

# Ontogeny of plasma proteins, albumin and binding of diazepam, cyclosporine, and deltamethrin

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**BACKGROUND:** To characterize the ontogeny of plasma albumin and total proteins, due to the lack of a comprehensive pediatric database. Secondly, to establish the magnitude and duration of maturational changes in binding of highly-bound drugs/chemicals.

**METHODS:** Anonymized plasma samples from 296 donors were pooled in 6 age brackets from birth to adolescence. Total protein and albumin levels were measured in each age group, as was the age-dependency of plasma binding of diazepam (DZP), cyclosporine (CYC), and deltamethrin (DLM), a pyrethroid insecticide.

**RESULTS:** Plasma levels of albumin and total proteins steadily increased for the first 1–3 y of life. Unbound DZP and CYC fractions were elevated three- to fourfold in neonates, but decreased to adult levels after 1 and 3 y, respectively. Unbound DLM levels exceeded those in adults for just 1 mo.

**CONCLUSION:** Neonates and infants under 1–3 y may be at risk from increased amounts of free drug, when given standard doses of some highly-bound drugs. Pyrethroid insecticides might be anticipated to pose increased risk for 1 mo.

The plasma protein binding of highly-bound drugs can have significant clinical implications (1–3). Binding limits the amount of compound that can exit the bloodstream and reach peripheral sites of action, storage, and elimination. Pharmacological activity and toxicity are generally assumed to be correlated with, and determined by unbound drug concentrations in plasma. An equilibrium exists between the concentration of unbound drug in the plasma and the site(s) of action, when drug distribution is governed by passive diffusion. Although therapeutic ranges for drug monitoring are frequently reported in terms of total drug concentration in blood or plasma, the unbound drug concentration provides a better index for monitoring highly-bound compounds. This is particularly true for patients for whom there are disease- or age-related alterations in plasma protein content or composition (4–6) Adverse effects of diazepam in newborns,

for example, have been attributed to significant elevations in serum concentrations of unbound parent compound and N-desmethyldiazepam, its major active metabolite (7).

There are number of factors that influence plasma binding of drugs and other chemicals, some of which can be age-related. These include the drug and its concentration, the presence of binding competitors, the type and quantity of plasma protein, and the proteins' affinity for the drug. Nau *et al.* (7) observed a twofold increase in the free fraction of diazepam in the serum of neonates that accompanied marked increases in free fatty acids and bilirubin, competitors for albumin-binding sites. Albumin levels progressively rose during a 6-d postgestation monitoring period, but remained significantly lower than in adults. Albumin is the major plasma protein, accounting for some 55% of total proteins. Many neutral and some basic drugs bind primarily to albumin by hydrophobic and ionic bonding, respectively (8).  $\alpha$ ,  $\beta$ , and  $\gamma$  globulins are the second major class, comprising ~38% of proteins. Globulins transport a variety of endogenous substances. The globulin  $\alpha_1$ -acid glycoprotein (AAG), accounts for only 2% of plasma proteins, but binds many basic, or cationic drugs (8). Lipoproteins play key roles in transporting endogenous lipids, as well as neutral and basic lipophilic drugs (9).

Pediatric patients, especially neonates, have been reported to have lower protein binding of a number of commonly prescribed drugs. A high degree of correlation has been seen between the early postnatal rise in plasma albumin levels and the fraction of drugs bound (10–12). The majority of investigations have been conducted with mixed cord blood immediately after parturition. Unfortunately, there are few and incomplete data on the ontogeny of plasma proteins and binding from infancy through adolescence. The size of study populations of infants and children is usually quite small, due to the difficulty of securing healthy subjects to serve as blood donors. Age brackets are typically too broad and the ages too diverse to accurately characterize the sequence of maturational changes (Table 1). In one such study of albumin levels and plasma protein binding of disopyramide, the wide age-brackets included

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**Table 1.** Studies of the ontogeny of total plasma proteins, albumin, and AAG

