Ontogeny of Drug Disposition in the Newborn: Studies Examining Valproic Acid and Diphenhydramine in Chronically Instrumented Lambs

by

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Abstract

Valproic acid (VPA, a low clearance carboxylic acid) and diphenhydramine (DPHM, a high clearance amine) were investigated in chronically instrumented lambs to examine developmental changes in their pharmacokinetics and metabolism.

In 10 day old, 1 month old and 2 month old lambs, and adult sheep, VPA clearance followed a pattern similar to humans. With a 10 mg/kg VPA *i.v.* bolus, both unbound and total VPA clearance increased significantly up to 2 months of age before decreasing to adult levels. Plasma protein binding was nonlinear in all ages examined with the area weighted unbound fraction being significantly higher in 10 day old lambs. A substantial (~2.5 fold) increase in urinary recovery of VPA-glucuronide in 2 month old lambs in comparison to 10 day old lambs suggested that changes in clearance may be largely related to post-natal development of enzymes involved in VPA glucuronidation. Finally, as with other organic acids, VPA renal clearance was initially low and increased with age. In a subsequent dose-ranging experiment in 10 day old lambs and adult sheep, the lambs appeared to have a lower binding capacity, but a higher binding affinity for VPA than adult sheep. Metabolic capacity was similar in 10 day old and adult sheep, however, the apparent *in vivo* K_m was lower in the adult. Differences in K_m appeared to account for the observed dose-dependent changes in unbound clearance for the two age groups. Estimates of apparent *in vivo* V_{max} and K_m appeared to largely reflect developmental differences in VPA glucuronidation.

The developmental disposition of DPHM was investigated in 15 day and 2 month old lambs. DPHM total body clearance in both groups of lambs was similar to previous non-placental clearance estimates in fetal lambs and significantly higher than observed for adult sheep. The renal clearance of DPHM and its acidic metabolite followed different and opposite trends with one (acidic metabolite) increasing with age and the other (DPHM) decreasing with age.

It appears that generalizations can be made regarding the post-natal development of Cl_r of acidic (increases) and basic (decreases) compounds. In contrast, the development of other drug clearance processes appears to be compound specific.

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List of Abbreviations

µ	Micron
µg	Microgram
µm	Micrometer
µM	Micromolar
~	Approximately
1 M	1 month old lambs
2 M	2 month old lambs
10 d	10 day old lambs
15 d	15 day old lambs
AIC	Akaike Information Criterion
ANOVA	Analysis of Variance
AR	AUC ratio [($AUC_{0-\infty}$ metabolite/$AUC_{0-\infty}$ VPA) × 1000]
AUC	Area under the plasma concentration *vs.* time curve
$AUC_{0-\infty}$	Area under the plasma concentration *vs.* time curve from zero to infinity
$AUC^{u}_{0-\infty}$	Area under the plasma concentration *vs.* time curve from zero to infinity for unbound drug
$AUMC_{0-\infty}$	Area under the first moment curve
B_{max}	Maximal binding capacity
°C	Degree Celsius
C_b	Plasma concentration of the protein bound drug
C_u	Plasma concentrations of the unbound drug
C^{u}_{mid}	Unbound drug concentration at the midpoint of the urine collection interval
Cl_{fo}	Non-placental clearance of drug from the fetal compartment based on total drug concentrations
Cl_{mo}	Non-placental clearance from the maternal compartment based on total drug concentrations
Cl_{int}	Intrinsic clearance
Cl_r	Renal clearance of the total drug

Cl^u_r	Renal clearance of the unbound drug
Cl_{tb}	Total body clearance based on total drug concentrations
Cl^u_{tb}	Total body clearance based on unbound drug concentrations
C_{max}	Maximal plasma concentration
CV	Coefficient of variation
Da	Dalton
DPHM	Diphenhydramine
DPMA	Diphenylmethoxyacetic acid
DPHMNO	Diphenhydramine-N-oxide
f_u	Unbound fraction
f_p	Area weighted free fraction of the drug ($=AUC_{unbound}/AUC_{total}$)
g	Gram
GC	Gas chromatography
GFR	Glomerular filtration rate
h	Hour
i.d.	Internal diameter
i.e.	*id est;* that is
i.v.	Intravenous
KCl	Potassium chloride
k_{cp}	Rate constant characterizing movement of drug from the central to the peripheral compartment (two-comparment model).
K_d	Dissociation constant for drug - plasma protein interaction
kg	Kilogram
K_m	A Michaelis-Menten parameter for enzymatic reactions; substrate concentration at which the reaction velocity is at half-maximal.
k_{pc}	Rate constant characterizing movement of drug from the peripheral to the central compartment (two-compartment model).
LC	Liquid chromatograph
LOQ	Limit of quantitation of the assay
M	Molar (moles/litre)
ml	Milliliter

mg	milligram
min	Minute
mm	Millimeter
mM	Millimolar
MRT	Mean residence time of the total drug
MRT^u	Mean residence time of the unbound drug
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MTBSTFA	N-methyl-N-(*tert*-butyldimethylsilyl)trifluoroacetamide
n	Number of subjects or animals
NADPH	Reduced β-nicotinamide-adenine dinucleotide tetrasodium salt
ng	Nanogram
o.d.	Outer diameter
pH	Negative logarithm of hydrogen ion concentration
r^2	Coefficient of determination
S.D.	Standard deviation
$t_{1/2}$	Half-life in a 1-compartment model based on total drug concentrations
$t^u_{1/2}$	Half-life in a 1-compartment model based on unbound drug concentrations
$t_{1/2\beta}$	Terminal elimination half-life in a 2-compartment model based on total drug concentrations
$t^u_{1/2\beta}$	Terminal elimination half-life in a 2-compartment model based on unbound drug concentrations
t_{max}	Time of occurrence of maximal plasma concentration
t_{mid}	Time at the midpoint of the urine collection interval
Tris	Tris(hydroxymethyl) aminomethane
V	Urinary excretion rate of VPA-glucuronide
Vd	Volume of distribution
Vd_{ss}	Apparent steady-state volume of distribution of the total drug

Vd_{ss}'	Apparent steady-state volume of distribution of the total drug corrected for the effects of saturable protein binding using area weighed free fraction
Vd^{u}_{ss}	Apparent steady-state volume of distribution of the unbound drug
VPA	Valproic Acid
x *g*	Times gravity (centrifugal force)
UDPGT	UDP-glucuronosyltransferase
V_{max}	Maximal velocity of an enzymatic reaction; a Michaelis-Menten parameter

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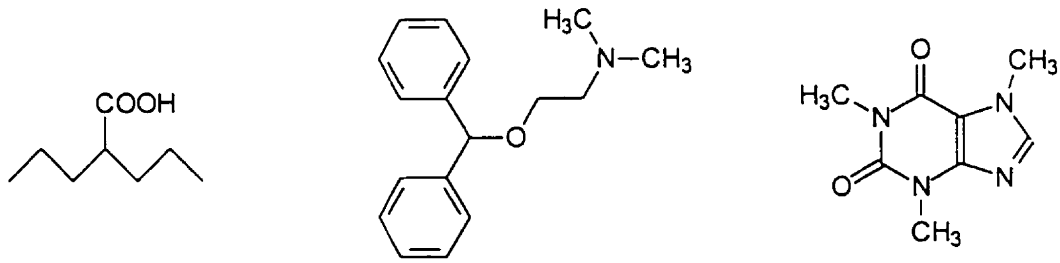

A Tribute to the Three Driving Forces of My Thesis:
Valproic Acid, Diphenhydramine, and Caffeine.

Chapter 1

Introduction

The period of development extending from the newborn to the adult is a time of rapid physiological and anatomical changes that can drastically affect drug disposition. Drug use during this dynamic period is often approached cautiously due to a poor understanding of age-related changes in pharmacokinetics. Traditionally, pediatric patients were considered to be "smaller adults", and thus were administered medication according to a body-weight adjusted "adult" dose (Morselli, 1976). However, depending on the therapeutic agent, administration using this approach often resulted in either over or under dosing. In order to determine appropriate dosing regimens for pediatric patients, an understanding of the distinct differences in drug ADME (i.e. absorption, distribution, metabolism, and excretion) that exist between the adult and pediatric populations, as well as within the pediatric population (i.e. neonates, infants, and children) is required (Moreselli, 1796; Moreselli *et al.*, 1980). Age-related alterations in the ADME of commonly used drugs in the pediatric population such as anti-infective agents, anticonvulsants, and cardiovascular drugs have been the topic of several reviews on developmental pharmacokinetics (Moreselli, 1976; Moreselli *et al.* 1980; Besunder *et al.*, 1988a and 1988b; Kearns and Reed, 1989; Morrow and Richens 1989; Butler *et al.*, 1994; Steinburg and Notterman, 1994; Battino *et al.*, 1995a and 1995b).

At the present time, there is a general understanding of the developmental changes in factors affecting drug disposition. Much of this understanding has been a result of data that have been gathered and "pieced together" from various literature sources. However, available information concerning drug disposition in newborns and infants is still quite limited when compared to what is known for the adult. This is largely due to the limited number of studies specifically designed to examine pharmacokinetics in the pediatric population (Butler *et al.*, 1994). Obvious ethical considerations have prevented such studies from being conducted in human neonates and infants. As well, detailed studies are often difficult to perform in common animal models (i.e. rats, guinea pigs) due to small size of newborns from these species. Thus, there is a need for studies that systematically investigate the ontogeny of drug disposition during the newborn period. This thesis is an investigation of the developmental disposition of two compounds, valproic acid (VPA) and diphenhydramine (DPHM) in chronically instrumented lambs. In the following sections, I will summarize relevant information about the pharmacokinetics of these two compounds and why they were chosen for study. It is hoped that information obtained from our studies will add to knowledge currently available in the literature and provide information for a more rational approach to drug therapy in newborns and infants.

1.1 Valproic Acid

Valproic acid (2-propylpentanoic acid, VPA) is a low molecular weight (144.2 Da) broad-spectrum anticonvulsant with a pKA of 4.8 and an octanol/water partition coefficient of 398 (Davis *et al.*, 1994; Baillie and Sheffels, 1995). Its branched-chain fatty acid structure is unrelated to the substituted heterocyclic ring structure common to other antiepileptic drugs (Figure 1.1) (Perny, 1991). VPA is available for use as the parent compound, its sodium salt, its amide derivative, and as a combination of the parent compound and its sodium salt (Davis *et al.*, 1994).

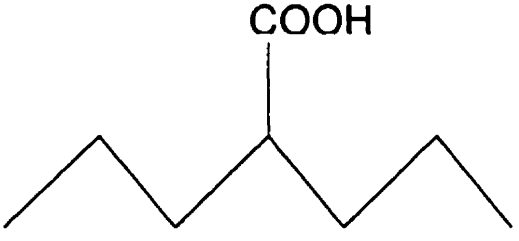

Figure 1.1 Chemical structure of valproic acid (VPA)

1.1.1 Therapeutic Use, Mechanisms of Action, Adverse Effects

As note above, VPA is a broad-spectrum anticonvulsant. It has clinical efficacy against several epileptic seizures types, including generalized seizures (i.e. tonic-

clonic, absence, and myoclonic) and certain partial seizures (i.e. simple, complex and secondarily generalized) (Davis *et al.*, 1994; Loscher, 1999). In addition, VPA has activity against compound/combination seizures including those that are refractory to other anticonvulants (Davis *et al.*, 1994).

The mechanism(s) of action of VPA is not well understood. The anticonvulsant activity of VPA is most likely related to its ability to potentiate the effects of the inhibitory neurotransmitter, γ-aminobutyric acid (GABA), by either increasing its production or inhibiting its degradation. VPA has been implicated in the activation of glutamic acid decarboxylase (GAD), an enzyme involved in the decarboxylation of glutamate which is required for GABA synthesis (Loscher, 1981; Loscher 1989). In addition, it appears to inhibit GABA-transaminase (GABA-T) and succinic semialdehyde dehydrogenase (SSADH), two enzymes that are involved in the successive degradation of GABA (Van der Laan *et al.*, 1979; Loscher and Vetter, 1985; Zeise *et al.*, 1991; Loscher, 1993). Finally, direct and indirect evidence suggests that VPA acts to enhance the potassium-induced release of GABA (Gram *et al.*, 1988). Other proposed mechanisms of action include inhibition of γ-hydroxybutyric acid (GHB; a minor degradation product of GABA that has epileptogenic effects) release *via* inhibition of aldehyde reductase (Vayer *et al.*, 1988) and inhibition of N-methyl-D-asparate (NMDA) receptor-mediated excitation (Zeise *et al.*, 1991). Moreover, VPA appears to have a nonspecific membrane stabilizing effect by reducing high frequency repetitive firing of neurons through action at sodium and/or potassium channels

(Slater and Johnson, 1978; McLean and MacDonald, 1986). Although there is ample evidence in the literature supporting all the proposed mechanisms mentioned above, none of the mechanisms alone can adequately explain VPA's broad-spectrum anticonvulsant effects. Thus, it is likely that there is more than one mechanism involved in its anticonvulsant activity.

