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Plasma Protein and Lipoprotein Binding of cis- and trans-Permethrin and Deltamethrin in Adult Humans and Rats

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Running Title: Binding of Pyrethroids to Human and Rat Plasma Proteins

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Non-standard Abbreviations:
cis-permethrin (CIS), trans-permethrin (TRANS), deltamethrin (DLM), National Health and Nutrition Examinaton Survey (NHANES), Persistent Organic Pollutant (POP), 3-Phenoxybenzoic Acid (3-PBA)

Standard Abbreviations: HSA, DMSO, GF, HDL, LDL, VLDL

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ABSTRACT

The majority of residents of the U. S., Canada and Europe are exposed to pyrethroids, the most commonly used class of insecticides. Surprisingly little is known about key aspects of their pharmacokinetics, including their mode of transport in the systemic circulation. This study tested the hypothesis that pyrethroids are transported by both plasma lipoproteins and proteins, similarly to other highly lipophilic environmental contaminants. Other aims were to characterize the binding of representative Type I and II pyrethroids, and to compare their binding to rat versus human plasma. Binding of ^{14}C-labeled cis- and trans-permethrin (CIS and TRANS) and deltamethrin (DLM) to proteins and lipoproteins was measured by sequential extraction of spiked plasma with isooctane, 2-octanol and acetonitrile. Binding of DLM, CIS and TRANS to plasma proteins and lipoproteins was linear from 250-750 nM, concentrations present in the plasma of orally dosed rats. Binding of DLM to high-density lipoprotein was twice that to low-density lipoprotein. Binding of DLM, CIS and TRANS was ~2-fold greater to proteins than to lipoproteins of rat and human plasma. Albumin was primarily responsible for protein binding. Higher total binding of each pyrethroid to human (~90%) than to rat (~80%) plasma resulted from higher protein binding in human plasma. This was attributable, in part to the higher albumin/protein content of human plasma. Rat albumin exhibited lower pyrethroid binding capacity than did human albumin. Results of this investigation indicate that albumin and lipoproteins play a major role in binding and transport of pyrethroids in the systemic circulation of both rats and humans.

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INTRODUCTION

Pyrethroids are the most commonly used insecticides in the U.S., Canada and much of Europe, since the phase out of organophosphates (Williams et al., 2008). Most pyrethroids have relatively low mammalian toxicity, and as such are widely utilized for indoor pest control. They are also applied in a variety of urban structural and landscaping settings, as well as agriculturally to a number of food crops. The chemicals are found in very low levels in some fruits, vegetables and grains (Lu et al., 2010). Pyrethroids are used in human and veterinary medicine as a pediculicide (Burgess et al., 2010; Frankowski and Bucchini, 2010). Members of this chemical class are also used to treat livestock and crops during their storage and transportation.

In light of the foregoing, it is not surprising that large segments of the U.S. and European populations are exposed to pyrethroids, albeit at quite low levels (Saillenfait et al., 2015). Barr et al. (2010) found 3-phenoxybenzoic acid (3-PBA), a metabolite common to many pyrethroids, in the urine of 70% of 5,046 persons ≥ 6 years old in the general U.S. population. Urine specimens from children contained higher PBA levels than did adult specimens. Morgan et al. (2012) summarized data from 15 published studies of pyrethroid exposure of children in homes and day-care centers. Permethrin was the most commonly detected pyrethroid, followed by cypermethrin. Permethrin (PER), a mixture of its cis (CIS) and trans (TRANS) isomers, is the most-widely used insecticide in household settings in the U.S.

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Pyrethroids, like several other classes of insecticides, are acute neurotoxicants. Tremors and paresthesia are elicited by high doses of PER and other Type I pyrethroids. Type II pyrethroids, such as deltamethrin (DLM) and cypermethrin, contain a cyano group. Type IIs are generally more acutely toxic and in sufficient doses can produce salivation, hyperexcitability and choreoathetosis. The primary mechanism of action of both types is interference with neuronal voltage-gated calcium and sodium channels (Cao et al., 2011; Soderlund, 2012). The parent compounds are the proximate neurotoxic moieties.

There is increasing concern that long-term, low level pyrethroid exposure may lead to adverse neurodevelopmental effects in children. Associations between pyrethroid exposure and neurobehavioral disorders have been described in reports of epidemiology study findings by several research groups (van Wendel de Joode et al., 2016; Viel et al., 2015; Wagner-Schumann et al., 2015; Richardson et al., 2015). Mice exposed to DLM during gestation and lactation developed several features of attention deficit hyperactivity disorder (Richardson et al., 2015).

There have been several definitive investigations of the metabolism and tissue deposition of pyrethroids, but relatively little information is available on their transport in the systemic circulation. The information published on their binding to plasma proteins is contradictory. Abu-Quare and Abu-Donia (2002) found no significant interaction of PER with human serum albumin (HSA). Cui et al. (2006), however, reported that cypermethrin could bind to bovine serum albumin and hemoglobin. CIS and TRANS distributed evenly between plasma and erythrocytes, in the physiologically/toxicologically relevant concentration range (Amaraneni et al., 2017). Sethi et al. (2016) observed substantial binding of DLM to adult human plasma albumin and lipoproteins. As pyrethroids are highly lipophilic, it is reasonable to assume they partition into chylomicrons and lipoproteins. Early in vitro and in vivo studies of lipophilic, persistent organic pollutants (POPs), such as benzo (a) pyrene, DDT and

hexachlorobenzene (Vost and McLean, 1984; Becker and Gamble, 1982) revealed that lipoproteins and albumin participated in transporting the pollutants in the blood of humans and laboratory animals. As most pyrethroids and POPs have very high log P values, it can be hypothesized that CIS, TRANS and DLM are also transported in the bloodstream by both albumin and lipoproteins.

The primary objectives of the current investigation were to test the foregoing hypothesis, to characterize the nature of the binding of representative Type I and II pyrethroids, and to contrast their binding in rat and human plasma. The extent of binding of highly-bound chemicals can significantly influence their toxic potential and systemic clearance. Only the unbound fraction is free to diffuse from the bloodstream to peripheral sites of action, metabolism and elimination (Yamasaki et al., 2013). Recent experiments revealed higher uptake of DLM into the brain of anesthetized rats when carotid artery perfusate contained low levels of HSA (Amaraneni et al., 2016). An important aim of the current study was to elucidate and compare key binding parameters of CIS, TRANS and DLM. These parameters are necessary for prediction of plasma and brain pyrethroid concentrations by physiologically-based pharmacokinetic (PBPK) models.