<b>Total proteins</b>						
Sethi et al. (41)	Birth—1 wk (31)	>1–4 wk (34)	>4 wk to 1 y (31)	>1–3 y (41)	>3–6 y (30)	>6–18 y (129)
Holt et al. (13)	<1 mo (13)		>4 wk to 2 y (19)		2–16 y (23)	
Smith et al. (42)	<3 d to 6 mo (5)		6 mo to 2 y (6)		2–12 y (4)	
Pickoff et al. (43)	Birth (6)		8–18 mo (8)		>2 y (12)	
<b>Albumin</b>						
Sethi et al. (41)	Birth—1 wk (31)	>1–4 wk (34)	>4 wk to 1 y (31)	>1–3 y (41)	>3–6 y (30)	>6–18 y (129)
Holt et al. (13)	<1 mo (13)		>4 wk to 2 y (19)		2–16 y (23)	
Meistelman et al. (44)	Birth (18)		2–12 mo (9)		3–10 y (11)	
Kanakoudi et al. (14)	Birth (153)	1 mo (96)	3 mo (96)	6 mo (57)		
Mazoit et al. (45)		1–2 mo (4)	3–4 mo (4)	6 mo (3)		
Nau et al. (7)	Birth—day 6 (8–12)					
<b>AAG</b>						
Holt et al. (13)	<1 mo (13)		>4 wk to 2 y (19)		2–16 y (23)	
Lerman et al. (15)	Birth (5)	1–12 mo (15)	1–5 y (16)		12–18 y (12)	
Kanakoudi et al. (14)	Birth (153)	1 mo (96)	3 mo (96)	6 mo (57)		
Meistelman et al. (44)	Birth (18)		2–12 mo (9)		3–10 y (11)	
Mazoit et al. (45)		1–2 mo (4)	3–4 mo (4)	6 mo (3)		

Number of subjects in parenthesis.  
AAG, albumin and  $\alpha_2$ -acid glycoprotein.

neonates (<1 mo), infants (1 mo to 2 y), children (2–16 y), and adults (13). The adults were healthy, but the young were all patients. Nau et al. (7) assessed 8–13 subjects, for whom five diazepam binding parameters were monitored the first 6 d of life. This study period was too short for albumin and bilirubin levels or drug binding to approach adult values. One of the most comprehensive studies of the ontogeny of albumin was conducted by Kanakoudi et al. (14). They measured serum levels of albumin, AAG and eight other proteins soon after birth and at the end of 1, 3, and 6 mo in relatively large numbers of subjects. Albumin and AAG concentrations remained lower at 6 mo than in adults. The resulting data were quite useful, but did not define increases during the first weeks of life nor subsequent changes in infancy or childhood. Lerman et al. (15) did characterize the ontogeny of AAG in groups of 10–20 subjects from birth to adolescence. These researchers found an inverse relationship between the serum concentrations of AAG and free fraction of lidocaine from birth to 1 y of age.

The current study was undertaken as part of an effort to determine whether neurological effects of pyrethroids are age-dependent. Children and adults are frequently exposed to low levels of pyrethroids, the most widely-utilized insecticides in the United States and the European Union (16,17). As our pilot experiments during analytical method development indicated that pyrethroids such as deltamethrin (DLM) are highly bound to plasma proteins and lipoproteins (18), we were concerned that lower binding in neonates, infants, or young children may contribute to increased deposition in the central nervous system (CNS). Children 1–2 y old are of concern to the United States EPA due to their potential for higher DLM ingestion and skin exposure. Although most pyrethroids are relatively

nontoxic to humans and other mammals, high doses of some congeners can be acutely neurotoxic. Clinical signs are primarily attributed to the binding of the parent (unmetabolized) compound to voltage-gated sodium channels in axonal membranes (19). Thus, an important study objective was to learn whether levels of unbound DLM are elevated during immaturity and for how long this condition persists.

