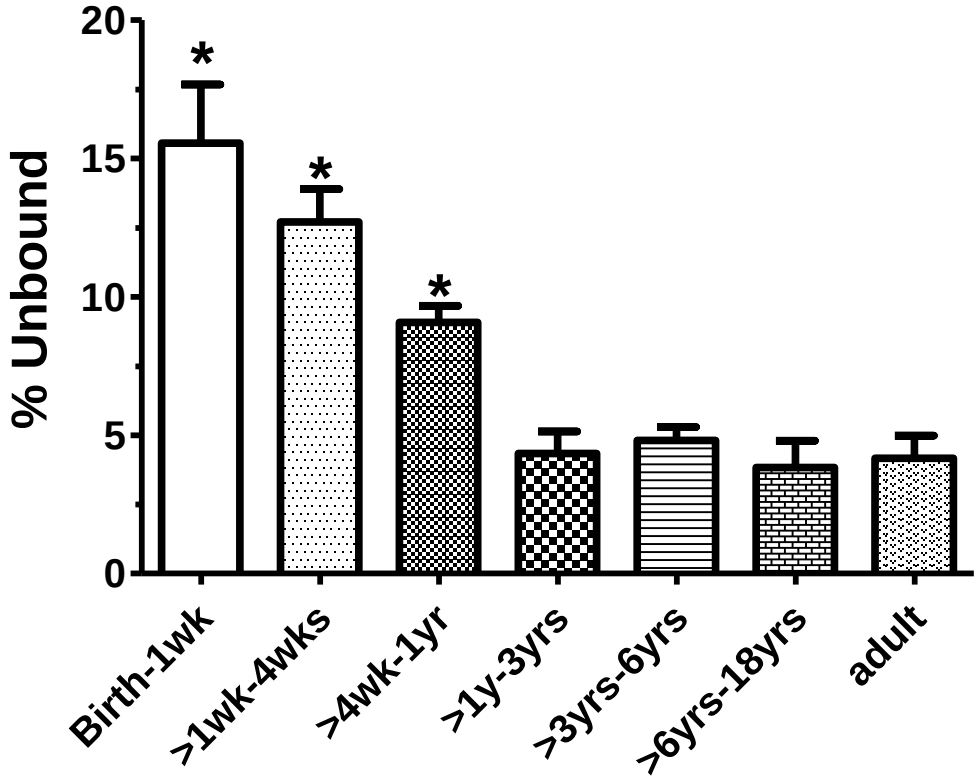

Figure 5-2. Effect of age on plasma protein binding of diazepam. Data represent mean ± SD, N=3. Statistical significance was evaluated by one-way ANOVA with post-hoc Dunnett's test, with a significance level of $p<0.05$ using Graphpad Prism. (*) Indicates statistically significant difference from adult.