Materials and Methods

Chemicals. ^{14}C-Deltamethrin (DLM) (99% purity) (54.1 mCi/mmol) was kindly supplied by Bayer Crop Science (Monheim, Germany), as was unlabeled analytical standard DLM (99.4% purity). ^{14}C- Permethrin (50:50 mixture of cis & trans) (61 mCi/mmol) was furnished by FMC Agricultural Products (Princeton, NJ, USA). The ^{14}C- permethrin was separated into its cis (CIS) and trans (TRANS) isomers, each of 61 mCi/mmol and 99% purity, by Symbiotic Research (Mount Olive, NJ, USA). Unlabeled analytical standard-grade CIS and TRANS of 99.3 and 99% purity were provided by FMC Agricultural Products (Princeton, NJ, USA). The chemical names, logP values, structures and ^{14}C- labeling positions of the three pyrethroids are shown in Fig. 1.

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Purified human serum albumin (HSA), dimethyl sulfoxide (DMSO), acetonitrile (ACN) (HPLC-grade), hexamethyldisilazane (HMDS) (reagent-grade) and sodium fluoride (NaF) and potassium bromide (KBr) (purity 99%) were purchased from Sigma Aldrich (St. Louis, MO, USA). Isooctane (99% purity) and 2-octanol (laboratory grade) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Frozen heparinized, pooled, adult human and rat plasma were provided by Innovative Research (Novi, MI, USA). The plasma was stored at -80°C until use. Glycerol formal (GF) (99+%) was purchased from Acros Organics (Geel, Belgium).

Measurement of Plasma Albumin and Total Proteins. Albumin levels in the pooled human (N=3) and rat (N=3)plasma were measured by use of a commercially available kit (QuantiChrom® BCG Albumin Assay Kit; BioAssay Systems, Hayward, CA, USA) according to the manufacturer's instructions. Total protein estimation of pooled human and rat plasma were performed using a BCA protein assay kit (Pierce/Thermo Scientific, Rockford, IL, USA) by following the directions of the manufacturer. **Plasma Protein and Lipoprotein Binding of DLM, CIS and TRANS.** Traditional methods to measure protein binding could not be used, due to the pyrethroids' very high lipophilicity. Poor solubility of pyrethroids in aqueous buffers that serve as a mobile phase, coupled with adherence, or nonspecific binding to glass, polymers, metals and filter membranes proved problematic. In order to minimize such difficulties, Sethi et al. (2014) developed a serial solvent extraction technique described below. Lobind® plastic pipette tips (Eppendorf, Hamburg, Germany) were used to avoid adherence. Furthermore, all clean glassware was silanized with 5% HMDS in toluene for at least 24 hours prior to the start of experiments.

Binding of DLM, CIS and TRANS to rat and human plasma components was quantified by the 3-step organic solvent extraction method developed by Sethi et al. (2014). Stock solutions of each unlabeled pyrethroid were prepared with DMSO. Appropriate quantities of ^{14}C-labeled DLM, CIS and TRANS were mixed with the respective stock solution to achieve final concentrations of 250 nM to 100

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µM. Aliquots of 80 µl of filtered human or rat plasma were spiked with 10 µl of each concentration of each pyrethroid in silanized glass vials. These samples were immediately combined with 10 µl of 0.64 M NaF to inhibit carboxylesterases (CaEs) and incubated in an orbital shaker run at 110 rpm for 3 hours at 37°C (N=4 per concentration). Binding of all three pyrethroids reached equilibrium within 3 hours. Each sample was then vortexed for 30 seconds with 200 µl of isooctane. The isooctane layer was removed, mixed with 3 ml of scintillation fluid, and counted in a Beckman Coulter LS 6500. The radiolabeled pyrethroid present in the isooctane was considered the unbound fraction. Samples were subsequently extracted in turn with 200 µl of 2-octanol and 200 µl of ACN. The amounts of radiolabel present in the 2-octanol and ACN extracts were considered the lipoprotein- and protein-bound pyrethroid fractions, respectively.

Albumin Binding of DLM, CIS and TRANS. The extent of binding of each pyrethroid to purified adult HSA was measured with the same 3-step solvent extraction procedure describe above. HSA (4 g/dl) was spiked with each radiolabeled pyrethroid to yield a 250 nM solution. These were immediately treated with 0.64 M NaF to inhibit serum CaEs, and incubated in an orbital shaker at pH 7.4 for 3 hours at 37°C (N =4). The solutions were extracted in turn with isooctane, 2-octanol and ACN. Total radioactivity in the ACN was determined by liquid scintillation counting and assumed to represent the pyrethroid bound to albumin.

Animal Maintenance and Dosing. Adult male CD Sprague-Dawley rats of ~250 g were purchased from Charles River Laboratories (Raleigh, NC, USA). The protocol for this study was approved by the University of Georgia Animal Care and Use Committee. The rats were housed in polycarbonate cages in an AAALAC-approved animal facility with a 12-hour light/dark cycle at 22 ± 2°C and 55 ± 5% relative humidity. Purina Irradiated Lab Diet 5053 (Brentwood, MO, USA) and tap water were provided ad libitum for an acclimation period of at least 10 days. DLM was diluted with GF, such that a dose of 30 mg DLM/kg body

weight could be administered orally in a total volume of 1 ml/kg. The solution was given by gavage with a curved, ball-tipped intubation needle. The rats were sacrificed 3 hours post dosing and blood collected by closed-chest cardiac puncture. Plasma was obtained by centrifugation, pooled and frozen at -20°C until analysis.

Binding of DLM to Lipoprotein Fractions. Pooled plasma was obtained as described previously from rats given 30 mg DLM/kg orally. High-density, low-density and very-low-density lipoprotein (HDL, LDL and VLDL, respectively) fractions were isolated from aliquots of the pooled plasma by a standard KBr density gradient ultracentrifugation procedure (Chapman et al., 1981). The DLM concentration in each fraction was measured by gas chromatography-negative chemical ionization mass spectrometry (Gullick et al., 2014). The final concentrations were expressed as ng DLM/ml.