An excellent source of pediatric plasma samples was identified, allowing a comprehensive characterization of the ontogeny of the plasma albumin and total proteins, as well as the binding of DLM, diazepam and cyclosporine. All three compounds are very lipophilic and extensively bound, but bind to different plasma components. Published Log *P* values for diazepam, DLM, and cyclosporine are 3.12, 4.6, and 6.92. Diazepam binds almost exclusively to albumin, notably to the protein's so-called benzodiazepine site (20). DLM binds hydrophobically to both albumin and lipoprotein. Albumin and lipoprotein binding account for 60 and 30% of the insecticide in adult human plasma (16). Cyclosporine the most highly lipophilic compound, associates largely with lipoproteins by partitioning into their nonpolar lipid core (9,21). In view of incomplete ontogeny data in the literature, it was deemed worthwhile to establish with greater certainty when plasma binding of each of the three compounds attained adult levels. These findings may be relevant, or applicable to other pharmaceutical agents with similar binding characteristics.

## RESULTS

### Ontogeny of Plasma Albumin and Total Proteins

Levels of albumin and total proteins progressively increased in the plasma during maturation [Table 2](#). Apparent increases

in albumin concentrations from one age group to the next were modest, such that no one group was significantly different from the next. Values for donors 3 y of age and younger were significantly lower than for adults. In contrast to albumin, total plasma protein levels rose substantially during the second, third, and fourth weeks. The contribution of albumin to total proteins diminished from ~65 to 55% during this period and remained constant through adolescence. The more pronounced rise in total plasma proteins indicates that proteins in addition to albumin are synthesized in increasing amounts during the first year of life.

**Plasma Drug Binding During Maturation**

The extent of plasma binding of diazepam (Figure 1, panel a) and cyclosporine (Figure 1, panel b) exhibited similar changes during maturation. The unbound fraction of each drug was highest in neonates (birth to 1 wk). The percentage of diazepam that was unbound was approximately threefold higher in neonatal (birth to 1 wk) than in adult plasma. Binding of diazepam decreased significantly in successive age groups during the first year of life. Differences from adults were no longer seen in infants and children older than 1 y. The percentage of unbound cyclosporine was approximately fourfold greater in neonates than adults. Although the unbound fraction of the

drug progressively decreased, 1- to 3-y olds still exhibited significantly lower binding than children, adolescents, and adults. The extent of plasma binding of both diazepam and cyclosporine remained quite consistent in these three oldest age groups.

**Plasma Pyrethroid Binding During Maturation**

The age-dependency of plasma binding of DLM differed from that of diazepam and cyclosporine (Figure 2). The unbound fraction of the pyrethroid insecticide, like the two drugs, was highest in neonates. This fraction remained elevated in the > 1-wk to 4-wk group, but matched adult values thereafter. It is apparent in Figure 2 that DLM binds to both plasma proteins and lipoproteins. Plasma protein binding of DLM rose significantly during the initial 4 wk, though there was relatively little increase in lipoprotein binding for the first 3 y. Once binding maturity was attained after 4 wk, protein and lipoprotein binding accounted for 55 and 35% of the total amount of DLM in plasma, respectively. Only ~10% remained unbound.

**DISCUSSION**

Our evaluation of plasma samples from 296 pediatric donors yielded the most comprehensive and detailed database, to our knowledge, that is available on the ontogeny of plasma albumin and total proteins, as well as maturation of plasma binding of