The incidence of side effects of valproic acid in humans is relatively low in comparison to that of other anticonvulsants (Loscher, 1999). Commonly observed adverse effects associated with VPA use include gastrointestinal disturbances (i.e. nausea, vomiting, and stomach upset), weight gain, neurological effects (i.e. tremor or sedation), and transient hair loss (Davis *et al.*, 1994). Use of VPA during pregnancy often results in an increased incidence of congenital abnormalities such as facial dysmorphia, neural tube defects and multiple malformations (Davis *et al.*, 1994). The most studied adverse effect of VPA, however, appears to be a rare but fatal idiosyncratic hepatotoxicity characterized by microvesicular steatosis and necrosis of the liver (Dreifuss *et al.*, 1989). The incidence of hepatotoxicity varies between 1 in 5,000 to 1 in 20,000 (Radatz and Nau, 1999), and is most likely to appear within 6 months from the start of therapy (Jeavons, 1984). In patients < 2 years of age and on anticonvulsant polytherapy, the incidence of hepatoxicity is higher being 1 in 600 (Bryant and Dreifuss, 1996). Although the exact mechanism of VPA induced liver toxicity is still unknown, its metabolites have been implicated (see below).

1.1.2 Pharmacokinetics

The absorption of VPA is rapid and complete in humans with an oral bioavailability of 90-100% for both immediate and sustained-release preparations (Klotz and Anton, 1977; Perucca *et al.*, 1978a). Peak plasma concentrations are achieved within 1-3 hours for immediate release preparations (i.e. syrup, capsules and tablets) and 3-8 hours for sustained-release formulations (i.e. enteric coated tablets) (Gugler *et al.*, 1977; Klotz and Anton, 1977; Davis *et al.*, 1994). VPA's high oral bioavailabilty stems from the fact that it readily crosses the intestinal mucosa and is not subject to a significant first pass effect (Levy and Shen, 1995). Absorption of VPA appears to occur throughout the intestine with no site specificity (Levy and Shen, 1995).

The volume of distribution of valproic acid ranges from 0.1-0.2 L/kg in adults, and 0.15-0.4 L/kg in neonates, infants and children (Gugler *et al.*, 1977; Perucca *et al.*, 1978a & b; Gugler and von Unruh, 1980; Irvine-Meek *et al.*, 1982; Hall *et al.*, 1985; Gal *et al.*, 1988; Herngren *et al.*, 1991; Cloyd *et al.*, 1993). This relatively small volume of distribution (slightly larger than the extracellular fluid volume) appears to be related to the compound's extensive plasma protein binding (~90% at therapeutic concentrations) and its high degree of ionization at physiological pH (pKa 4.8) (Levy and Shen, 1995). These two factors act to confine VPA largely to the vascular space and extracellular fluids. VPA is largely bound to albumin in plasma (Kober *et al.*, 1980). Plasma protein binding of VPA at

therapeutic concentrations appears to be saturable/nonlinear (Scheyer *et al.*, 1990; Cloyd *et al.*, 1993; Levy and Shen, 1995). Thus, the unbound fraction of the drug increases at higher total plasma concentrations. Despite its high degree of ionization, the entrance of VPA into the central nervous system appears to be rapid (Levy and Shen, 1995). The concentration of VPA in cerebrospinal fluid (CSF) in humans as well as other animals ranges between 60-100% of the unbound fraction in plasma (Levy and Shen, 1995). In fact, the plasma unbound fraction appears to influence VPA levels in the central nervous system (Levy and Shen, 1995). There is evidence that transporters may play a role in the influx and efflux of VPA across the blood-brain and the blood-CSF barriers. It has been suggested that a medium chain fatty acid transporter and a carrier-mediated, probenecid-sensitive transport process are involved in the transport of VPA into and out of the CNS (Levy and Shen, 1995).

In human adults, VPA is a low clearance compound with plasma clearances ranging from 1.0-3.0 ml/min/kg (based upon unbound drug concentrations) and 0.1-0.3 ml/min/kg (based upon total drug concentrations), and elimination half-lives ($t_{1/2\beta}$) ranging from 9-18 h (Gugler *et al.*, 1977; Perucca *et al.*, 1978a & b; Bowdle *et al.*, 1980; Schapel *et al.*, 1980; Hoffman *et al.*, 1981; Nau *et al.*, 1982; Bialer *et al.*, 1985; Zaccara *et al.*, 1988; Davis *et al.*, 1994). In children and adults on polytherapy, the clearance of VPA is generally higher than that of adults on monotherapy (Gugler *et al.*, 1977; Perucca *et al.*, 1978b; Bowdle *et al.*, 1980; Schapel *et al.*, 1980; Hoffman *et al.*, 1981; Bialer *et al.*, 1985). VPA clearance is

dose-dependent with nonlinearities in clearance resulting from either saturable protein binding and/or saturable metabolism (Bowdle *et al.*, 1980; Gómez Bellver *et al.*, 1993). Elimination of VPA is almost entirely *via* hepatic metabolism with only 1 to 3 % of the dose excreted as the parent compound in urine (Gugler *et al.*, 1977; Gugler and von Unruh, 1980; Dickinson *et al.*, 1989).

1.1.3 Metabolism

The metabolism of valproic acid has been the topic of many reviews. Although the structure of VPA is relatively simple, its metabolic fate is extremely complex with approximately 50 metabolites identified to date in man (Baillie and Scheffels, 1995). Of these metabolites, 16 are consistently observed in humans (Kassahun *et al.*, 1990; Baillie and Scheffels, 1995). The major routes of VPA metabolism can be divided into three main pathways, glucuronidation, mitochondrial β-oxidation, and P-450 catalyzed oxidative metabolism (Figure 1.2). The glucuronidation pathway plays the most significant role in VPA elimination with approximately 10-70% of the administered dose being recovered in human urine (Gugler *et al.*, 1977; Dickinson *et al.*, 1989; Levy *et al.*, 1990) as the 1-O-acyl-β-D-ester linked glucuronide (VPA-glucuronide) (Dickinson *et al.*, 1979). β-oxidation of VPA results in the formation of the metabolites, 2-n-propyl-2-pentenoic acid (2-ene VPA) (formed predominately as the E-isomer), 2-n-propyl-3-pentenoic acid (3-ene VPA) (formed predominantly as the E-isomer), 2-n-propyl-3-hydroxypentanoic acid (3-OH VPA) and 2-n-propyl-3-oxopentanoic acid

(3-keto VPA) (Bjorge and Baillie, 1991; Li *et al.*, 1991). The formation of 3-OH VPA does not appear to be exclusive to β-oxidation and is also be formed *via* direct P-450 mediated microsomal hydroxylation of the parent compound

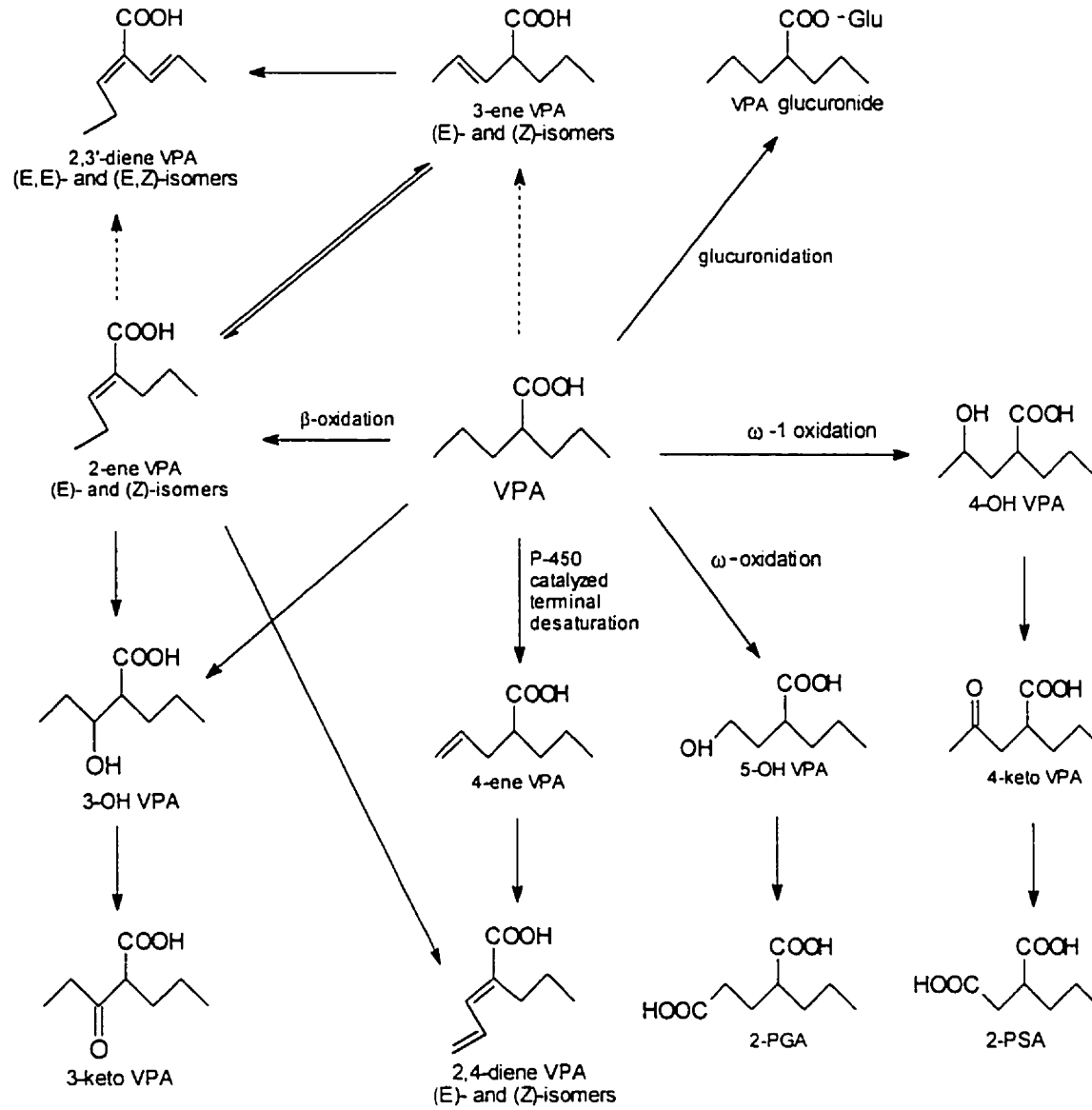

Figure 1.2 Metabolic pathways of VPA in man. Dotted arrows indicate pathways for which direct experimental evidence is lacking.

(Rettenmeier *et al.*, 1987). Isomerization of (E)-2-ene VPA gives rise to (E)-3-ene VPA which can undergo further β-oxidation to result in the formation of the diunsaturated metabolite, (E,E)-2, 3'-diene VPA (Bjorge and Baillie, 1991). Of the β-oxidation metabolites, 3-keto VPA is the most prominent accounting for 10-60% of the dose recovered in urine (Dickinson *et al.*, 1989; Levy *et al.*, 1990; Sugimoto *et al.*, 1996). VPA undergoes P-450 catalyzed ω and ω-1 oxidation to form 2-n-propyl-5-hydroxypentanoic acid (5-OH VPA) and 2-n-propyl-4-hydroxypentanoic acid (4-OH VPA), respectively (Rettenmeier *et al.*, 1987). Further oxidation of 5-OH VPA results in 2-propylglutaric acid (2-PGA) while oxidation of 4-OH VPA leads to the formation of 2-n-propyl-4-oxopentanoic acid (4-keto VPA) and 2-propylsuccinic acid (2-PSA) (Granneman *et al.*, 1984). Unlike 2-ene and 3-ene VPA, 2-n-propyl-4-pentenoic acid (4-ene VPA) arises from a distinct P-450 mediated desaturation reaction (Rettie *et al.*, 1987). Subsequent β-oxidation of this metabolite results in the formation of (E)-2, 4,-diene VPA (Kassahun and Baillie, 1993). Both 4-ene VPA and (E)-2, 4-diene VPA have been hypothesized to be associated with the hepatotoxic effects of VPA, thus the study of their formation and subsequent fate have received considerable attention (Kassahun and Abbott, 1993; Kassahun and Baillie, 1993; Tang and Abbott 1996).