Data Analyses. Binding capacity (Bmax) and dissociation constant (Kd) were determined using nonlinear regression analysis of saturation binding curves (Prism 6 GraphPad Software, Inc., San Diego, CA). Data were fitted with the following equation:

$$B = (Bmax*C)/(Kd + C)$$

where Bmax is the maximum number of binding sites, expressed as nmol bound per gm of protein, C is the pyrethroid concentration and Kd is the equilibrium dissociation constant. Kd is the inverse of the binding affinity (Ka).

The statistical significance of differences in mean values was assessed with a two-way analysis of variance test, followed by Tukey's Multiple Comparison Test ($p < 0.05$) or by use a Student's t-test ($p<0.05$) using Prism 6 (GraphPad Software, Inc., San Diego, CA)

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Results

Plasma Albumin and Total Protein Levels. Albumin and total protein concentrations in pooled human and pooled rat plasma were analyzed in triplicate. The mean albumin concentrations in the adult human and rat plasma were 4.0 ± 0.2 and 3.0 ± 0.2 g/dl (mean ± S. D.), respectively. Mean total protein concentrations in human and rat plasma were 6.4 ± 0.5 and 5.4 ± 0.3 g/dl (mean ± S. D.), respectively. The human albumin and total protein values were significantly higher than those for rats. Albumin comprised ~62% of total human plasma proteins versus 55% of rat plasma proteins.

Plasma Binding of Pyrethroids. Total binding of a wide range of concentrations of DLM, CIS and TRANS to human plasma is illustrated in Fig. 2A. It is apparent in the figure insert that the extent of binding of the three pyrethroids is quite similar and linear over the concentration range of 250-750 nM. Some 90% of each pyrethroid was bound at 250 nM, a concentration found in plasma of rats given quite low oral doses of the insecticides (Kim et al., 2008; Tornero-Velez et al., 2012). Fraction unbound steadily increased as pyrethroid concentrations exceeded 750 nM indicating a shift from linear to nonlinear binding. The extent of binding of the three pyrethroids is comparable at higher concentrations, except at 25 and 50 μM, where the bound fraction of TRANS exceeded those of CIS and DLM by ~10%.

Total binding of DLM, CIS and TRANS to rat plasma is shown in Fig. 2B. As with human plasma, binding of all three pyrethroids is linear from 250-750 nM. Some 80% of each compound is bound in this concentration range which has been observed in vivo (Kim et al., 2008; Tornero-Velez et al., 2012). The unbound fractions of DLM, CIS and TRANS progressively increase in parallel with increasing concentrations ≥ 1 μM which is indicative of nonlinear binding in the plasma. Binding of CIS is significantly lower than that of TRANS and DLM at 5, 10, 25 and 50 μM.

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Saturation binding curves for DLM, CIS and TRANS for human and rat albumin and total protein are presented in Fig. 3, and Bmax and Kd values are given in Table 1. Bmax values for human albumin and protein were consistently higher than for rat albumin and protein for all three compounds. Human and rat albumin had the lowest binding capacity for DLM. Binding capacity for CIS was approximately 10% (human) and 20% (rat) lower than for TRANS. The dissociation constant for DLM was significantly lower than for CIS and TRANS for both rat and human albumin, and total protein indicating that DLM has a higher binding affinity for albumin. No significant difference in Kd values was noted for CIS and TRANS.

Plasma Protein and Lipoprotein Binding of Pyrethroids. DLM, CIS and TRANS were found to bind similarly to proteins, as well as to lipoproteins of human plasma. Binding of each test compound was approximately 2-fold greater to proteins than to lipoproteins at the lowest pyrethroid concentration evaluated. It can be seen in the figure inserts that the proportion of DLM (Fig. 4A), CIS (Supplemental Data) and TRANS (Supplemental Data) bound to proteins progressively diminished with increase in pyrethroid concentration. It is apparent that the decrease in % bound to lipoprotein is less pronounced than the decrease in % bound to protein with the 40-fold increase (0.25 -10 μM) in concentration. The binding to lipoproteins is approximately 30% in humans and rats until concentrations exceed 10 μM and 5 μM, respectively. Nonlinear binding to albumin is observed in both species when concentrations exceed 1 μM. The extent of both lipoprotein and protein binding drops substantially with a further 10-fold increase (10 -100 μM). These concentrations are lethal and far in excess of what would be encountered in vivo.

The three test compounds were also much alike in their binding to rat plasma proteins and lipoproteins. The percentages of DLM (Fig. 4B), CIS (Supplemental Data) and TRANS (Supplemental Data) bound to proteins at 250 nM were about 1.7-fold higher than that bound to lipoproteins (Fig. 4B). As with human plasma, decrease in the extent of binding to lipoprotein was less pronounced than the

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decrease in protein binding with the increase in concentration from 0.25 - 10 μM. The extent of binding to protein and lipoprotein was comparable at 100 μM, the highest pyrethroid concentration tested. Protein binding was inversely proportional to concentration over the wide range from 750 nM to 100 μM. Percent bound to lipoproteins was inversely proportional to concentration when concentrations exceeded 10 μM in humans and 5 μM in rats.

The binding characteristics of a toxicologically-relevant range of concentrations of DLM (250-750 nM), CIS and TRANS were comparable. The pyrethroids were bound primarily (50-60%) by plasma proteins, although a substantial (30-35%) was associated with the lipoprotein fraction. The pattern of distribution was consistent for all three pyrethroids examined in the concentration range of 250-750 nM. Binding of DLM in this range is shown in Fig. 5A, while binding of 250 nM of each compound is shown in Fig. 5B. Relative binding to human and rat plasma of 250 nM DLM, CIS, and TRANS is presented in Fig. 6. In human plasma 10-12% of each pyrethroid in this linear concentration range was found to be unbound, 28–33% associated with lipoproteins, and 58-60% bound to proteins. Protein binding of 750 nM DLM appeared to be slightly lower and lipoprotein binding slightly higher than for 500 nM DLM, although the apparent difference in lipoprotein binding was not statistically significant (Fig. 5A). The unbound fraction of each pyrethroid was approximately twice as high in rat as in human plasma (Fig. 6). In rat plasma the unbound fraction of the pyrethroids ranged from 17–24%. The fractions bound to lipoproteins and proteins were 28–31% and 47–50%, respectively. Binding of each pyrethroid to purified human serum albumin consistently appeared to be slightly lower than to total human plasma proteins, though the differences were not significant (Fig. 5B).