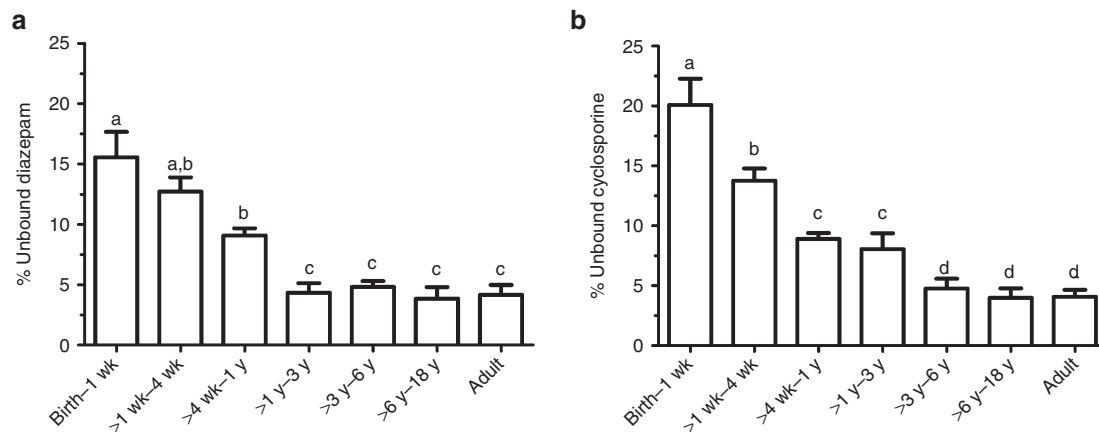
**Table 2.** Plasma albumin and total protein concentrations in different age groups

Age	Number of donors	Albumin concentration (g/dl)	Total protein concentration (g/d)	Albumin concentration (% of total protein)
Birth-1 wk	31	2.8±0.1*	4.3±1.0*	65
>1 wk to 4 wk	34	2.9±0.05*	5.3±0.1*	55
>4 wk to 1 y	31	2.9±0.4*	5.4±0.2*	54
>1 y to 3 y	41	3.1±0.2*	5.9±0.1	52
>3 y to 6 y	30	3.1±1.1	6.2±0.1	50
>6 y to 18 y	129	3.2±0.6	5.9±0.1	54
Adult	NS	4.0±0.2	6.3±0.1	63

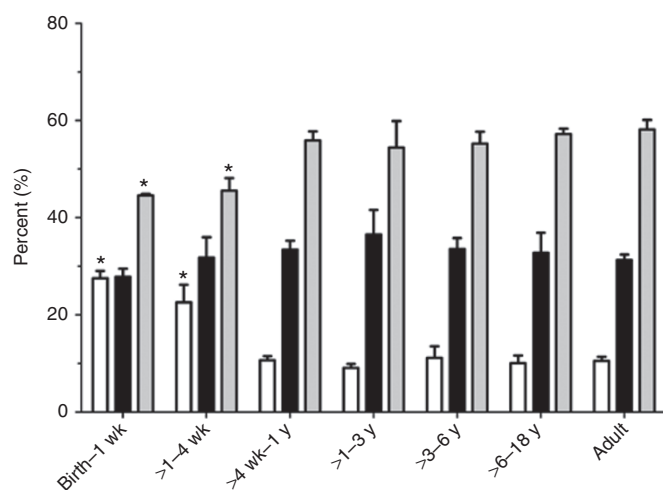
Data represents mean ± SD. N = 4.

NS, not specified by commercial source.

\* Indicates statistically significant difference from adult (P ≤ 0.05).



**Figure 1.** Effect of age on plasma binding of diazepam (a) and cyclosporine (b). Bars represent mean % unbound drug (SD). Values for age-groups with the same letter (a, b, and c) are not statistically different.



**Figure 2.** Effect of age on plasma binding of Deltamethrin (DLM). Bars represent mean (SD). Asterisks indicate statistically significant difference from unmarked values for the same parameter in other age groups. No fill = Unbound. Filled solid = Lipoprotein bound. Filled gray = Albumin bound.

selected drugs and a common insecticide. Albumin concentrations rose progressively, initially attaining values that were not significantly different from adults in the 3- to 6-y age group. Other pediatric albumin data sets we found were incomplete, lacked sufficient numbers of healthy subjects, or had broad age brackets. A survey by Kanakoudi *et al.* (14) included a large number of donors who were monitored for just the first 6 mo of life. Albumin levels remained lower in their 6-mo-old infants than in adults, as did prealbumin. Although albumin is the most abundant protein in plasma, its concentrations increased modestly during infancy and early childhood in the current study. Total plasma protein levels increased during the first year of life, as did binding of diazepam, cyclosporine, and DLM. Inverse relationships have been observed by several other researchers between the postnatal rise in albumin levels and the free fraction of drugs that bind to it (10–12).