1.1.4 Valproic Acid Pharmacokinetics in the Pediatric Population

Despite cases of VPA-induced idiosyncratic hepatoxicity, VPA remains the drug of choice for treating seizures of various etiologies in neonates, infants, and children due to its broad spectrum of activity and minimal cognitive side effects (Sarisjulis and Oliver, 1999). Compiled data from the literature reveals that VPA exhibits age-related alterations in drug clearance (Levy and Shen, 1995). Information on the pharmacokinetics of VPA during the immediate newborn period is limited to data gathered from studies where neonates were exposed to VPA *in utero* as a result of maternal administration (Dickinson *et al.*, 1979b; Ishizaki *et al.*, 1981; Nau *et al.*, 1981; Nau *et al.*, 1984; Kondo *et al.*, 1987). In addition to these studies, there are two other studies where neonates were given VPA for the treatment of intractable seizures (Irvine-Meek *et al.*, 1982; *Gal et al.*, 1988). The range of elimination half-lives compiled from these studies are 2 to 8 fold longer than in adults and range from 15.1-80 h. Weight-adjusted VPA clearance based upon total drug concentrations in newborns (~14.4 ml/h/kg) is within the range of values for adult epileptics on polytherapy (~15 ml/h/kg), but less than values observed for children (~25-30 ml/h/kg) (Gal *et al.*, 1988). However, unbound VPA clearance in neonates (~108.9 ml/h/kg) is less than estimates for both children (~216 ml/h/kg) and adults (~127 ml/h/kg) (Gal *et al.*, 1988). The lower unbound clearance in neonates may be a result of a lower intrinsic metabolic clearance due to immature drug-metabolizing enzyme activities (Levy and Shen, 1995). Two separate reports on VPA protein binding

have reported free fractions in neonates (~19%) that were higher than values observed for adults (≤10%) at therapeutic concentrations (Nau *et al.*, 1984; Gal *et al.*, 1988). A study in infants (2-36 months of age) reports VPA clearances that were higher than observed in newborns especially for patients on polytherapy (~36 ± 11 ml/h/kg) (Hall *et al.*, 1985). Weight-normalized VPA clearance in children is intermediate between values reported for infants and adults (Levy and Shen, 1995).

More systematic *in vivo* studies on the developmental disposition of valproic acid have been conducted in rats (Haberer and Pollack, 1994) and guinea pigs (Yu *et al.*, 1985; Yu *et al.*, 1987). Similar to humans, lower unbound VPA clearance and longer half-lives were observed in newborns from these two species. Therefore, it appears that the elimination capacity for VPA is lower during the immediate newborn period for all the species examined thus far.

1.1.5 VPA Pharmacokinetics in Sheep: Previous Studies

In the past, our laboratory has conducted studies examining the disposition of valproic acid in pregnant sheep and 1 day old lamb (Kumar, 1998). Aside from the diunsaturated metabolites (*e.g.*, (E,E)-2,3'-diene VPA and (E)-2,4-diene VPA), the common VPA metabolites observed in humans (see Figure 1.2) were found in sheep. Clearances of both unbound (Cl^{u}_{tb}, 0.66 ± 0.28 ml/min/kg) and total VPA (Cl_{tb}, 0.25 ± 0.07 ml/min/kg) in newborn lambs were much lower than

observed in pregnant sheep (Cl^{u}_{tb}, 5.4 ± 2.7 ml/min/kg; Cl_{tb}, 1.5 ± 0.6 ml/min/kg) suggesting impaired VPA elimination in the newborn lamb. In addition, the elimination half-life of both unbound and total drug was ~2-3 fold longer than observed for pregnant sheep. Furthermore, estimates of a fetal clearance component (i.e. fetal non-placental clearance; Cl_{fo}) were obtained from the pregnant sheep experiments through the use of a modified two-compartment model with Michealis-Menten elimination (Kumar, 1998). This Cl_{fo} parameter is considered to be a measure of the fetal lamb's intrinsic ability to eliminate VPA. Surprisingly, fetal Cl_{fo} estimates for total (6.8 ± 3.2 ml/min/kg) and unbound VPA (25.9 ± 17.3 ml/min/kg) were significantly higher than corresponding values in newborn lambs and pregnant sheep (Kumar, 1998). This phenomenon was attributed to possible placental utilization of valproic acid, which would result in an overestimation of Cl_{fo}. An interesting finding from these studies was that the VPA-glucuronide metabolite recovered in the urine accounted for a significantly smaller portion of the dose in newborn lamb (~28% of the dose) in comparison to pregnant sheep (~ 62% of the dose) (Kumar, 1998). In contrast, a larger percentage of the dose was recovered in newborns as the 3-keto VPA metabolite (~11.6% in newborn lambs vs. ~2.4% in pregnant sheep). These results suggest that differences exist in the contribution of the main metabolic pathways (i.e. glucuronidation and β-oxidation) responsible for VPA elimination in newborn lambs. Finally, the renal clearance of VPA was substantially higher in pregnant sheep when compared to both fetal and newborn lambs (Kumar, 1998).

1.2 Diphenhydramine

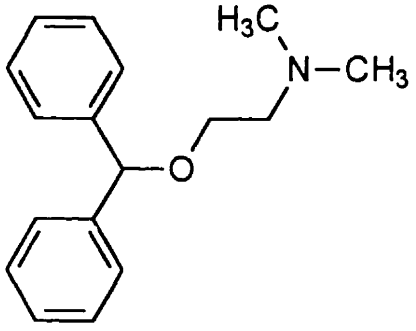

Figure 1.3 Chemical Structure of diphenhydramine (DPHM)

Diphenhydramine (2-(diphenylmethoxy)-N,N-dimethylethylamine, DPHM) is a classical first-generation H_1-receptor antagonist of the ethanolamine class with molecular weight of 255.4 Da (Garrison, 1991). It is a weak base with a pK_a of 9.0, and is considerably more lipophilic than VPA with an octanol/water partition coefficient of 1862 (Figure 1.3) (de Ross *et al.*, 1970). DPHM is marketed as its hydrochloride salt.

1.2.1 Therapeutic Use, Mechanisms of Action, Adverse Effects

DPHM is a competitive H_1-receptor antagonist with little or no activity on H_2-receptors. (Cooper *et al.*, 1990). As a result, DPHM inhibits the actions of histamine during allergic and anaphylactic reactions (i.e. smooth muscle

contraction, increased capillary permeability, itch, and edema, wheal, and flare formation) (Garrison, 1991). Therefore, DPHM is used therapeutically to relieve symptoms of allergy which include hay fever, allergic rhinitis, cough urticaria, dermatoses and pruritis (Garrison, 1991). In addition, DPHM has been used as a hypnotic, and for the management of post-operative, pregnancy, and cancer chemotherapy related nausea and vomiting and motion sickness (Witiak and Cavestri, 1989; Garrison, 1991; Bologa *et al.*, 1994; Smith *et al.*, 1994). Common side effects resulting from DPHM use include dizziness, nervousness, drowsiness and fatigue. In addition, DPHM has anticholinergic properties that result in a "drying" of respiratory and nasal passages, and antitussive effects (Witiak and Cavestri, 1989; Garrison, 1991). At higher doses, DPHM can result in convulsions, cardiovascular and pulmonary collapse, and death (Koppel *et al.*, 1987; Garrison, 1991).

1.2.2 Pharmacokinetics

The absorption of DPHM in humans is rapid achieving peak plasma levels 2-4 hours following oral administration (Carruthers *et al.*, 1978; Blyden *et al.*, 1986; Luna *et al.*, 1989; Simons *et al.*, 1990). Following the administration of a 50 mg therapeutic dose, peak plasma concentrations between 40-100 ng/ml are achieved in healthy human adults (Albert *et al.*, 1975; Carruthers *et al.*, 1978; Blyden *et al.*, 1986; Luna *et al.*, 1989). DPHM undergoes an extensive hepatic first-pass effect following oral administration with oral bioavailability ranging from

40 to 70 % (Albert *et al.*, 1975; Carruthers *et al.*, 1978; Spector *et al.*, 1980; Berlinger *et al.*, 1982; Blyden *et al.*, 1986).

The apparent volume of distribution of DPHM ranges from ~3-7 L/kg suggesting extensive distribution within the body (Carruthers *et al.*, 1978; Spector *et al.*, 1980; Berlinger *et al.*, 1982; Blyden *et al.*, 1986). Tissue distribution studies conducted in rats and guinea pigs show that following oral, subcutaneous, intraperitoneal, or intravenous administration, the highest tissue concentrations of the drug are in the lung followed by the spleen, kidney, brain, and liver (Glazko and Dill, 1949). Similarly, DPHM concentrations were highest in lung tissue from a human patient following a fatal over dosage of the substance (Hausmann *et al.*, 1983). The high concentrations observed in lung tissue may be a result of DPHM binding to lung monoamine oxidases (Yoshida *et al.*, 1989; Yoshida *et al.*, 1990). DPHM is approximately 70-85% protein bound in plasma (Spector *et al.*, 1980; Meredith *et al.*, 1984; Zhou *et al.*, 1990). There is evidence of ethnic variations in the degree of plasma protein binding with unbound fractions being significantly higher in Orientals when compared to Caucasians (Spector *et al.*, 1980; Zhou *et al.*, 1990). Binding of DPHM to plasma albumin is low (Drach *et al.*, 1970) which suggests that like other basic amine drugs, it may bind to α_1-acid glycoprotein (Kremer *et al.*, 1988; Zhou *et al.*, 1990).

DPHM systemic total body clearance in humans ranges between 6-15 ml/min/kg with terminal elimination half-lives ranging from 3-9 h. (Carruthers *et al.*, 1978;

Spector *et al.*, 1980; Meredith *et al.*, 1984; Blyden *et al.*, 1986). There appears to be an ethnic variation in the clearance of this compound that is likely related to inter-racial differences in plasma protein binding (Spector *et al.*, 1980; Zhou *et al.*, 1990). Adult oral clearance of DPHM (20-30 ml/min/kg) is substantially higher than systemic total body clearance (Luna *et al.*, 1989; Simons *et al.*, 1990; Scavone *et al.*, 1998). The high oral clearance of DPHM is a result of the substantial first-pass effect of the compound. DPHM clearance exhibits age-dependence being the highest in children (~2 fold higher than adults) (Simons *et al.*, 1990). Studies examining differences in clearance between adults (~30 years of age) and elderly (~65-70 years of age) are inconclusive as the findings from these studies are conflicting (Simons *et al.*, 1990; Scavone *et al.*, 1998). The urinary excretion of DPHM accounts for only 2-4% of the administered dose in humans (Hald, 1947; Albert *et al.*, 1975). In rats, ~4-6% of the dose is excreted unchanged in urine (Glazko and Dill, 1949) while this value is ~21 % in rabbits (Hald, 1947). Thus far, only ~50-60% of the oral dose has been recovered in urine as either DPHM or one of its identified metabolites (Glazko *et al.*, 1974). In rats, following subcutaneous administration of ^{14}C-DPHM, only ~33% of the total administered radioactivity was excreted in urine (Glazko *et al.*, 1949). The remaining radioactivity was recovered in feces suggesting the possibility of DPHM biliary excretion and/or intestinal exsorption of the drug.

1.2.3 Metabolism

The liver appears to be the primary site for DPHM metabolism in rat, guinea pig, and rabbit as it is rapidly degraded in liver homogenate from these species (Glazko *et al.*, 1949). In addition, lung and kidney homogenates from these species have some metabolic activity, although to a much lesser extent than is observed for the liver (Glazko *et al.*, 1949). The liver appears to play a role in DPHM elimination in humans as clearance is reduced for patients with chronic liver disease (Meredith *et al.*, 1984). Evidence from experiments where the drug was incubated with liver microsomes from rat, guinea-pig and rabbit suggest that DPHM is metabolized *via* a N-demethylation followed by subsequent deamination rather than direct oxidative deamination of the dimethylamino group (Yamada *et al.*, 1993). However, it is still unclear as to whether the direct oxidative deamination of N-demethyl DPHM or oxidative deamination of N,N-didemethyl DPHM (product of further N-demethylation of N-demethyl DPHM) is quantitatively the more important pathway. Oxidative deamination of either metabolite results in the formation of the acidic metabolite, diphenylmethoxyacetic acid (DPMA).