Binding of DLM to Lipoprotein Fractions. DLM was found to be associated largely with the HDL fraction of plasma from DLM-dosed rats. Two-ml aliquots of each lipoprotein fraction were divided into 1-ml portions for analysis of their DLM content. DLM concentrations in the two HDL aliquots were 24.8 and

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21.4 ng/ml. DLM concentrations in the two LDL aliquots were 9.5 and 10.1 ng/ml. DLM was not detected in the VLDL aliquots.

DISCUSSION

Findings in this investigation demonstrate that ~90% of physiologically-relevant concentrations of DLM, CIS and TRANS bind to human versus ~80% to rat plasma. Binding of each pyrethroid is linear in the concentration range of 250-750 nM. Peak plasma levels of 500-1,000 nM were measured in rats gavaged with 10 mg DLM or PER/kg body weight (Kim et al., 2008; Tornero-Velez et al., 2012). The 10 mg/kg dose of DLM produced only mild, transient salivation in adult rats, but tremors and death in pups (Anand et al., 2008). As would be anticipated, the percent bound was inversely proportional to pyrethroid concentration over the very wide range of concentrations used in the current study. The fraction of each compound bound to plasma proteins/lipoproteins progressively decreased as its concentration exceeded the linear binding range. The distribution of each pyrethroid in the plasma also changed. The ratios of DLM, CIS and TRANS bound to human plasma proteins versus lipoproteins diminished from approximately 2:1 at 0.25 μM to 1:1 at 100 μM. This shift reflected saturation of protein binding in the higher, non-physiological range of the pyrethroid concentrations examined. Under these extreme conditions, the lipoprotein fraction appeared to serve as a “reservoir” with a finite capacity to accommodate these highly lipophilic compounds. Modest, but statistically significant differences in binding of TRANS (human) and CIS (rat) occurred at the higher concentrations. This is consistent with TRANS exhibiting a higher Bmax than DLM and CIS in human plasma and CIS having a lower binding affinity and Bmax in rat plasma.

The current data indicate that albumin is primarily responsible for protein binding of pyrethroids in adult plasma. The extent of binding of DLM, CIS and TRANS to purified HSA approached that to total human plasma protein (Fig. 5B). In an early paper, Helmer et al. (1968) reported that the binding of a

wide variety of organic compounds to bovine serum albumin (BSA) correlated well with their octanol: water partition coefficient. Becker and Gamble (1982) described a cylinder-shaped "hole" in BSA lined with hydrophobic residues that interacted with hexachlorobenzene, a highly lipophilic POP. Hydrophobic binding, or interaction at this site was reported to be non-specific, yet effective and slowly reversible. Albumin exhibited the highest affinity of any component of human serum for DDT and dieldrin in vitro (Skalsky and Guthrie, 1978). The binding was demonstrated to be of low affinity, slowly reversible and hydrophobic in nature. It is generally accepted that HSA has two primary binding sites, as well as numerous secondary binding sites distributed across the molecule (Yang et al., 2014; Pongprayoon and Gleeson, 2014). Primary binding site I preferentially binds bulky heterocyclic drugs (e.g., phenylbutazone, warfarin) with a delocalized negative charge, although hydrophobic interactions occur there. A large hydrophobic cavity, that accommodates lipophilic ligands, is present in subdomain IIa (Ghuman et al, 2005; Sulkowska, 2002). It appears likely that pyrethroids will interact similarly with this site.

As noted earlier, the previously published information about binding of pyrethroids to plasma proteins is conflictual. Cui et al. (2006) quantified fluorescence quenching and enhancement as their index of protein binding. They reported that cypermethrin was bound extensively to BSA and less so to bovine hemoglobin. Abu-Quare and Abou-Donia (2002), however, concluded that PER did not interact significantly with HSA. They incubated PER with HSA for 1 hour, after which they added ACN to precipitate the proteins. Their ACN supernatant contained 93% of the spiked PER, which they assumed was unbound. Sethi et al. (2014), however, determined that ACN disrupts protein binding of pyrethroids, releasing them so they are effectively extracted and represent what was formerly bound. Sethi et al. (2016), in a subsequent study of the ontogeny of binding of xenobiotics to human plasma, observed that protein and lipoprotein binding accounted for 90% of DLM in adult plasma.

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Findings in the current investigation indicate that DLM, CIS and TRANS are transported in the bloodstream by both plasma proteins and lipoproteins. Since these compounds distribute evenly between plasma and red blood cells (Amaraneni et al. (2017), erythrocytes also play an important role in pyrethroids' disposition. The extent of binding of the insecticides to proteins (i.e., 50-60%) was higher, but the fraction associated with lipoproteins was substantial (i.e., 30-35%). DLM was primarily associated with HDL isolated from plasma of rats dosed orally with 30 mg DLM/kg. DLM levels were ~2-fold higher in the HDL than the LDL fraction. DLM was not detectable in VLDL. Findings of several research groups have demonstrated that other very lipophilic chemicals interact with apolipoproteins, rather than merely partitioning into lipoprotein triglycerides and cholesterol (Hjelmborg et al., 2008). Becker and Gamble (1982) described several nonspecific binding sites on human LDL for hexachlorobenzene. Spindler-Vomachka et al. (1984) observed a marked shift of ^{14}C-hexachlorobiphenyl from LDL to HDL within 1 to 3 hours in rats injected i.v. with the POP. Vost and McLean (1984) reported HDL to be the major accepter of DDT and benzo (a) pyrene in rats fed the lipophiles in chylomicrons. Gomez-Catalan et al. (1991) pointed out that binding of POPs to lipoproteins was a dynamic process influenced by apolipoproteins, the lipid core composition, and lipoprotein turnover and abundance. It is to be expected that lipoproteins serve as a mode of transport for pyrethroids and POPs, as they do for fatty acids, cholesterol, androgens and other endogenous lipophiles. In the present study, the extent of distribution of each of the three pyrethroids was quite similar to the lipoprotein fraction. It is noteworthy that their chemical structures and logP values are also very similar (Fig. 1).