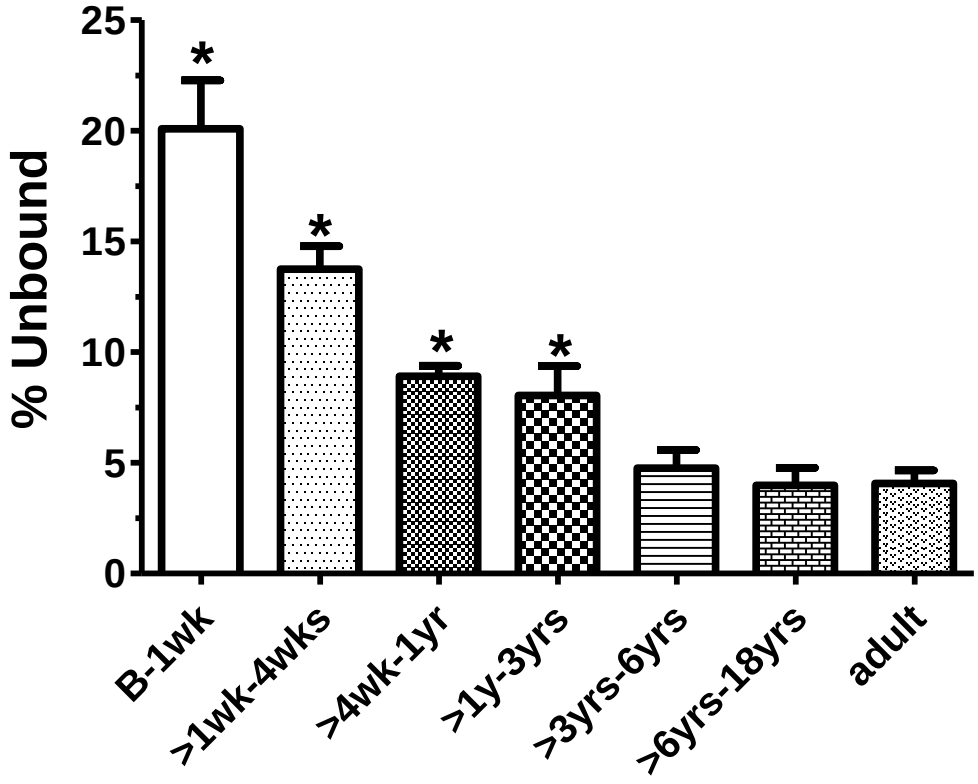

Figure 5-3. Effect of age on plasma protein binding of cyclosporine. Data represent mean ± SD, N=3. Statistical significance was evaluated by one-way ANOVA with post-hoc Dunnett's test, with a significance level of $p<0.05$ using Graphpad Prism. (*) Indicates statistically significant difference from adult.

Table 5-1. Albumin and Total Protein Concentration in Different Age Groups of Humans

Age	Albumin Conc. (g/dl)	Total Proteins conc. (g/dl)
Bith-1week	2.9 ± 0.1*	4.3 ± 0.1*
>1week-4weeks	3.0 ± 0.1*	5.3 ± 0.1*
>4weeks-1year	3.2 ± 0.1	5.4 ± 0.2*
>1year-3years	3.3 ± 0.2	5.9 ± 0.1
>3year-6years	3.2 ± 0.3	6.2 ± 0.1
>6years-18years	3.4 ± 0.4	5.9 ± 0.1
Adult	4.0 ± 0.2	6.3 ± 0.1

Data represent Mean ± SD, N=4. * Indicates statistically significant difference from adult ($P \leq 0.05$).

Table 5-2. Free Fraction of Diazepam and Cyclosporine in Different Age Groups of Humans

Age	Unbound (%) Diazepam	Unbound (%) Cyclosporine
Bith-1week	15.6 ± 2.1*	20.1 ± 2.2*
>1week-4weeks	12.7 ± 1.2*	13.8 ± 1.0*
>4weeks-1year	9.1 ± 0.6*	8.9 ± 0.5*
>1year-3years	4.3 ± 0.8	8.1 ± 1.3*
>3year-6years	4.8 ± 0.5	4.8 ± 0.8
>6years-18years	3.8 ± 0.5	4.0 ± 0.8
Adult	4.2 ± 0.8	4.1± 0.6

Data represent Mean ± SD, N=4. * Indicates statistically significant difference from adult ($P \leq 0.05$).

CHAPTER 6

SUMMARY

Pyrethroids are the most common insecticides used in the United States with the phase out of the organophosphates. Deltamethrin (DLM), Cispermethrin (CIS) and Transpermethrin (CIS), the compounds chosen for the current study, are widely-used type I (CIS and TRANS) and type II (DLM) pyrethroids. They exert neurotoxicity in mammals. Laboratory studies have suggested that the toxicity is due to the parent compound. Large segments of the population in the U.S. are exposed to these pyrethroids, although at quite low levels. The overall objective of this research was to characterize the plasma protein binding of these representative pyrethroids. A related objective was to learn about the relative distribution of DLM, CIS and TRANS in human and rat plasma. We also wanted to determine the binding parameters (e.g., maximum binding capacities, dissociation rate constants) for input into physiological based pharmacokinetic models. Further we wanted to determine whether DLM, CIS and TRANS plasma protein binding are species- and age-dependent, and identify factor(s) responsible for difference(s).

Although acute toxicity of these compounds is well characterized, remarkably little is known about the plasma protein binding of these pyrethroids. It was necessary to develop an assay for plasma proteins and lipoprotein binding of pyrethroids, as they are too lipophilic for standard binding techniques (e.g., equilibrium dialysis, ultrafiltration) employing aqueous media. It was found that the parent compounds were freely soluble in glycerol formal, but precipitated when water was added. A significant portion (~ 55%) of the compounds adhered to plastic vials.