There are obvious species differences in DPHM metabolism. In humans, rhesus monkeys, and dogs, DPHM appears to undergo successive N-demethylations to give N-demethyl DPHM and N,N-didemethyl DPHM followed by subsequent deamination to DPMA (Drach and Howell, 1968; Drach *et al.*, 1970; Chang *et al.*,

1974; Glazko *et al.*, 1974). Although all these metabolites were identified *in vivo*, the portion of the DPHM dose recovered in urine as these metabolites varies with species. In rhesus monkey, DPMA is conjugated with glutamine. DPMA and its glutamine conjugate are the main urinary metabolites in this species accounting for 4-20% and 35-59% of the dose, respectively (Drach *et al.*, 1970). In contrast, DPMA is conjugated with glycine in dogs, and the resulting glycine conjugate is the major metabolite accounting for ~33% of the dose. Furthermore, a DPHM-N-oxide metabolite was recovered in the urine of both these species accounting for 7-15% of the dose in rhesus monkey and ~25% of the dose in dogs (Drach *et al.*, 1970). In rats, DPHM, N-demethyl DPHM, N,N-didemethyl DPHM, and DPHM-N-oxide are present in rat urine. However, unlike dogs and rhesus monkeys, no trace of DPMA or its conjugates were detectable in rat urine (Drach *et al.*, 1970). In this species, most of the dose (~80%) was recovered in urine as an uncharacterized conjugate (Drach *et al.*, 1970). Thus far, DPHM, N-demethyl DPHM, N,N-didemethyl DPHM, and DPMA have been identified in human urine (Chang *et al.*, 1974). The major metabolite in human plasma and urine appears to be DPMA which is excreted in urine as both its free and conjugated form. The exact nature of the DPMA conjugate(s) is not known and remains to be identified (Chang *et al.*, 1974). More recently, a quaternary ammonium glucuronide conjugate of DPHM has been identified in human urine that may account for 2-15% of the administered dose (Luo *et al.*, 1991; Luo *et al.*, 1992; Fischer and Breyer-Pfaff, 1997). Breyer-Pfaff *et al.* (1997) have shown that this quaternary ammonium glucuronide can be formed in human liver microsomes.

Presently, it is unclear which enzymes are responsible for the metabolism of DPHM. It has been demonstrated that DPHM and its structural analogues (e.g. orphenadrine) form metabolic-intermediate complexes with cytochrome P-450's and may act as inhibitors for these enzymes (Reidy *et al.*, 1989; Rekka *et al.*, 1989; Bast *et al.*, 1990). Consistent with this hypothesis is the observation that DPHM inhibits the clearance of diltiazam in isolated perfused rat liver (Hussain *et al.*, 1994). In addition, DPHM has been observed to bind to monoamine oxidases in rat lung (Yoshida *et al.*, 1989; Yoshida *et al.*, 1990). The exact contribution to DPHM elimination of these enzymes is not known.

1.2.4 DPHM Disposition in the Pediatric Population

DPHM is commonly administered to children in cough, cold, and anti-motion sickness medications (Simons *et al.*, 1990). It is also used for the treatment of allergic reactions in children and infants (Canadian Medical Association, 1996). In pregnancy, DPHM is used frequently for severe nausea, vomiting, and urticaria (Bologa *et al.*, 1994; Smith *et al.*, 1994). Its high concentration in breast milk provides a route of exposure to the newborn (Dostal *et al.*, 1989). Thus, newborns, infants and children can be exposed to DPHM *via* various routes/ therapeutic uses. There is limited information on the pharmacokinetics of DPHM in the pediatric population. As mentioned above, DPHM clearance is higher in

children in comparison to adults (Simons *et al.*, 1990). However, there appears to be no information on DPHM disposition in human neonates and infants.

1.2.5 DPHM Pharmacokinetics in Sheep: Previous Studies

Because of its common use during pregnancy, our laboratory has previously investigated the disposition of DPHM and its metabolites, diphenylmethoxyacetic acid (DPMA) and DPHM-N-oxide (DPHMNO), in the maternal-fetal unit using chronically instrumented pregnant sheep (Yoo *et al.*, 1993; Kumar *et al.*, 1997; Kumar *et al.*, 1998; Kumar *et al.*, 1999a). As well, we have attempted to assess fetal drug elimination capacity for this compound (Kumar *et al.*, 1997). Our pregnant sheep investigations have involved the use of a two-compartment model which allows for the separation of placental and non-placental components of total drug clearance from both the maternal and fetal compartments (Szeto *et al.*, 1982). Thus, as mentioned for VPA, estimates of fetal non-placental clearance (Cl_{fo}) are considered to be a measure of the fetal lamb's intrinsic ability to eliminate the compound in question. In these studies, we found that maternal non-placental clearance (Cl_{mo}) contributed to ~95% of total maternal clearance (Kumar *et al.*, 1999a). In contrast, Cl_{fo} accounted only for ~40% of total fetal clearance (Kumar *et al.*, 1999a). Furthermore, both weight normalized fetal non-placental and renal clearances of DPHM were higher compared to the adult. In fact, weight normalized Cl_{fo} was ~3 times that observed in the ewe (i.e. Cl_{mo}) and is the highest yet found of all the drugs previously

examined in the fetal lamb (Kumar *et al.*, 1997). Our estimates of maternal (i.e. Cl_{mo}) and fetal (Cl_{fo}) non-placental clearance could be accounted for entirely by the efficient hepatic extraction of DPHM in the maternal and fetal liver, respectively (Kumar *et al.*, 1997). Although DPHM clearance in adult sheep can be attributed entirely to hepatic elimination, we have recently found evidence of significant DPHM gut uptake (Kumar *et al.*, 1999c). However, the role of the gut in fetal DPHM disposition remains to be assessed. Finally, another finding from our pregnant sheep studies was that fetal DPMA renal clearance was negligible apparently due to the limited organic acid secretion by the fetal kidney (Kumar *et al.*, 1999a). This is in contrast to the situation mentioned above for renal clearance of the parent compound.

1.3 Rationale

Development is a dynamic period during which factors affecting drug disposition are in rapid change. The use of drugs in the pediatric population sometimes cannot be avoided, and thus an understanding of developmental drug disposition will help to improve therapeutic outcomes. There are documented changes in factors such as plasma protein binding, body composition, and organ size that can influence drug distribution (Moreselli *et al.*, 1980). Also, drug elimination *via* metabolism and/or renal excretion differs between the pediatric population and the adult, and often within the pediatric population itself. The current information concerning age-related changes in drug disposition is largely gathered from

clinical observations and/or studies (Moreselli, 1976; Moreselli *et al.* 1980; Besunder *et al.*, 1988a and 1988b; Kearns and Reed, 1989; Morrow and Richens 1989; Butler *et al.*, 1994; Steinburg and Notterman, 1994; Battino *et al.*, 1995a and 1995b). There are few studies that systematically examine changes in pharmacokinetics with age as well as the nature of these changes. In fact, recently in the United States, the Food and Drug Administration (FDA) has recognized a need for information on developmental pharmacokinetics. In their "Final rule of 1998", the FDA has mandated that pediatric studies be a component of preclinical research and clinical trials of all drugs that could be of benefit to children. Thus, detailed studies investigating the ontogeny of drug disposition are warranted. Valproic acid and diphenhydramine are two drugs with contrasting pharmacokinetic and physical/chemical characteristics that we can take advantage of in our investigation of developmental pharmacokinetics.

Valproic acid is a low clearance, acidic compound with complex metabolism and exhibits saturable protein binding. An investigation of the developmental pharmacokinetics of VPA will allow us to examine the relative contribution of the pathways responsible for its metabolism (i.e. glucuronidation, β-oxidation, and P-450 metabolism), and changes that occur in their relative contributions with age. As VPA-glucuronide appears to be the primary metabolite in sheep, we expect to observe developmental alterations in total body clearance that are largely influenced by age-related changes in glucuronidation. In addition, the influence of developmental changes in plasma protein binding on total body clearance will

be investigated. Furthermore, VPA is an acidic compound and can be used to examine the development of renal excretion of organic acids. We expect to observed a gradual increase in VPA renal excretion with age similar to what has been observed for the organic acid, para-aminohippurate, in other species (Calcagno and Rubin, 1963; Kleinman and Lubbe, 1972; Horester and Lewy, 1970).

In contrast, DPHM is a high clearance compound with basic properties. A developmental study in post-natal lambs would allow us to determine if the high fetal Cl_{fo} values persist into the post-natal period. As well, the developmental disposition of DPMA and DPHMNO will be assessed. Furthermore, this study provides us with a unique opportunity to simultaneously examine developmental changes in the renal excretion of acidic (i.e. DPMA) and basic (i.e. DPHM) compounds. Data on the secretion of organic bases such as cimetidine (Mihaly *et al.*, 1983; Czuba *et al.*, 1990), meperidine (Szeto *et al.*, 1980), tetraethylammonium (Elbourne *et al.*, 1990), and ranitidine (Czuba *et al.*, 1990) in fetal lamb suggests that a decrease in renal clearance of DPHM will be observed with increasing age. The renal clearance of DPMA is expected to follow a pattern similar to what is described for VPA above as it is an acidic metabolite.

Finally, the pediatric population is exposed to both VPA and DPHM either through their direct use or indirectly through breast milk (i.e. newborns). Thus, a study of their developmental pharmacokinetics is warranted. Chronically

catheterized lambs will be used in the proposed studies in order to overcome limitations in the available sampling volume of biological fluids associated with smaller animal models, and thus allows for more detailed studies.

1.4 Objectives

The objectives of the proposed research are as follows:

1. To investigate developmental changes in factors affecting VPA drug disposition.

   More specifically we wish to investigate developmental changes in:

   A. The role of glucuronidation and β-oxidation to overall VPA metabolic elimination.

   B. VPA plasma protein binding.

   C. VPA renal excretion.

2. To study the developmental disposition of DPHM and its metabolites, DPMA and DPHMNO in post-natal lambs.

3. To examine the ontogeny of renal excretion of an acid (DPMA) *vs.* a base (DPHM).

Chapter 2

Study A: Developmental Alterations in VPA Disposition with Age

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The purpose of study A presented in this chapter is to systematically investigate alterations in the disposition of VPA and its metabolites that occur with age in post-natal lambs. Specifically, we wished to study developmental changes in VPA glucuronidation and β-oxidation as they appear to be the primary pathways responsible for VPA elimination in essentially all species previously examined (Nau and Loscher, 1984). As well, developmental changes in renal excretion were examined. In the past, we have studied VPA pharmacokinetics in pregnant and newborn (1 day old) sheep, and have found that the main metabolic pathways (i.e. glucuronidation, β-oxidation, and P-450 catalyzed pathways) and metabolites (i.e. VPA-glucuronide, 3-keto VPA) observed in humans are present in sheep (Kumar, 1998).

2.1 Methods

2.1.1 Animals and Surgical Preparation

All studies were approved by the University of British Columbia Animal Care Committee, and the procedures performed on sheep conformed to the guidelines of the Canadian Council on Animal Care.

Adult Sheep: Five non-pregnant Dorset Suffolk cross-bred ewes, with a body weight of 61.9 ± 7.3 kg (mean ± S.D.) were surgically prepared at least three days prior to experimentation. Food was withheld for ~18 h prior to surgery, but free access to water was provided. Aseptic techniques were used throughout the surgical procedure. Approximately 20-30 min before surgery, a 6 mg *i.v.* dose of atropine (Glaxo Laboratories, Montreal, Canada) was administered *via* the jugular vein to control salivation. Anesthesia was induced by *i.v.* administration of 1 g pentothal sodium (Abbott Laboratories, Montreal Canada) *via* the jugular vein. The ewe was immediately intubated and maintained on isoflurane (1%) anesthesia (Ayerst Laboratories, Montreal, Canada). Polyvinyl catheters (Dow Corning, Midland, MI) were implanted in a femoral artery and vein (catheter i.d. 1.02 mm and o.d. 2.16 mm). Catheters were tunneled subcutaneously and exteriorized *via* a small incision on the flank of the ewe. When not in use, catheters were stored in a pouch held in place with bandages. All catheters were flushed daily with approximately 2 ml of sterile 0.9% sodium chloride containing 12 units of heparin/ml to maintain catheter patency. Ampicillin (500 mg) (Novopharm, Toronto, Canada) was administered intramuscularly on the day of the surgery and for 3 days post-operatively

Following a recovery period of at least three days, the sheep were moved to a monitoring pen adjacent to and in full view of the holding pen for experimentation purposes. On the morning of the experiment, a Foley® bladder catheter (French 12) was inserted *via* the urethra of the ewe and attached to a sterile polyvinyl bag for cumulative urine collection.

Post-natal Lambs: A total of 17 Dorset Suffolk cross-bred lambs were employed in this study. Lambs were divided into a 10 day old group (n=8), a 1 month old group (n=4), and a 2 month old group (n=5). All lambs were surgically prepared at least 3 days prior to the experiment under isoflurane (1%) anesthesia (Ayerst Laboratories, Montreal, Canada). Briefly, polyvinyl catheters (Dow Corning, Midland, MI) were implanted in a carotid artery and a jugular vein (catheter i.d. 1.02 mm and o.d. 2.16 mm). A third larger diameter catheter (i.d. 3.0 mm and o.d. 4.5 mm) was implanted in the urinary bladder *via* a lower abdominal midline incision. The catheters were tunneled subcutaneously and exteriorized *via* a small incision either between the shoulder blades (carotid artery and jugular vein catheters) or on the flank of the lamb (urinary bladder catheter). When not in use, catheters were stored in a pouch held in place with bandages. As with the adult animals, catheters were flushed daily with ~2 ml of heparinized saline. Ampicillin (500 mg) (Novopharm, Toronto, Canada) was administered intramuscularly on the day of the surgery and for 3 days post-operatively. Following surgery, animals were returned to holding pens with their mothers and allowed three days to recover prior to experimentation.