The patterns of distribution of the three test chemicals between plasma proteins and lipoproteins were qualitatively similar in both species, though there was a quantitative difference in protein binding. The unbound fraction of each pyrethroid was ~2-fold higher in rat plasma, whereas the extent of binding to rat and human lipoproteins was similar. Higher total binding to human plasma was the result of higher protein binding, which in turn can be attributed in part to the higher albumin and

total protein levels. Rat albumin and total protein also had lower Bmax values than human albumin and protein, indicating lower pyrethroid binding capacity and lower plasma protein binding. Despite their structural similarities, Mandula et al. (2006) described different binding characteristics of rat and human albumins. Human albumin Site II bound lipophilic ligands (e.g., diazepam, ibuprofen) more strongly by hydrophobic interactions. Despite similar amino acid sequences, the tertiary structure of rat albumin differs from human albumin, in that the former lacks a deep hydrophobic cleft (Kosa et al., 1997). Thus, hydrophobic interactions with ligands such as diazepam are weaker with rat albumin, resulting in somewhat lower binding capacity (i.e., 92% for rats versus 98% for humans) (Laznicek et al., 1982). Our results showed that protein binding of DLM, CIS and TRANS was also modestly, but consistently higher to human plasma protein. The dissociation constant for DLM was significantly lower than for CIS and TRANS for both rat and human albumin, indicating albumin has and total protein have a higher binding affinity for DLM. Although some of the apparent differences were not sufficient to be statistically significant, CIS exhibited a lower binding capacity and higher dissociation constant than TRANS with rat and human albumin and total protein. A higher unbound fraction of CIS available for brain uptake may contribute to the observation that CIS is more acutely neurotoxic to rats than TRANS (Mortuza et al., 2018).

The plasma binding of highly-bound compounds can significantly influence their disposition, and in turn their pharmacological and toxicological properties (Yamasaki et al., 2013). It has been observed in the current investigation that some 90% of toxicologically-relevant concentrations of DLM, CIS and TRANS is bound to adult human plasma. Thus, binding of pyrethroids appears to be one determinant of their modest neurotoxic potential. Limited amounts of the lipophilic chemicals are free to diffuse from the blood into the brain and other organs. In addition, pyrethroids are sequestered in adipose tissue and extensively hydrolyzed by CaEs and oxidized by cytochrome P450s in humans and rats (Anand et al., 2006; Ross et al., 2006; Scollon et al., 2009). In vitro experiments with hCMEC cells, a human brain

microvascular endothelial cell line, revealed cellular uptake of DLM was dependent upon the free fraction of the chemical, which was inversely related to the concentration of HSA in the culture media (Amaraneni et al., 2016). It follows that conditions that influence the affinity or quantity of albumin may alter the free fraction, and thus the intensity and/or duration of action of well-bound chemicals. Such conditions include immaturity, malnutrition, dyslipidemia, liver and kidney disease, drug interactions and aging (Anger and Piquette-Miller, 2010; Tesseromatis and Alevizou, 2008; Verbeeck, 2008). Sethi et al. (2016) recently reported significant elevations of unbound diazepam and cyclosporine in plasma of infants younger than 1- 3 years old. Levels of unbound DLM exceeded those in adults for only 4 weeks after birth.

Plasma binding parameters have not previously been available for inclusion in PBPK models for pyrethroids. Mirfazaelian et al. (2006) and Tornero-Velez et al. (2010) simply utilized an erythrocyte: plasma ratio in their modeling of DLM kinetics in adult and maturing rats. Subsequently, erythrocytes and plasma were combined into a whole blood compartment for modeling DLM (Godin et al., 2010) and CIS and TRANS (Tornero-Velez et al., 2012; Willemin et al., 2016) in rats and humans. K_d and B_{max} values, estimated by standard Scatchard analysis, were utilized in a PBPK model of bisphenol A in rats and humans (Teeguarden et al., 2005). Inclusion of the binding parameters was necessary for accurate prediction of dose-dependent estrogenic action in rats. Loccisano et al. (2011) successfully predicted the plasma kinetics of perfluorooctanoate and perfluorooctane sulfonate, highly lipophilic chemicals that are > 97% bound to plasma proteins. It was necessary to estimate the free fraction of each of these chemicals in plasma from kinetic data, in order to obtain the best fits for monkeys and humans. K_d and B_{max} values obtained in the current study for DLM, CIS and TRANS were used to calculate the free fraction of each isomer for PBPK modeling of the pyrethroids in maturing rats (Song et al., 2019).

Authorship Contributions

Participated in research design: Sethi, White, Bruckner, Cummings

Conducted experiments: Sethi, Muralidhara, Mortuza

Performed data analysis: Sethi, White, Bruckner, Muralidhara, Mortuza

Wrote or contributed to the writing of the manuscript: Sethi, Bruckner, White, Cummings

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FOOTNOTE

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Table 1

Albumin and total protein binding in rats and humans

Data are mean ± S.D.

	Albumin				Total Protein			
	Rat		Human		Rat		Human	
	Bmax (nmol/mg)	Kd (μM)	Bmax (nmol/mg)	Kd (μM)	Bmax (nmol/mg)	Kd (μM)	Bmax (nmol/mg)	Kd (μM)
DLM	2.6 ± 0.1^{a,*}	3.8 ± 0.3^{a}	4.0 ± 0.1^{a,*}	4.6 ± 0.3^{a}	3.4 ± 0.4^{a,*}	6.4 ± 0.5^{a}	4.1 ± 0.1^{a}	6.7 ± 0.7^{a}
CIS	3.2 ± 0.7^{a,c,*}	10.3 ± 4.2^{b}	4.5 ± 0.2^{b}	7.7 ± 1.4^{b}	3.7 ± 0.4^{a,*}	12.4 ± 2.6^{b}	4.7 ± 0.2^{b}	10.4 ± 1.5^{b}
TRANS	4.1 ± 0.7^{b,c,*}	8.1 ± 2.8^{b}	4.9 ± 0.1^{c}	6.4 ± 0.6^{b}	4.5 ± 0.1^{b,*}	11.3 ± 0.3^{b,*}	5.2 ± 0.3^{b}	7.3 ± 1.3^{a,c}

Binding capacity (Bmax) is expressed as nmol/mg albumin or total protein

Disassociation constant (Kd) is expressed as μM

Significant differences between pyrethroids are indicated by different subscript letters. $P < 0.05$

* Denotes significant difference from corresponding human value

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FIGURE LEGENDS

Figure 1. Chemical structures and nomenclature for deltamethrin (DLM), cis-permethrin (CIS) and trans-permethrin (TRANS). Asterisk designates position of [^{14}C] incorporation.