To avoid non-specific binding glass vials were used. A three step solvent extraction method was developed and validated to determine the plasma protein binding of these compounds. The procedure produced high recoveries (>95%) and good both inter- and intraday precision (below 15 % R.S. D.). Eliminating dialysis membranes and plastic enabled us to overcome the nonspecific binding that often serves as a hindrance to measure true plasma protein binding of these highly lipophilic compounds. The suitability of this method was examined through measurement of plasma protein binding values for two well-studied drugs in the therapeutic range. Diazepam and cyclosporine were selected based on their binding properties in the plasma. Although both diazepam and cyclosporine are highly bound to plasma proteins, diazepam primarily binds to albumin whereas cyclosporine binds primarily to lipoprotein. Solvent extraction method proved efficient and good correlations with literature values were obtained for diazepam and cyclosporine. This method has enabled our laboratory to characterize the plasma protein binding in both human and rat plasma.

Albumin and lipoproteins are important determinants of plasma protein binding of hydrophobic drugs. Albumin differs in amino acid sequence and protein structure between humans and rats. Further the distribution and composition of lipoproteins are different in rat and human plasma. These changes might cause species difference in binding of these pyrethroids. Therefore, we investigated the species differences in the binding of DLM, CIS and TRANS to plasma proteins. Albumin is the major plasma protein and lipoproteins interact with hydrophobic dugs. Hence, we also determined relative contribution of plasma albumin and lipoproteins in the transport of DLM, CIS and TRANS in blood.

All three pyrethroids exhibited similar binding properties in human and rat plasma. DLM, CIS and TRANS binding were linear over ranges of 250 nM –750 nM in adult human and rat

plasma. Binding was inversely proportional to concentration >750 nM. We found that DLM, CIS and TRANS were bound to both albumin and lipoproteins in the plasma. The binding of DLM, CIS and TRANS to human and rat plasma was quantitatively different. The binding of all three compounds were higher in human than in rat. In human plasma total binding of DLM, CIS and TRANS were in the range of 88-90 % in concentrations below 750 nM. However, in adult rat, total binding of all three compounds were in the range of 76-83 %. Rat plasma exhibited approximately twice the free fraction when compared to human plasma. Although the levels of DLM, CIS and TRANS distributed to lipoproteins were slightly higher in human than rat plasma, they were not significantly different from each other.

All three pyrethroids were bound more to albumin (approximately 54-60%) compared to lipoproteins (30-35%) in both human and rat plasma at concentrations below 750 nM. However, at highest concentration (100 µM) there was an equal distribution of all three compounds to albumin and lipoprotein fraction. This indicates that at lower concentrations lipoproteins have to compete with albumin for binding to pyrethroids. At higher concentration lipoproteins might play more important role in transport of these compounds in vivo. The higher free fraction in rat plasma might result in greater toxicity of DLM, CIS and TRANS in rats.

There is a great deal of concern that exposure of infants and children to pyrethroids may result in acute toxicity and possible neurological dysfunction later in adulthood. The therapeutic and toxic effects of a drug are often linked to the amount of free drug in the blood. The plasma protein binding of a drug is dependent on several factors, age is an important one. To understand effect of maturity on plasma protein binding of these compounds, we conducted studies in four different age groups (PND 10, 15, 21 and 90) in rats and seven different age groups (birth-1week, >1week-4week, >4weeks-1year, >1year-3years, >3years-6years, >6years-18years and

adult) in humans. In all age groups of rat and human binding were linear over the concentration of 0.25 µM to 0.75 µM and inversely proportional to concentrations >0.75µM.