Following the recovery period, the lambs were moved to monitoring pens adjacent to and in full view of their mothers. The urinary bladder catheter was allowed to drain by gravity into a sterile reservoir. While in the holding pens, lambs were fed Deluxe Lamb Milk Replacer (Canadian Nurs-ette Distributor Ltd., Canrose, AB, Canada) and had free access to hay, grain, and water.

2.1.2 Experimental Protocols

All sheep experiments involved administration of an *i.v.* bolus dose of VPA (Sodium Valproate, Sigma Chemical Co., St. Louis, MO) equivalent to 10 mg VPA/kg body weight. All doses were prepared in sterile water for injection and were sterilized by filtering through a 0.22 μm nylon syringe filter (MSI, Westboro, MA) into a capped empty sterile injection vial. The dose was administered over 1 min *via* the jugular vein (lambs) or femoral vein (ewes). Post-natal lamb experiments were initiated at approximately 10 days, 1 month, or 2 months following birth depending on which group the lambs had been assigned to. For post-natal lambs, serial blood samples (~2 ml) were collected from the carotid artery at 5, 15, 30, 45, 60 min, and 2, 4, 6, 9, 12, 24, 36, 48, 60, 72 h following drug administration. Serial blood samples (~3 ml) were collected for adult sheep from the femoral artery at 5, 15, 30, 45, 60 min, and 2, 4, 6, 9, 12, 15, 24, 36 h following drug administration. Cumulative urine was also collected for both adult sheep and post-natal lambs for the full duration of the experiment (i.e. 72 h for lambs and 36 h for adult sheep). The only exceptions were the entire 1 month group (n=4), four lambs in the 10 day old group, and one lamb in the 2 month old group where urine collection

was incomplete due to catheter failure; these were excluded from analysis of the urinary data.

All blood samples collected were placed into heparinized Vacutainer® tubes (Becton-Dickinson, Rutherford, NJ) and centrifuged at 2000 × *g* for 10 min. The plasma supernatant was removed and placed into clean borosilicate test tubes with polytetrafluoroethylene-lined caps. Plasma and urine samples were stored frozen at -20°C until the time of analysis.

2.1.3 Determination of VPA Plasma Protein Binding

Unbound plasma concentrations of VPA were determined *ex vivo* in all adult sheep and post-natal lamb plasma samples by an ultrafiltration procedure at 1000 x *g* for 30 min using Centrifree® micropartition devices (Amicon, Inc., Danver, MA). Plasma samples (~1 ml) for the determination of unbound VPA concentrations were stored in separate aliquots so as to avoid repetitive thawing that could result in lipolysis and release of free fatty acids and hence competitive displacement of bound VPA from plasma binding sites (Haberer and Pollack, 1994).

2.1.4 Drug and Metabolite Assay

Concentrations of VPA and 12 of its metabolites (i.e. (E)-2-ene VPA, (E)-3-ene VPA, 4-ene VPA, (E,E)-2,3'-diene VPA, (E)-2,4-diene VPA, 3-keto VPA, 4-keto VPA, 3-OH VPA, 4-OH VPA, 5-OH VPA, 2-PSA and 2-PGA) in all biological fluids and plasma

ultrafiltrate were measured using an established gas chromatographic-mass spectrometric (GC-MS) analytical method (Yu *et al.*, 1995). Briefly, the procedure involves acidification of biological fluid samples (with appropriate internal standards added) to pH 3.0-3.5 with 1*M* HCl. Next, the samples are extracted twice with 3 ml ethyl acetate (Caledon Laboratories, Georgetown, Canada) on a rotary mixer for 30 min each. Any absorbed water from the combined ethyl acetate extract is removed by vortexing with anhydrous sodium sulfate (BDH Chemical Co, Toronto, Canada). The dry extract is then concentrated to ~100 µl under a gentle stream of nitrogen followed by the addition of a 50 µl aliquot of MTBSTFA (Pierce Chemical Co., Rockville, IL) and derivatization by heating at 60°C for 1 h. Following derivatization, a 1 µl aliquot is injected into the GC-MS (Hewlett-Packard, Avondale, PA) in splitless mode. Chromatographic separations were performed on a DB1701 (30 m x 0.25 mm i.d., 0.25 µm film thickness) fused silica capillary column (J&W Scientific, Folsom, CA) with helium as the carrier gas at a 15 psi column head pressure. GC operating conditions were as follows. The injection port temperature was 250°C. Oven temperature programing consisted of an initial temperature of 80°C (0.1 min hold time), a 10°C/min ramp to 100°C (0.1 min hold time), a 2°C/min ramp to 130°C (0.1 min hold time), and a 30°C/min ramp to 260°C (8 min hold time). The resulting total run time was 29.5 min. The mass spectrometer was operated in electron impact ionization mode (electron ionization energy 70eV) with selected ion monitoring (EI-SIM) at transfer line and ion source temperatures of 280°C and 180°C, respectively. Calibration curve concentration ranges for VPA and various metabolites, internal standards utilized for each compound, and the ions monitored are presented in Table 2.1. Previous assay validation studies have established that the

variability and bias of this assay for all the compounds within these concentration ranges does not exceed 15% (Yu *et al.*, 1995). VPA metabolites used for the standard calibration curves were synthesized and purified as reported elsewhere (Acheampong *et al.*, 1983; Acheampong and Abbott, 1985; Lee *et al.*, 1989; Yu *et al.*, 1995).

Table 2.1 Calibration curve concentration range of VPA and its metabolites and their corresponding internal standards that were used for their quantitation using the GC-MS assay. Ions that were monitored for each compound during selected ion monitoring are also presented.

Analytes to be quantitated			**Internal Standards**	
Analyte	Calibration curve range (μg/ml)	Ion monitored (m/z)	Compound	Ion monitored (m/z)
VPA	0.025-20.0	201	[$^2H_7$]VPA	208
(E)-2-ene VPA	0.01-8.0	199	(E)-2-ene[$^2H_7$]VPA	206
(E)-3-ene VPA	0.0025-2.0	199	(E)-2-ene[$^2H_7$]VPA	206
4-ene VPA	0.0025-2.0	199	(E)-2-ene[$^2H_7$]VPA	206
(E,E)-2,3'-diene VPA	0.0025-2.0	197	(E)-2-ene[$^2H_7$]VPA	206
(E)-2,4-diene VPA	0.0075-6.0	197	(E)-2-ene[$^2H_7$]VPA	206
3-keto VPA	0.02-4.0	329	3-keto[$^2H_7$]-VPA	336
4-keto VPA	0.0025-2.0	215	4-keto[$^2H_7$]- VPA	222
3-OH VPA	0.02-2.0	217	3-OH[$^2H_7$]- VPA	224
4-OH VPA	0.04-4.0	100	[$^2H_7$]-VPA	208
5-OH VPA	0.02-2.0	331	5-OH [$^2H_7$]-VPA	338
2-PSA	0.01-2.0	331	2-MGA	317
2-PGA	0.01-2.0	345	2-MGA	317

VPA glucuronide concentrations in both adult and lamb urine were measured using a base hydrolysis procedure described as follows. Urine samples were adjusted to pH 12.5, incubated at 60° C for 1 h, and the total VPA (unconjugated + conjugated) was quantified by the above GC-MS analytical method. The concentration of VPA glucuronide was estimated as the difference between total and unconjugated (unhydrolyzed) VPA concentrations. This procedure was preferred over hydrolysis with β-glucuronidase because VPA glucuronide has been shown to rearrange to at least six β-glucuronidase-resistant structural isomers *via* migration of the acyl moiety away from the C-1 position and subsequent ring opening, mutarotation and lactone formation (Dickinson *et al.*, 1984). These rearrangements are pH, temperature and storage time dependent (Dickinson *et al.*, 1984). Hydrolysis with alkali, however, is capable of measuring total VPA-glucuronide in spite of these possible rearrangements (Dickinson *et al.*, 1984).

2.1.5 Pharmacokinetic Analyses

Pharmacokinetic parameters were calculated by standard methods as described in Gibaldi and Perrier (1982). Systemic total body clearance of total and unbound drug was calculated non-compartmentally as the *i.v.* dose divided by the $AUC_{0-\infty}$ of total and unbound VPA, respectively. Renal clearance of total and unbound drug was calculated as the cumulative amount of unchanged drug excreted in urine divided by the $AUC_{0-\infty}$ of total and unbound VPA, respectively. Terminal elimination half-life ($t_{1/2\beta}$) of the total and unbound VPA in plasma was obtained from a 2-compartment model fitting of the data

using the nonlinear least-squares regression software WinNonlin (Scientific Consulting, Inc., Apex, NC). Model fittings were carried out using a weighting factor of 1/predicted y^2. Plasma $AUC_{0-\infty}$ and $AUMC_{0-\infty}$ of total and unbound drug were calculated by the linear trapezoid rule.

Due to the nonlinear/saturable nature of VPA plasma protein binding, the parameters f_p (area weighted unbound fraction of the drug) and Vd_{ss}' (steady state volume of distribution parameter corrected for the effects of saturable protein binding) were also calculated as follows:

$$f_p = \frac{AUC_{0-\infty}(unbound\ VPA)}{AUC_{0-\infty}(total\ VPA)} \qquad \textbf{(Equation 2.1)}$$

$$Vd_{ss}' = f_p * Vd^u_{ss}\ (steady\text{-}state\ volume\ of\ distribution\ based\ upon\ unbound\ drug\ concentrations) \qquad \textbf{(Equation 2.2)}$$

For drugs exhibiting saturable protein binding, the steady-state volume of distribution parameter (Vd_{ss}) when calculated using the 'model independent' approach (Gilbaldi and Perrier, 1982) overestimates the "true" Vd_{ss} and is concentration dependent (McNamara *et al.*, 1983). Similarly, Vd^u_{ss} is constant only for a particular f_p value and can be used to relate steady-state plasma concentrations to the amount of the drug in the body if steady-state unbound fraction of the drug is equal to f_p (McNamara *et al.*, 1983). Thus, both Vd_{ss} and Vd^u_{ss} are poor indicators of drug distribution. Instead, the Vd_{ss}' parameter is more reflective of shifts in drug mass into or out of the vascular space (i.e. information traditionally provided by the Vd_{ss} parameter) (McNamara *et al.*, 1983). As with Vd^u_{ss}

above, this Vd_{ss}' parameter is also constant only for a particular f_p or a steady-state plasma unbound fraction equivalent to f_p.

2.1.6 Statistical Analysis

All data are reported as mean ± S.D. Pharmacokinetic parameters were compared using ANOVA followed by a Fischer's LSD multiple comparison test. The significance level was $p < 0.05$ in all cases.

2.2 Results

2.2.1 Pharmacokinetics of VPA in Post-natal Lambs and Adult Sheep

Average age of the post-natal lamb groups on the day of their experiments was 10.9 ± 1.4 days for the 10 day old group (10 d), 30 ± 1.4 days for the one month old group (1 M), and 60.0 ± 1.0 days for the two month old group (2 M). Mean adult ewe body weight was 61.9 ± 7.3 kg, and mean lamb body weights were 5.8 ± 1.4 kg (10 d), 9.4 ± 1.7 kg (1 M), and 13.2 ± 2.1 kg (2M). Figures 2.1 A-D are semilogarithmic plots of mean VPA (unbound and total) concentration *vs.* time for all age groups. Following the administration of a 10 mg/kg VPA *i.v.* bolus, mean plasma levels at the first sampling time (i.e. 5 min following drug administration) were at or near the human therapeutic range (i.e. 50-100 μg/ml). Total and unbound VPA plasma profiles in lambs and adult sheep appeared to be biexponential in nature. In addition, VPA plasma protein binding was saturable (nonlinear) for all age groups. At the first sampling point, where measured VPA concentrations were the greatest (i.e. mean VPA concentration range 48-73 ug/ml), the mean unbound fraction was 0.37 ± 0.11 in 10 d lambs, 0.34 ± 0.12 in 1 M lambs, 0.22 ± 0.04 in 2 M lambs, and 0.22 ± 0.05 in adult sheep. Characteristic of nonlinear plasma protein binding, unbound fractions proceeded to decline with decreasing VPA concentrations in all animals such that at 12 h following drug administration (mean VPA concentration range 2-15 μg/ml), the mean unbound fraction in plasma was 0.11 ± 0.09 in 10 d lambs, 0.06 ±0.01 in 1 M lambs, and 0.04 ± 0.02 in 2 M lambs and adult sheep.