Newborns are particularly sensitive to diazepam's CNS-depressant effects (22–24). High maternal doses during labor can result in elevated neonatal blood levels of free drug, which can produce inadequate respiration, apnea, "floppiness," hypothermia and poor suckling. There is a limited basis on which to judge the age at which this is no longer of concern. One well-conducted study showed a 40% decrease in the free fraction of diazepam during the first week of life, but did not assess neonates older than 11 d of age (7). We found elevated unbound diazepam levels in plasma from infants younger than 1 y. The free fraction was threefold higher in the birth to 1-wk age group than in adults. The unbound fraction was still twofold higher in the > 4 wk to 1 y olds. There was no difference, however, between adults and children 1–3 y of age or older. The decreased binding at <1 y might be attributable to lower affinity of albumin for diazepam, as well as lower albumin levels. Adult and fetal albumin, however, are reported to have the same binding affinity for diazepam (25). Both albumin and total plasma protein levels increased in concert with the extent of diazepam binding in the present study during the

initial year after birth. Assuming an absence of metabolism and elimination, a high unbound fraction of diazepam would result in more drug free to diffuse from the bloodstream into tissues, resulting in enhanced and prolonged CNS effects.

Clinical investigations clearly show that neonates' and infants' ability to metabolize diazepam is quite limited. The drug undergoes oxidative hydroxylation and demethylation to bioactive metabolites by cytochrome P450 (CYP) enzymes, and is subsequently glucuronidated and excreted. Treluyer *et al.* (26) found that urinary excretion of diazepam's major metabolites was very low in the 1- and 2-d olds. There was a substantial increase in metabolite excretion during the initial week, although metabolism of the drug by hepatic microsomes remained lower than in adults in the 3- and 12-mo age groups. Hepatic CYP2C19 is largely responsible for demethylation of diazepam. This enzyme's expression and activity approach adult levels within ~6 mo (27). Levels of CYP3A4, the enzyme largely responsible for catalyzing diazepam hydroxylation, rise gradually during the first 6 mo, but remain lower in children up to 2 y of age than in adults (28). Thus, increased amounts of free diazepam in the bloodstream, coupled with low metabolic clearance, can enhance CNS deposition. Pharmacodynamic factors may also contribute to the susceptibility of newborns and infants, as may the immature CNS and blood–brain barrier.

The extent of plasma binding of cyclosporine was also shown to be age-dependent in the present investigation. The unbound fraction of the drug was approximately fourfold higher in neonates than adults, but progressively decreased, reaching adult levels in the 3- to 6-y-old group. Sethi *et al.* (18) previously confirmed that cyclosporine binds primarily to the lipoprotein fraction of adult human plasma. The lipoprotein levels in plasma samples were not quantified, as others have characterized the ontogeny of major lipoproteins. In one such investigation, serum was taken from infants at 2, 4, 6, 9, and 12 mo (29). Cholesterol levels rose markedly for the first 2 mo and more slowly thereafter. Low-density lipoprotein, high-density lipoprotein, very low-density lipoprotein, and triglyceride levels increased slowly during the first 12 mo of life. Another research group measured cholesterol content of high-density lipoproteins in serum of subjects in six age groups ranging from newborns to adolescents and adults (30). high-density lipoprotein cholesterol increased quite modestly in males from birth to age 6–10 y. Somewhat larger increases were observed in females until they reached 11–15 y. It is likely that the progressive increase in cyclosporine binding we observed during the first 3 y in pooled male and female plasma samples was due in part to rising plasma lipoproteins.