Figure 2. Concentration-dependent total binding of DLM, CIS and TRANS to human (A) and rat (B) plasma. The lower, toxicologically-relevant range is expanded in the insert. Symbols represent mean ± S.D. for 4 replicates. Values for each pyrethroid are connected point to point.*Indicates statistically significant difference from other pyrethroid values at the same concentration.

Figure 3. Saturation binding curves for DLM, CIS and TRANS for human (A) and rat (B) albumin. Symbols represent mean ± S.D. for 4 replicates. Solid lines are fitted curves.

Figure 4. Concentration-dependent total binding, protein binding and lipoprotein binding of DLM in human (A) and rat (B) plasma. Insert shows change and relative distribution of DLM over a 400-fold range of concentrations. Statistically significant differences between concentrations are indicated by different superscript letters.

Figure 5. (A) Binding of DLM to human serum albumin (HSA) and to plasma protein and lipoprotein within the linear range of total plasma binding. Asterisk indicates statistically significant difference from binding at 500 nM. % Unbound and % bound to lipoprotein did not vary significantly with DLM concentration (B). 5BRelative binding of 250 nM DLM, CIS and TRANS to human plasma protein, plasma lipoprotein, and purified human serum albumin (HSA). Bar heights represent mean ± S.D. for 4 replicates.

Figure 6. Species-dependent binding of 250 nM DLM (A), CIS (B) and TRANS (C) to plasma protein and lipoprotein fractions. Bar heights represent mean ± S.D. for 4 replicates. Statistically significant species differences are indicated by different superscript letters for corresponding parameters.

Supplemental Data FIGURE LEGENDS

Figure I. Concentration-dependent total binding, protein binding and lipoprotein binding of CIS in human (A) and rat (B) plasma. Insert shows change and relative distribution of DLM over a 400-fold range of concentrations. Statistically significant differences between concentrations are indicated by different superscript letters.

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Figure II. Concentration-dependent total binding, protein binding and lipoprotein binding of TRANS in human (A) and rat (B) plasma. Insert shows change and relative distribution of DLM over a 400-fold range of concentrations. Statistically significant differences between concentrations are indicated by different superscript letters.

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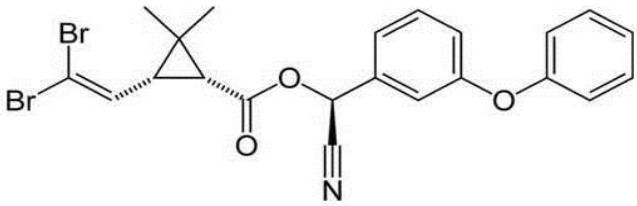
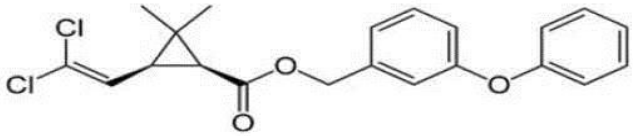
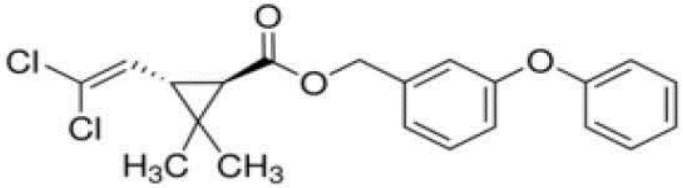


Deltamethrin: (s)-cyano (3-phenoxyphenyl)(^{14}C)methyl (1R,3R)-3-(2,2-dibromovinyl)-2,-2 dimethylcyclopropanecarboxylate; logP=6.1



Cis-Permethrin: 3- Phenoxybenzyl (^{14}C)(1RS)-cis-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate; logP=6.2



Trans-Permethrin: 3-Phenoxybenzyl (^{14}C) (1RS)-trans -3-(2, 2-dichlorovinyl)2,2-dimethylcyclopropanecarboxylate; log P=5.8

Figure 1

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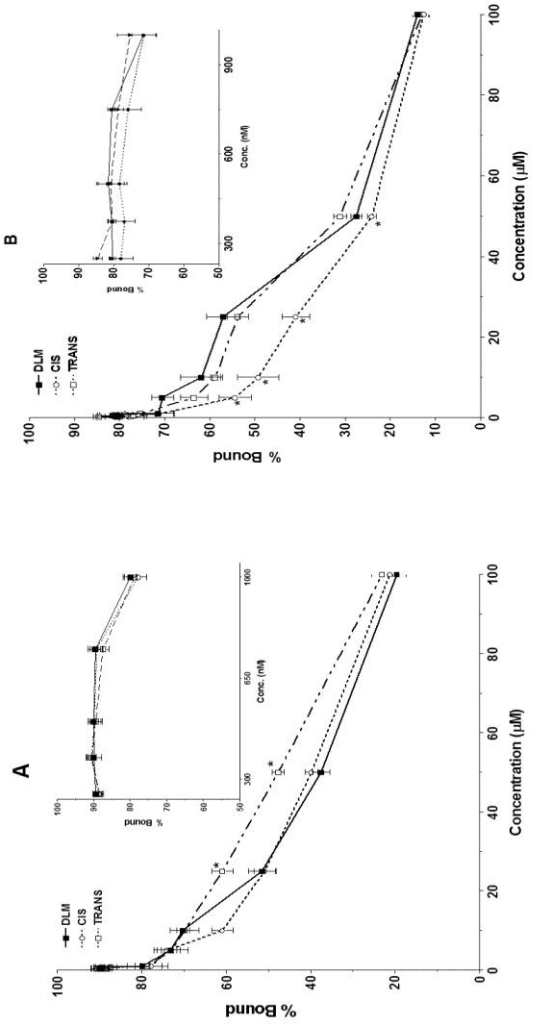