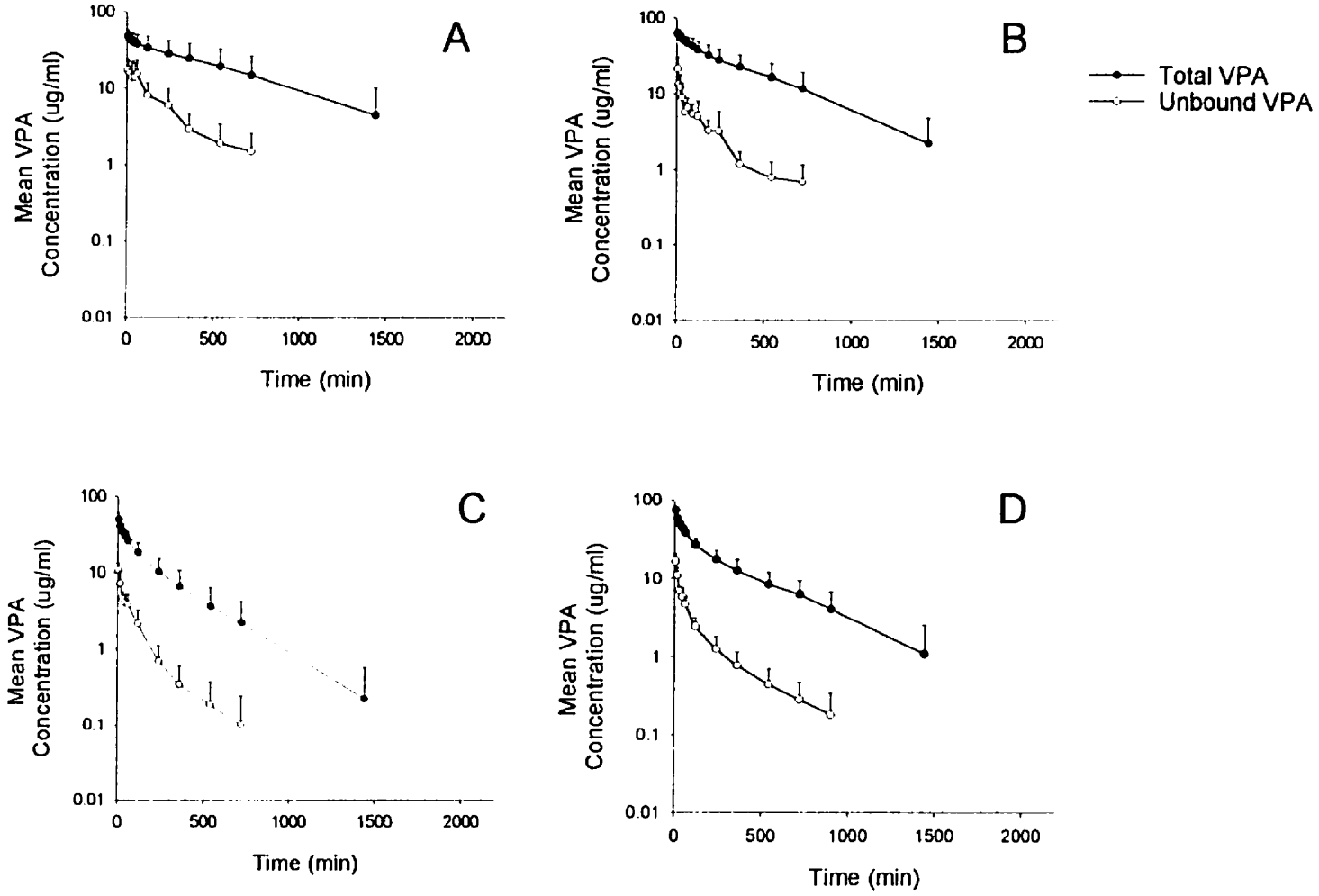

Figure 2.1 Mean VPA (total and unbound) plasma concentrations *vs.* time profiles in **(A)** 10 day old lambs, **(B)** 1 month old lambs, **(C)** 2 month old lambs, and **(D)** adult sheep following VPA *i.v.* bolus administation.

Figure 2.2. depicts age-related changes in VPA total body clearance for both total and unbound drug. VPA clearance for total (Cl_{tb}) and unbound (Cl^{u}_{tb}) drug increased significantly from 10 days to 2 months of age, before decreasing to adult levels (Figure 2.2, Table 2.2). Due to the nonlinear nature of VPA plasma protein binding, differences in clearance were more evident when examining unbound drug concentrations (Figure 2.2). Additional pharmacokinetic parameters for unbound and total VPA for all age groups are presented in Table 2.2. As with total body clearance, age-related alterations in terminal elimination half-life ($t_{1/2\beta}$) and mean residence time (MRT) were more pronounced for unbound VPA. Both, $t^{u}_{1/2\beta}$ (terminal elimination half-life of unbound drug) and MRT^{u} (mean residence time of unbound drug) were significantly longer for 10 d lambs than for other age groups. No differences in Vd_{ss} and Vd_{ss}' were observed between age groups; however; Vd^{u}_{ss} was significantly lower in the 10 d lambs. Furthermore, the area weighted unbound fraction (f_p) was significantly higher in 10 d lambs when compared to the corresponding values from the other age groups.

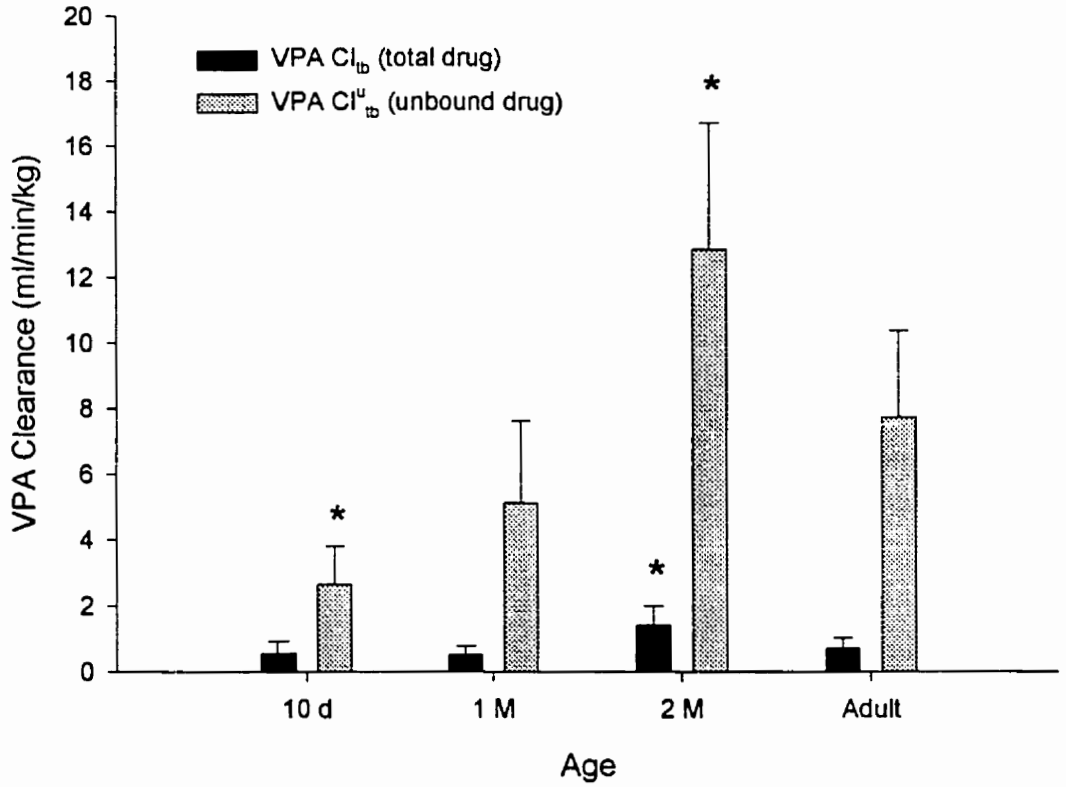

Figure 2.2 Alterations in mean VPA (total and unbound) total body clearance with age. * denotes significant difference from adult values ($p<0.05$).

Table 2.2 Valproic acid pharmacokinetic parameters in 10 day old, 1 month old, 2 month old and adult sheep.

AGE	TOTAL VPA					UNBOUND VPA				
	Cl_{tb} (ml/min/kg)	$t_{1/2\beta}$ (h)	MRT (h)	Vd_{ss} (L/kg)	Vd_{ss}' (L/kg)	Cl^{u}_{tb} (ml/min/kg)	$t^{u}_{1/2\beta}$ (h)	MRT^{u} (h)	Vd^{u}_{ss} (L/kg)	f_p
10 day (n = 8)	0.55 ± 0.38	7.0 ± 3.4	10.0 ± 4.9[a]	0.22 ± 0.08	0.14 ± 0.06	2.65 ± 1.16[a]	10.6 ± 8.5[b]	6.7 ± 2.8[b]	0.79 ± 0.32[b]	0.21 ± 0.12[b]
1 month (n = 4)	0.50 ± 0.27	5.2 ± 1.1	7.2 ± 1.9	0.19 ± 0.0.7	0.14 ± 0.07	5.11 ± 2.49	2.5 ± 1.2	3.2 ± 1.6	1.48 ± 0.32	0.10 ± 0.01
2 month (n = 5)	1.40 ± 0.58[a]	2.6 ± 0.7	3.5 ± 1.1	0.30 ± 0.06	0.16 ± 0.03	12.84 ± 3.88[a]	1.5 ± 0.5	1.8 ± 0.6	1.54 ± 0.39	0.11 ± 0.03
Adult (n = 5)	0.70 ± 0.31	4.2 ± 2.0	5.7 ± 2.8	0.24 ± 0.03	0.13 ± 0.02	7.73 ± 2.64	1.7 ± 0.7	2.1 ± 0.9	1.47 ± 0.51	0.09 ± 0.02

[a] statistically different from adult age group ($p<0.05$).
[b] statistically different from 1 month, 2 month, and adult age groups ($p<0.05$).

2.2.2 VPA Metabolites in Post-natal Lamb and Adult Sheep Plasma

Plasma samples collected from post-natal lambs and adult sheep were analyzed for VPA metabolites generated from fatty acid β-oxidation ((E)-2-ene, (E)-3-ene, and 3-keto VPA), and cytochrome P-450-mediated desaturation (4-ene VPA) and hydroxylation and subsequent oxidation (3-OH VPA, 4-OH, 5-OH, and 4-keto VPA, 2-PSA, and 2-PGA). The diunsaturated metabolites (see Figure 1.2) were not observed in both preliminary experiments in this study or in previous studies with fetal, newborn, and pregnant sheep (Kumar, 1998), and thus were not monitored. The main metabolites observed in lamb and adult sheep plasma were the β-oxidation metabolites 3-keto and (E)-2-ene VPA. This was followed by the cytochrome P-450 generated, 4-OH VPA. Figures 2.3 A-C show mean plasma profiles of these three metabolites for lambs and adult sheep following *i.v.* bolus administration of the drug. The maximal plasma concentration (C_{max}), time of occurrence of the maximal plasma concentration (t_{max}), and $AUC_{0-\infty}$ for the three mentioned metabolites are presented in Table 2.3. In general, metabolite profiles appeared to be more similar between 10 d and 1 M lambs in comparison to 2 M lambs and adult sheep. All three metabolites could be detected for a longer period of time in the younger animal groups (10 d and 1 M). The C_{max} of 3-keto and (E)-2-ene VPA were both significantly higher in 10 d and 1 M lambs when compared to 2 M lambs and adult sheep (Table 2.3). Also, the mean t_{max} values of 3-keto, (E)-2-ene, and 4-OH VPA were significantly longer for the 10 d group than for all the other age groups. Mean 3-keto $AUC_{0-\infty}$ values were significantly larger (~ 7 to 10 fold) for the two younger age groups (i.e. 10 d and 1 M).

Similarly, $AUC_{0-\infty}$ estimates for (E)-2-ene and 4-OH VPA were also significantly larger in the two younger groups (i.e. ~13 to 16 fold for (E)-2-ene VPA and ~3 to 5 fold for 4-OH VPA). Accurate estimates of $AUC_{0-\infty}$ could not be obtained for 5-OH, (E)-3-ene, 4-ene, and 4-keto VPA due to their low plasma concentrations (i.e. C_{max} values of < 0.2 μg/ml). Plasma concentrations of 3-OH VPA, 2-PGA, and 2-PSA were almost always below the limit of quantification (LOQ) of the assay (Yu *et al.*, 1995) (see p. 32).