Age-related differences in plasma binding have the greatest effect on systemic disposition of compounds that are highly bound to a single plasma component. Diazepam and cyclosporine are both highly (>95%) bound. As described previously diazepam binds primarily to albumin (20), while cyclosporine is largely associated with the lipoprotein fraction (21). Deficits of albumin or lipoproteins in plasma of neonates and infants thus contribute to the significant elevations seen



in the unbound fraction of the corresponding drug in these age groups. Although the total binding of DLM to plasma is high (~90%), binding to each of its major components, lipoproteins (~30%) and proteins (~60%), is low to moderate. One of these plasma components serves to buffer/bind excess DLM resulting from age-dependent deficit in the other component. Accordingly, an age-related increase in unbound DLM is manifest only in the group with the lowest plasma protein and lipoprotein levels.

The finding that the amount of free DLM available for brain uptake and/or metabolic clearance is no higher for those older than 4 wk than for adults rules out plasma binding as a contributor to potential age-related differences in the insecticide's CNS deposition in infants older than 4 wk. Neonates would be predicted to be at greater risk due to lower protein binding; however neonatal pyrethroid exposures should be negligible. In contrast, infants and children may ingest pyrethroids in house dust and from pets by hand to mouth activity, as well as in foods (31). As pyrethroids are very lipophilic chemicals, it appears likely the age-dependency of the plasma binding of highly lipophilic, persistent environmental pollutants (POPs), such as DDT, phthalates, polychlorinated biphenyls and dioxins, may be similar to DLM. Early investigators reported such chemicals are also transported in the bloodstream by albumin and lipoproteins (32,33). Neonatal exposures to POPs can be substantial, as these compounds cross the placenta and are concentrated in breast milk.

The binding data in this investigation can be used in the development of pediatric physiologically-based pharmacokinetic (PBPK) models for diazepam, cyclosporine, DLM and other pyrethroid insecticides. PBPK models are used to predict the time-course of a chemical in the blood or specific organs (i.e., the internal dose) affected by the agent (34). These computer-enabled models take into account key anatomical, physiological and biochemical features of laboratory animals and humans, as well as physicochemical properties of the compound of interest. Inclusion of accurate information about the chemical's absorption, transport in the bloodstream, uptake by different tissues, metabolism, and elimination is essential. Input of complete and accurate data significantly reduces uncertainty in the models' pharmacokinetic and pharmacodynamic simulations. PBPK models have been widely utilized for three decades to assess the toxicity of chemical agents (35,36). Such models are finding increasing use in drug development and regulation (37–39). Very few models, however, have yet been adapted and utilized to forecast the systemic uptake and disposition of therapeutic agents in infants and children. The nature and age-dependency of plasma binding of diazepam, cyclosporine and DLM can now be accounted for in PBPK models, whereas little to no information was previously available.

## METHODS

### Blood and Plasma Donors

Anonymized plasma samples from a total of 296 individuals were obtained in the fall of 2012, from a College of American

Pathologists-certified clinical testing laboratory in Children's Hospital and Health System in Milwaukee, WI. Frozen plasma samples not used for clinical tests were retrieved, divided into appropriate age brackets, thawed, and equivalent volumes pooled by trained laboratory personnel. Unused whole blood samples were also pooled, plasma separated, and combined with thawed plasma samples of the same age bracket. The age brackets and number of samples in each bracket are shown in **Table 2**. Specimens, from donors suffering from a condition anticipated to adversely impact albumin or lipoprotein levels, were excluded. The health of the subjects was not otherwise verified. Gender and ethnicity were not inclusion criteria. The pooled aliquots for each age bracket were frozen at  $-20^{\circ}\text{C}$  until analyses could be performed. The collection and use of the anonymous plasma samples were classified as exempted by the Children's Hospital and Health System Institutional Review Board.

Frozen heparinized, pooled adult human plasma was purchased from Innovative Research (Novi, MI). The name, ethnicity, gender and number of donors were not specified, although the donors were 18–65 y old. Thawed plasma samples were filtered through a 0.45- $\mu\text{m}$  Millipore filter to remove precipitated fibrinogen.