Figure 2

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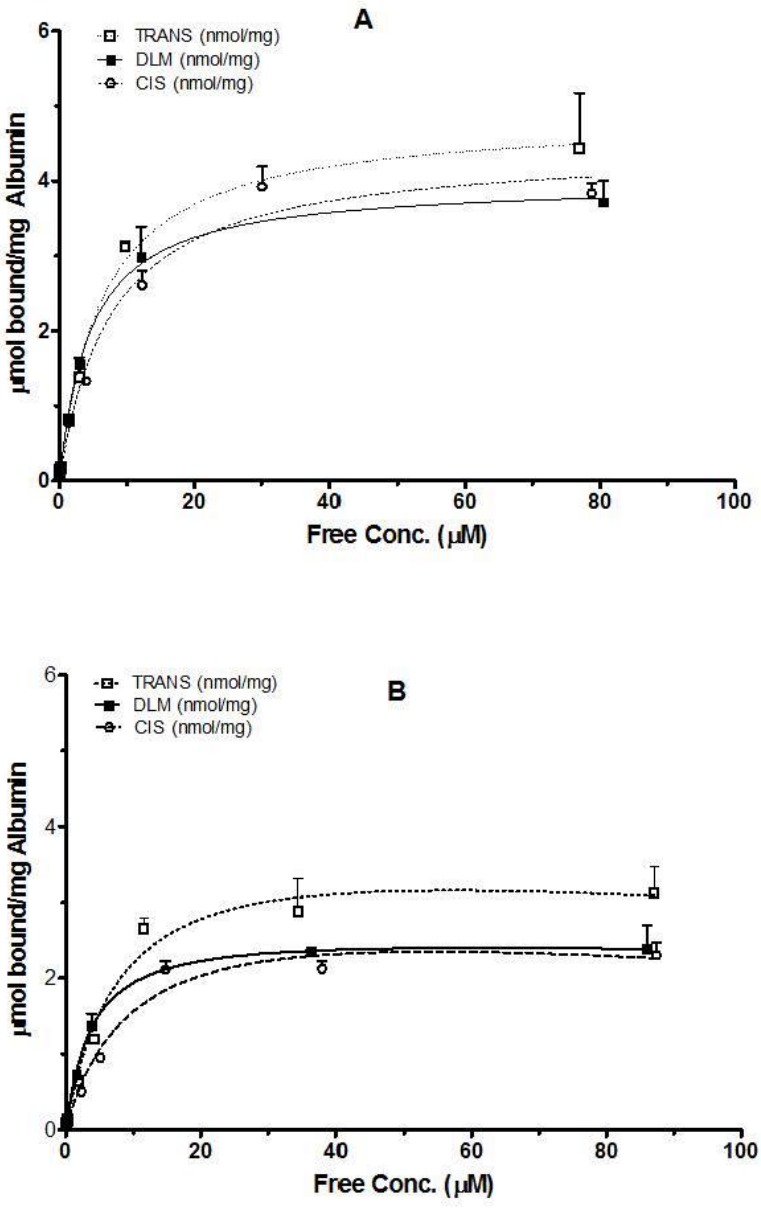