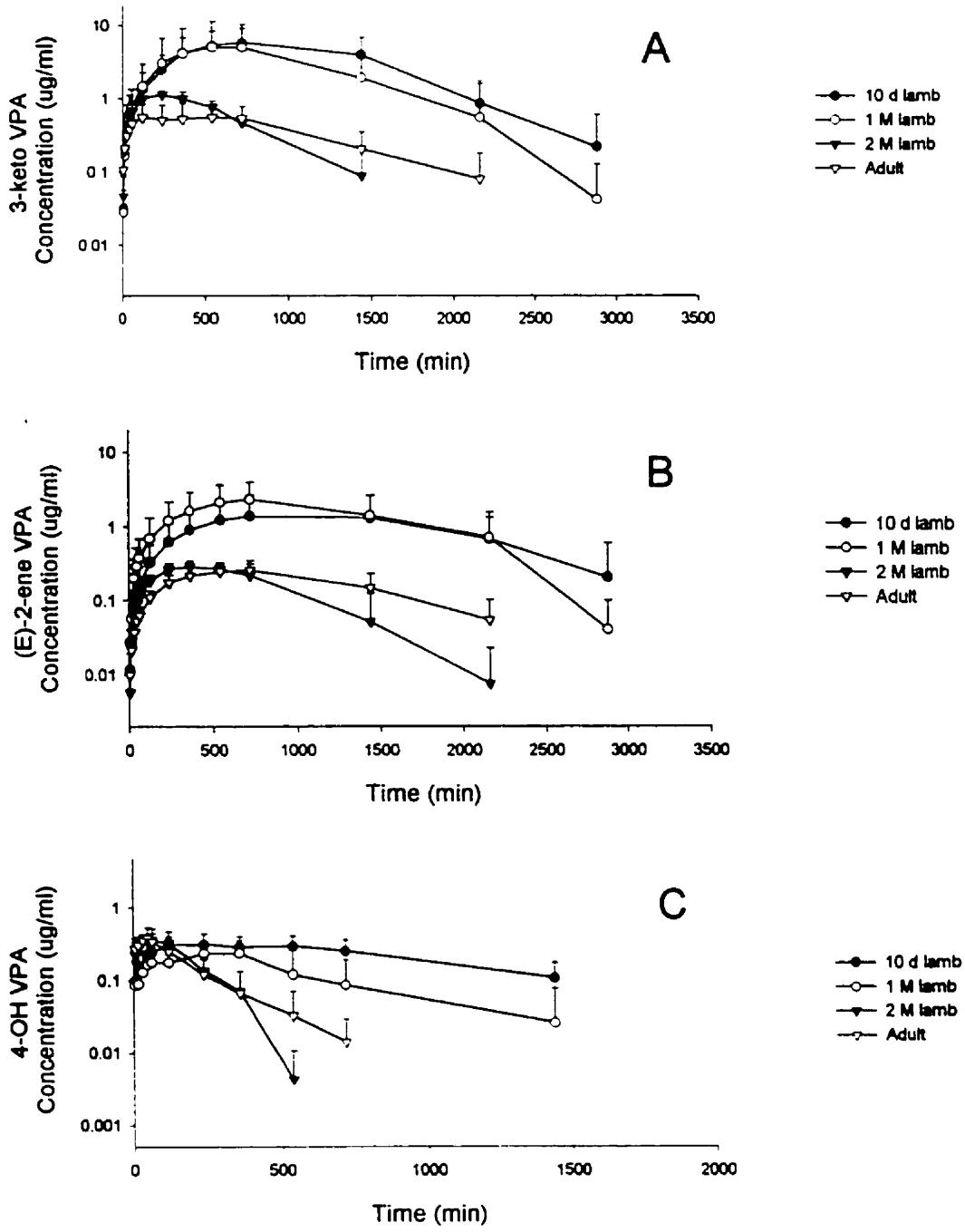

Figure 2.3 Mean metabolite plasma concentration *vs.* time profiles in 10 day old (10 d), 1 month old (1 M), 2 month old (2 M), and adult sheep for **(A)** 3-keto VPA, **(B)** (E)-2-ene VPA, and **(C)** 4-OH VPA.

Table 2.3 C_{max}, t_{max}, and $AUC_{0-\infty}$ of VPA metabolites in plasma following a 10 mg/kg VPA *i.v.* bolus.

METABOLITE		AGE			
		10 day (n = 8)	**1 month** (n = 4)	**2 month** (n = 5)	**Adult** (n = 5)
3-keto VPA	C_{max} (μg/ml)	6.83 ± 3.05[a]	5.66 ± 6.10[a]	1.20 ± 0.12	0.67 ± 0.26
	t_{max} (h)	13.3 ± 4.9[a]	8.5 ± 4.1	3.6 ±1.7	7.0 ± 3.7
	$AUC_{0-\infty}$ (μg*h/ml)	150.4 ± 69.7[a]	103.7 ± 103.3[a]	14.4 ± 4.1	13.8 ± 6.7
(E)-2-ene VPA	C_{max} (μg/ml)	1.76 ± 1.45[a]	2.32 ± 1.60[a]	0.30 ± 0.05	0.27 ± 0.08
	t_{max} (h)	18.4 ± 7.0[b]	11.3 ± 1.5	6.8 ± 3.0	12.0 ± 2.1
	$AUC_{0-\infty}$ (μg*h/ml)	47.2 ± 44.1[a]	57.1 ± 39.6[a]	3.5 ± 1.4	6.8 ± 2.8
4-OH VPA	C_{max} (μg/ml)	0.34 ± 0.14	0.28 ± 0.10	0.42 ± 0.09	0.40 ± 0.14
	t_{max} (h)	4.6 ± 4.2[a]	3.3 ± 2.2	0.75 ± 0.25	0.55 ± 0.21
	$AUC_{0-\infty}$ (μg*h/ml)	7.4 ± 2.7[a]	4.7 ± 2.8[a]	1.5 ± 0.5	1.6 ± 0.8

[a] statistically different from 2 month and adult age groups ($p<0.05$).
[b] statistically different from 1 month, 2 month, and adult age groups ($p<0.05$).

2.2.3 Urinary Excretion of VPA and its Metabolites

Urinary recovery of the VPA dose as the parent compound and its metabolites for 10 d, 2 M, and adult sheep is presented in Table 2.4. The majority of the administered dose was recovered in urine as VPA, VPA-glucuronide, 3-keto VPA, and 4-OH VPA. All other metabolites (3-OH, 5-OH, (E)-2-ene, (E)-3-ene, 4-ene, and 4-keto VPA, 2-PSA and 2-PGA) individually accounted for less than 1% of the administered dose in all age groups. When added together these minor metabolites combined to account for 1.1 $\pm$ 0.6 % of the dose in 10 d lambs, 1.4 $\pm$ 0.2 % of the dose in 2 M lambs, and 2.2 $\pm$ 0.9 % of the dose in adult sheep. For both 2 M lambs and adult sheep, essentially the entire dose was recovered in urine during the experimental period with the major component being in the form of the glucuronide metabolite (Table 2.4). However, for 10 d lambs only ~50% of the dose could be accounted for. The percent of the dose recovered as the parent compound was significantly lower in lambs when compared to adult sheep. Similarly, recovery of the parent compound as VPA-glucuronide and 4-OH VPA was significantly less in 10 d lambs. No differences were found in the percent of the dose excreted in urine as 3-keto VPA.

Figure 2.4 depicts age–related alterations in renal clearance for unbound (Cl^u_r) and total VPA (Cl_r). As with total body clearance, changes in VPA renal clearance were more apparent when examining unbound drug concentrations. Consistent with the mass balance data mentioned above, renal clearance estimates for unbound VPA from both lamb groups (10 d Cl^u_r = 0.28 $\pm$ 0.22 ml/min/kg and 2 M Cl^u_r = 0.39 $\pm$ 0.21 ml/min/kg) were

significantly lower than adult values (0.97 $\pm$ 0.43 ml/min/kg). Figure 2.5 displays changes in Cl_r of 3-keto and 4-OH VPA with age. Similar to unbound VPA, Cl_r of both these metabolites increased significantly with age reaching adult levels by 2 months after birth. We could not determine unbound concentrations of VPA metabolites due to limited plasma samples, therefore Cl^u_r could not be calculated for 3-keto and 4-OH VPA. Renal clearance values were not calculated for other metabolites due to either their low plasma concentrations and/or trace levels excreted in urine.

Table 2.4 Recovery of VPA and its metabolites as a percentage of the total dose in 10 day old, 2 month old, and adult sheep after administration of a 10 mg/kg *i.v.* bolus.

DRUG OR METABOLITE	% OF ADMINISTERED DOSE RECOVERED IN URINE		
	10 day (n = 4)	2 month (n = 4)	Adult (n = 5)
VPA	5.6 ± 3.1[a]	3.0 ± 1.3[a]	12.2 ± 2.2
VPA-glucuronide	29.2 ± 16.0[b]	74.6 ± 3.2	73.8 ± 5.3
3-keto VPA	12.6 ± 9.8	13.2 ± 5.8	11.3 ± 6.5
4-OH VPA	0.6 ± 0.4[b]	1.6 ± 0.3	2.0 ± 0.6
Other[c]	1.1 ± 0.6	1.4 ± 0.2	2.2 ± 0.9
% of Dose Recovered	49.1 ± 25.6	93.7 ± 9.6	101.5 ± 8.5

[a] statistically different from adult age group ($p<0.05$).
[b] statistically different from 2 month and adult age groups ($p<0.05$).
[c] includes 3-OH, 5-OH, (E)-2-ene, (E)-3-ene, 4-ene, and 4-keto VPA, 2-PSA and 2-PGA.

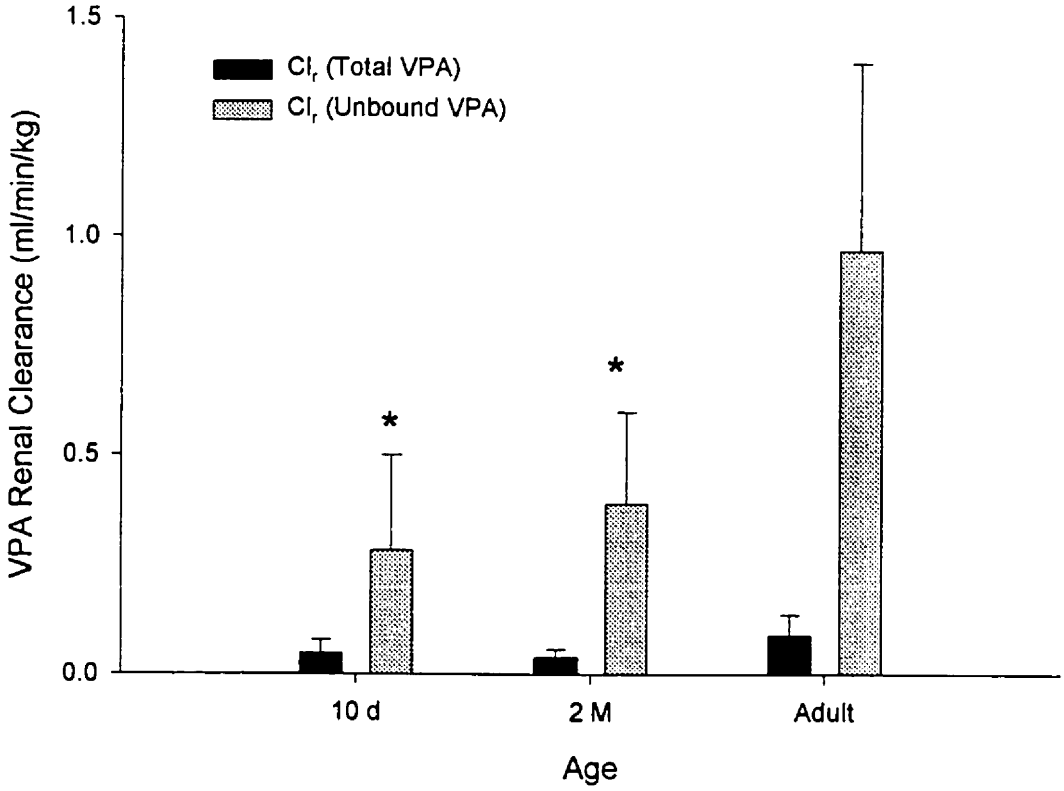

Figure 2.4. Changes in VPA renal clearance with age in 10 day old (10 d), 2 month old (2 M), and adult sheep for total and unbound drug. *denotes significant difference from the adult value ($p<0.05$).