### Test Substances/Materials

Radiolabeled  $^{14}\text{C}$ -deltamethrin (DLM) (57.9 mCi/mmol) of 99% purity was kindly provided by Bayer CropScience (Stilwell, KS), as was unlabeled DLM of 98.9% purity. N-Methyl- $^{14}\text{C}$ -diazepam (55 mCi/mmol) and  $\beta$ - $^3\text{H}$ -cyclosporine (20 Ci/mmol) were obtained from American Radiolabeled Chemicals (St. Louis, MO). Glycerol formal, acetonitrile (HPLC-grade), hexamethyldisilazane (Reagent-grade), and sodium fluoride (NaF) (purity, 99.0%) were purchased from Sigma Aldrich (St. Louis, MO). Isooctane (purity, 99.0%) and 2-octanol (laboratory grade) were purchased from Fisher Scientific (Pittsburgh, PA).

### Albumin and Total Plasma Protein Measurement

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

### DLM Binding Experiment Precautions

Standard plasma binding measurement techniques, such as equilibrium dialysis and ultracentrifugation, are not suitable for highly lipophilic compounds such DLM. DLM and other pyrethroids are practically insoluble in the aqueous media used. Pyrethroids adhere avidly to glass, metal and many polymer components of separation devices. Thus, it was essential to utilize clean glassware silanized with 5% hexamethyldisilazane in toluene, at least 24 h before use in experiments. LoBind plastic pipette tips (Eppendorf, Hamburg, Germany) were used to avoid pyrethroid adherence.

### Plasma Protein and Lipoprotein Binding of DLM

Binding of DLM to plasma components was quantified by the three-step organic solvent extraction procedure of Sethi *et al.* (18). Each 80- $\mu\text{l}$  aliquot of plasma was spiked with 10  $\mu\text{l}$  of  $^{14}\text{C}$ -DLM in a silanized glass vial to yield a final concentration of 250 nmol/l. Eighty microliters of HSA (40 mg/ml) were similarly spiked with  $^{14}\text{C}$ -DLM to produce a 250 nmol/l solution. This concentration was found in the plasma of DLM-dosed rats that exhibit signs of neurotoxicity (40). The spiked plasma solutions were immediately treated with 10  $\mu\text{l}$  of 0.64 M NaF to inhibit serum carboxylesterases, and incubated in an orbital shaker at pH 7.4 for 3 h at  $37^{\circ}\text{C}$ . The samples were then extracted in turn with 200  $\mu\text{l}$  of isooctane, 2-octanol and acetonitrile, which extracted the unbound, lipoprotein-bound and protein-bound pyrethroid fractions, respectively. DLM concentrations were measured by liquid scintillation counting using a Beckman Coulter LS 6500 instrument.

### Plasma Binding of Diazepam and Cyclosporine

Plasma binding of diazepam and cyclosporine were determined by equilibrium dialysis, using a Dispo Equilibrium Dialyzer purchased

from Harvard Apparatus (Holliston, MA,). Each 75- $\mu$ l aliquot of plasma was spiked with  $^{14}$ C-diazepam to yield a 200 ng/ml solution. This was dialyzed against isotonic phosphate buffer at 37 °C for 4 h to establish equilibrium. A 75- $\mu$ l aliquot of plasma was spiked with  $^3$ H-cyclosporine to yield a 1  $\mu$ g/ml solution. This solution was dialyzed against isotonic phosphate buffer at 37 °C for 18 h to reach equilibrium. No fluid shifts occurred during the incubation. After the specified times, 100- $\mu$ l aliquots of plasma and buffer were removed simultaneously and mixed with 3 ml of scintillation fluid (MP Biochemicals, Solon, OH). Radioactivity in each sample was quantified in a Beckman LS 6500 scintillation counter. The extent of binding was calculated using the following formula:

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

$$\% \text{ free fraction} = 1 - \% \text{ bound fraction}$$

### Statistical Analysis

Data were analyzed using ANOVA coupled with Tukey's multiple comparison's test using Prism 6.4 (GraphPad, San Diego, CA).

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