We also observed significantly lower albumin levels in PND 10 and 15 from adult plasma in rat. Total proteins levels were significantly higher in PND 90 compared to PND 10, PND 15 and PND 21. Similarly, albumin levels in birth-1 week and >1 week-4 weeks were significantly lower from adults. Total proteins levels in birth-1 week, > 1 week-4 weeks, and > 4 weeks-1 year were significantly lower than adult levels. In rat plasma we observed a significant difference in total binding of all three compounds in PND 10 (70-73%) and PND 15 (70-75 %) from PND 90 (77-83 %) at concentrations lower than 750 nM. However, no significant differences in binding of all three compounds were observed between PND 21 (78-80 %) from PND 90 plasma. Binding of all three compounds to albumin was significantly lower at PND 10 (35-36 %) and PND 15 (39-42 %) from PND 90 (47-50%). Lipoprotein binding of DLM, CIS and TRANS was significantly higher in PND 10 (35-36 %) from PND 90 (28-31%).

Similarly, in human plasma total binding of all three compounds in birth-1 week (74-77%) and >1 week-4 weeks (75-77%) were significantly different from the adult at concentration below 750 nM. However, after 4 weeks no significant difference in binding of all three compounds was observed in other age groups from adult plasma. Binding to albumin for all three compounds was significantly lower at birth-1 week (27-30%) and >1 week-4 weeks (45-48%) from adults. No significant difference in binding of all three compounds to lipoproteins was observed in any other age groups in human plasma.

It is widely accepted that in newborn rats and humans, several factors like quantity and quality of albumin, plasma lipoproteins, and endogenous substances (bilirubin and free fatty

acids) may result in reduced plasma protein binding. Therefore, it was not surprising to see a higher free fraction in PND 10, and PND 15 in rats and birth-1 week, >1 week-4 weeks in humans from their respective adult levels. In rats although levels of albumin and total proteins at PND 21 were lower by ~ 23% and ~ 32%, respectively, from the adults, the binding had reached the adult levels. This suggests that by PND 21 the rat plasma attains the adult properties. No difference in binding of all three compounds from adult was observed in any age groups after 4 weeks of age in humans. Several previous studies with other compounds have shown similar binding in children and adults. Thus it is reasonable to conclude that higher free fractions of DLM, CIS and TRANS at PND 10, PND 15 in rats and birth-1 week, >1 week-4 weeks in humans may result in greater toxicity in these age groups.

Saturation binding studies were conducted in human and rat plasma to determine the values of Bmax (maximum binding capacity) and Kd (dissociation constant) for all three compounds in all age groups. There were significant difference in Bmax for all three compounds between adult human and rat at PND 90. Kd values were similar in both adult human and PND 90 rat plasma. Bmax values in rat were significantly lower in PND 10 and PND 15 as compared to PND 90. In human plasma Bmax values were significantly lower in birth-1 week and >1 week-4 weeks as compared to adults. Kd values were similar in all age groups of rat. However, Kd values for all three pyrethroids in birth-1week were significantly different as compared to adults. The binding parameters obtained in our study will be used for development of a physiologically-based pharmacokinetic model for children's risk assessment.

Plasma protein binding data along with other parameters such as volume of distribution and clearance are used to determine the dosing regimen of a drug. However, limited studies are conducted in newborns, infants and children due ethical considerations and small volumes of

available samples. We were fortunate to have enough plasma volume to conduct plasma protein binding studies for two drugs. We used micro equilibrium dialysis technique to determine the plasma protein binding of diazepam and cyclosporine in human plasma in different age groups. These drugs were chosen for their ability to bind extensively and reversibly to different plasma components. Diazepam binds mainly to albumin, whereas cyclosporine primarily binds to lipoproteins. We observed the free fraction of diazepam was significantly higher in infants up to 1 year of age as compared to adults. The percent free fraction of cyclosporine was significantly higher the younger age brackets. The binding of cyclosporine in children older than 3 was not significantly different from binding in adult plasma. As explained earlier lower albumin, lipoproteins and total protein levels as well as the presence of endogenous substances like bilirubin and free fatty acids might be responsible for higher free fraction of diazepam. The binding characteristics of diazepam and cyclosporine were different from pyrethroids. Binding of diazepam at > 4 weeks – 1 year age was significantly different as compare to adult. Similarly binding of cyclosporine at > 1 year – 3 years age was significantly different as compared to adult. This might be due to the exclusive binding of diazepam (> 90%) and cyclosporine (> 90%) to albumin and lipoprotein respectively. However, pyrethroids are bond ~55 % to albumin and ~35 % to lipoproteins.