Figure 3

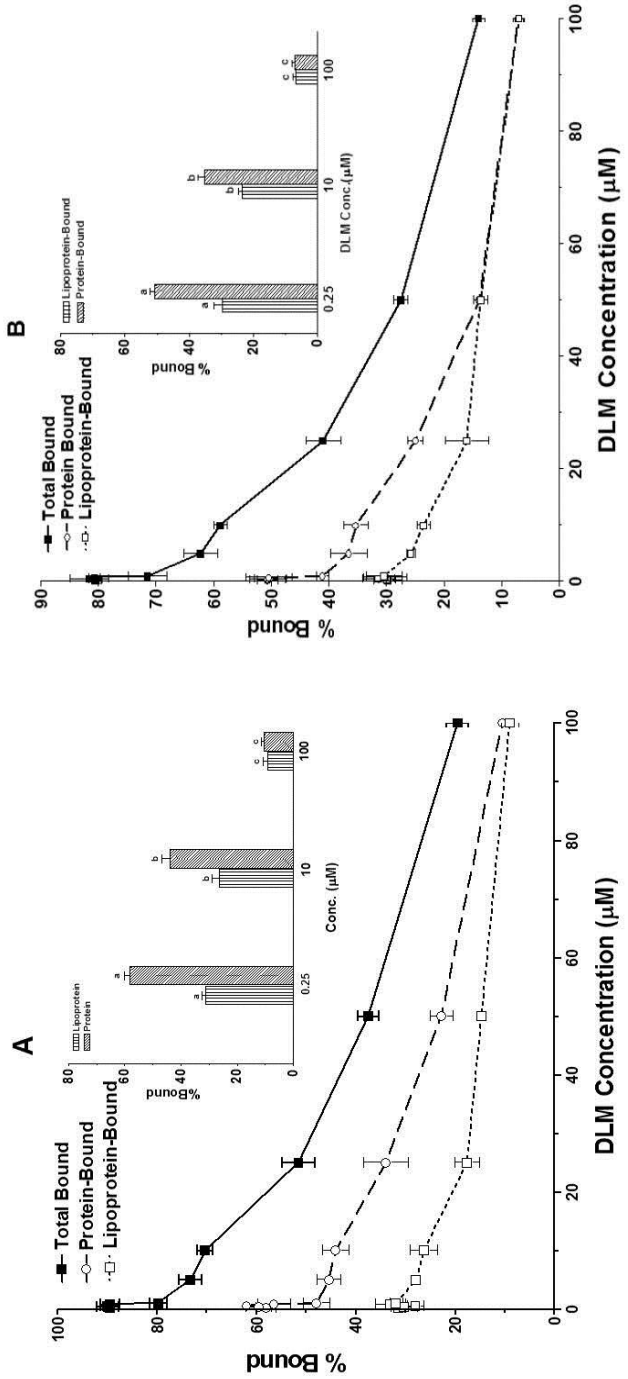

Figure 4

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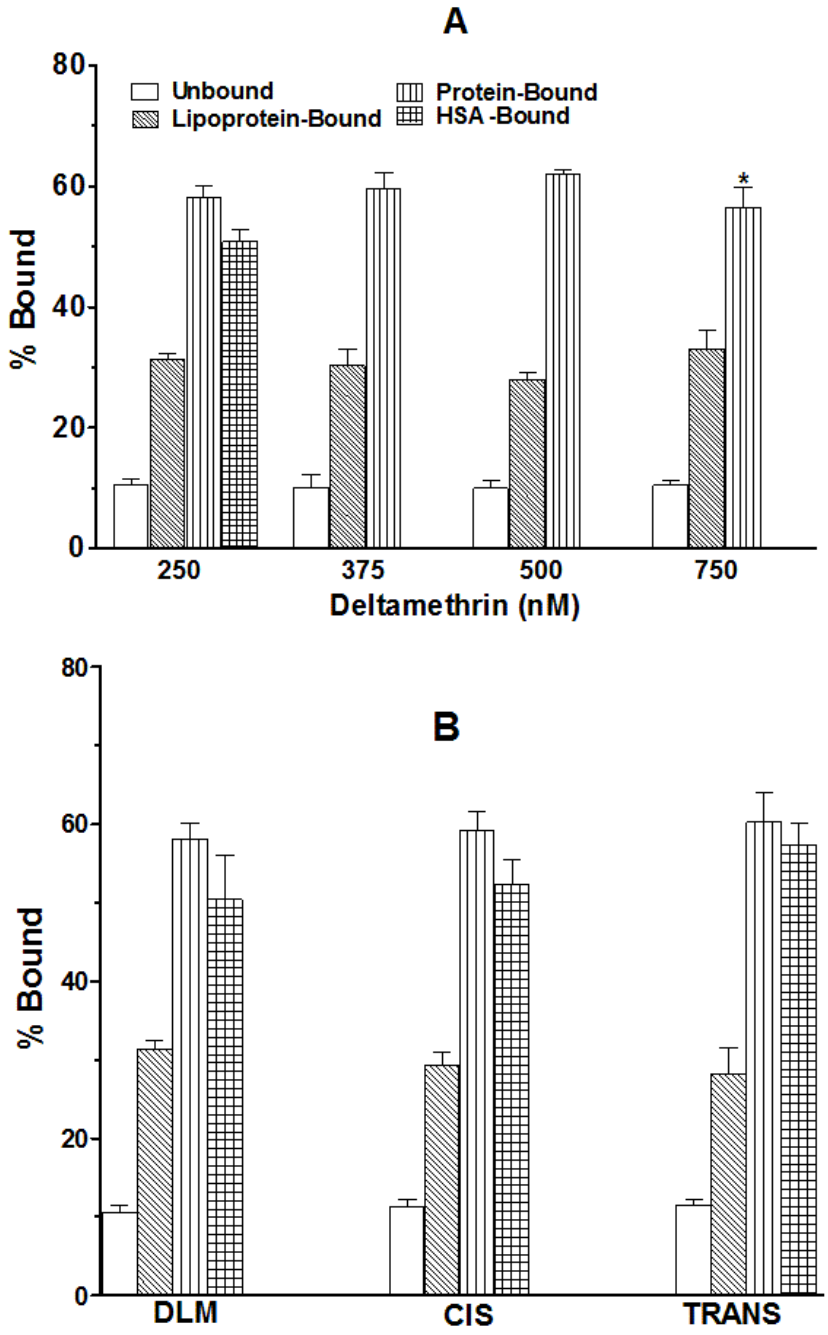

Figure 5

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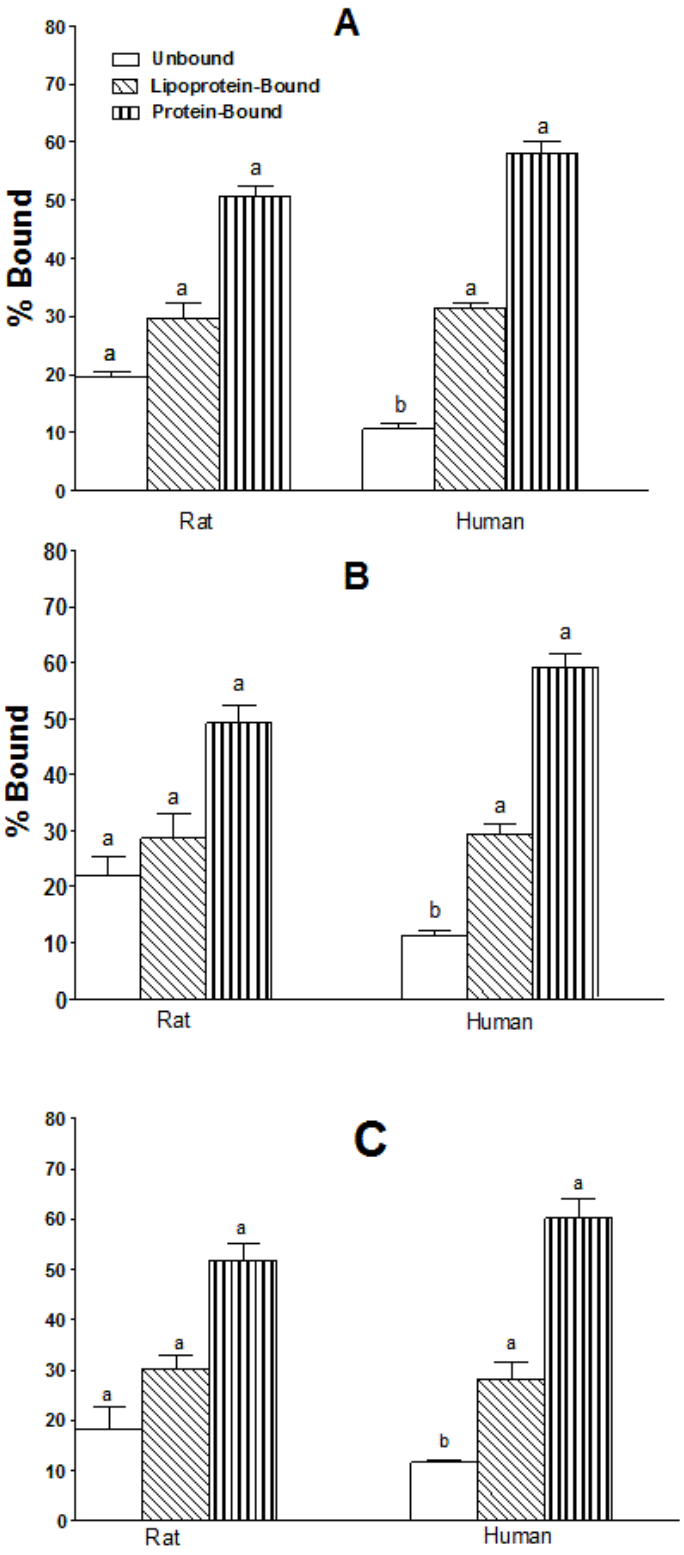

Figure 6

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