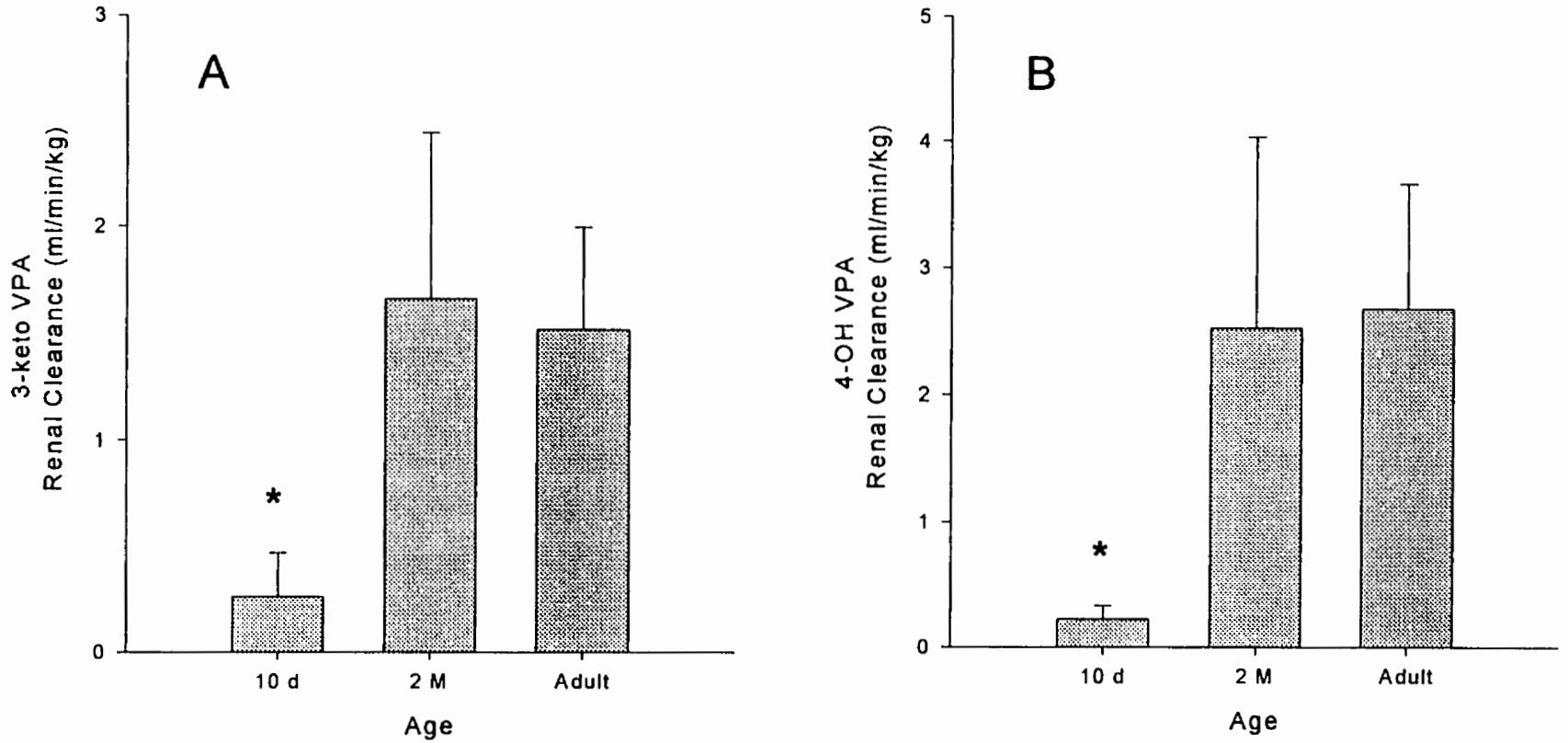

Figure 2.5 Changes in renal clearance with age in 10 day old (10 d), 2 month old (2 M), and adult sheep for **(A)** 3-keto VPA and **(B)** 4-OH VPA. *denotes significant difference from the adult value ($p<0.05$).

2.3 Discussion

2.3.1 Developmental Changes in VPA Pharmacokinetics

The results of this study indicate that VPA plasma protein binding is nonlinear in nature at therapeutic concentrations for all age groups. This is consistent with previous observations in sheep (Kumar, 1998), rats (Haberer and Pollack, 1994), guinea pigs (Yu and Shen, 1992), and humans (Scheyer *et al.*, 1990). Thus, for VPA, unbound drug clearance is more reflective of metabolic clearance. A species comparison of pharmacokinetic parameters revealed that in general, VPA clearance is slightly higher in sheep when compared to humans. Clearance estimates from human neonates 6 to 9 days of age (Cl_{tb}: 0.1-0.2 ml/min/kg and Cl^{u}_{tb}: 0.7-1.2 ml/min/kg), and newborns ~ 1 month of age (Cl_{tb}: 0.2-0.5 ml/min/kg and Cl^{u}_{tb}: ~4 ml/min/kg) were lower than their corresponding lamb values (Table 2.2) (Irvine-Meek *et al.*, 1982; Gal *et al.*, 1988). Furthermore, similar findings were observed in comparisons between adult humans (Cl_{tb}: 0.1-0.3 ml/min/kg and Cl^{u}_{tb}: 1-3 ml/min/kg (Davis *et al.*, 1994; Levy and Shen, 1995)) and sheep (Table 2.2). The trend observed in previous investigations in developing rats (Haberer and Pollack, 1994) and guinea pigs (Yu *et al.*, 1985; Yu *et al.* 1987), was a detectable increase in VPA metabolic clearance with age. In contrast, we observed a rise in Cl_{tb} and Cl^{u}_{tb} until 2 months of age, before decreasing to adult levels. This pattern of change is similar to what is observed in humans where metabolic clearance is lowest in newborns, reaches a maximum from 2-36 months of age, and then decreases to adult values (Levy and Shen, 1995). The relatively lower VPA clearance in 10 d lambs is reflected in the longer $t_{1/2}$ and

MRT estimates observed for both unbound and total drug. Similarly, reported VPA elimination half-lives in human neonates (17-80 h) are substantially longer than for adult subjects (9-18 h) (Davis *et al.*, 1994; Levy and Shen, 1995). Vd_{ss} and Vd_{ss}' estimates for all age groups were similar to reported values in humans (0.13-0.20 L/kg) (Davis *et al.*, 1994; Levy and Shen, 1995). The significantly lower Vd^{u}_{ss} value for the 10 d lambs is likely related to their higher f_p value since the two parameters are inversely related (McNamara *et al.*, 1983).

2.3.2 Age-Related Differences in VPA Metabolites in Plasma

By far the most prevalent metabolites observed in sheep plasma were the β-oxidation metabolites, 3-keto and (E)-2-ene VPA followed by the P-450 mediated hydroxylation metabolite, 4-OH VPA. The presence of 3-keto and (E)-2-ene VPA as the two most prominent metabolites in plasma is similar to previous observations in humans, dogs, rats and mice (Nau and Loscher, 1984). All three metabolites appeared to persist longer in the two younger animal groups as evident by their significantly larger $AUC_{0-\infty}$ values. Also, 3-keto and (E)-2-ene VPA C_{max} values for 10 d and 1 M lambs were significantly greater (~5-10 fold) when compared to 2 M lambs and adult sheep (Table 2.3). Although detailed data on VPA metabolism is unavailable in human neonates, similarities exist between our data and information available on plasma serum profiles from human epileptic children (Nau *et al.*, 1991; Siemes *et al.*, 1993). Similar to the two younger animal groups, higher concentrations of β-oxidation metabolites (i.e. 3-keto, 2-ene, 2,3'-diene and 3-ene VPA) were observed in children < 2 years of age in comparison to children > 2 years of age.

Also, 4-OH VPA concentrations, although not significant, were slightly higher in children < 2 years old. The higher observed $AUC_{0-\infty}$ values for the 3-keto, (E)-2-ene, and 4-OH VPA in the younger lambs may be related to either an increased formation and/or a reduced elimination of these metabolites.

2.3.3 Developmental Changes in Renal Excretion of VPA and its Metabolites

As a component of this study, we examined post-natal development in VPA renal elimination. As mentioned, VPA Cl^u_r increased progressively with age. The mechanisms responsible for the tubular secretion of organic acid are not fully functional in late gestational lamb (Jones and Stapleton, 1992). We have observed in previous studies, using pregnant sheep, that the urinary excretion of acidic compounds such as indomethacin (Krishna *et al.*, 1995) and VPA (Kumar, 1998) by the fetal lamb is limited. Post-natal increases in the tubular secretion of organic acids have been attributed to the development of renal organic anion transporters in the kidney (Jones and Stapleton, 1992). Studies examining this area have largely focused on the renal tubular transport of para-aminohippurate (Jones and Stapleton, 1992). Observations in newborn humans (Calcagno and Rubin, 1963), dogs (Kleinman and Lubbe, 1972) and rats (Horster and Lewy, 1970) have demonstrated a gradual increase in renal tubular extraction of para-aminohippurate with age. The post-natal increase in VPA Cl^u_r is likely due to the development of similar mechanisms. As well, age-related increases in renal blood flow and glomerular filtration rate may also play a role (Jones and Stapleton, 1992). Our current study indicates that by 2 months of age VPA Cl^u_r has not yet reached adult

levels (Figure 2.4). The significantly lower Cl^{u}_{r} in 10 d and 2 M lambs is reflected in the lower percentage of the dose recovered as the parent compound in post-natal lamb urine when compared to adult sheep (Table 2.4). Renal excretion of unchanged VPA accounts for a larger percentage of the administered dose in sheep (~ 12% in adult) in comparison to humans (~2-3%) (Levy and Shen, 1995). This observed difference has been previously attributed to species differences in VPA renal clearance (Kumar, 1998). Despite playing a larger role in sheep, renal excretion of the unchanged drug remains a minor route of elimination.

VPA-glucuronide is by far the major metabolite recovered in urine accounting for ~74% of the dose in adult sheep. This is at the high end of the range of values previously reported for humans (10-70%) (Gugler *et al.*, 1977; Dickinson *et al.*, 1989; Levy *et al.*, 1990). The percentage of the dose metabolized *via* glucuronidation appears to increase with age. Our data in 10 d lambs indicates that a significantly smaller portion of the dose is recovered as VPA-glucuronide (i.e. ~29%) in younger animals. This is in excellent agreement with previous studies in 1 day old lambs where ~28% of the administered dose was recovered as the glucuronide metabolite (Kumar, 1998). A similar phenomenon was observed in studies involving acetaminophen where the percentage of the dose that was glucuronidated in newborn lambs (46 $\pm$ 11%) was less in comparison to the adult (64 $\pm$ 4%) (Wang *et al.*, 1990). By 2 months of age, recovery of VPA-glucuronide in urine accounts for ~75% of the administered dose suggesting a rapid postnatal development of the glucuronidation pathway responsible for VPA metabolism (Table 2.4). This increase in VPA glucuronidation coincides with the

significant increases in unbound and total VPA clearance mentioned above. Although the effects of advancing age on VPA glucuronidation have been investigated previously using rat hepatic S9 fractions (age range: 2.5 - 24 month old rats; Chen *et al.*, 1996), there are no detailed studies examining the role of glucuronidation in overall VPA elimination early on in development. Our data suggests that glucuronidation may play an important role in changes in VPA disposition that occur during the first two months of life.

Approximately 11% of the administered dose was recovered in adult sheep urine as the β-oxidation metabolite, 3-keto VPA (Table 2.4). In contrast to VPA-glucuronide, this is at the lower end of the range of values previously reported for humans (10-60%) (Dickinson *et al.*, 1989; Levy *et al.*, 1990; Sugimoto *et al.*, 1996). Recovery of the dose in non-pregnant sheep urine as 3-keto VPA is substantially larger than corresponding values in pregnant sheep (~1.6%) (Kumar, 1998). This difference may be related to reductions in β-oxidation activity associated with pregnancy (Grimbert *et al.*, 1993). No developmental changes with age were observed when examining mass balance data for the 3-keto metabolite. In fact, a similar percent of the administered VPA dose was recovered in studies involving 1 day old lambs (~11%) (Kumar, 1998). Thus, it appears that β-oxidation activity is substantially developed early on in life. This observation is consistent with findings that β-oxidation activity increases dramatically during the first few hours after birth and may reach levels exceeding that of the adult within one day (Krahling *et al.*, 1979; Duee *et al.*, 1985; De Vivo *et al.*, 1991). In contrast, the percentage of the dose recovered in urine as 4-OH VPA increased ~3 fold from 10 d

lambs to adult sheep suggesting post-natal development of cytochrome P-450 mediated metabolism. Despite the observed increases, the cytochrome P-450 pathway remains relatively unimportant in terms of overall VPA elimination.

Overall, the entire administered dose was essentially recovered in 2 M lambs and adult sheep. In contrast only ~50% of the dose was excreted in urine by 10 d lambs. This is similar to observations in 1 day old lambs where ~30-50% of the total VPA dose could not be accounted for (Kumar, 1998). Thus, it is possible that additional routes of metabolism are present during the newborn period, which are responsible for the elimination of a significant portion of the administered VPA dose.

Examination of the Cl_r of 3-keto and 4-OH VPA revealed age-related increases in this parameter. By 2 months of age, the Cl_r of these two metabolites appear to be at adult levels (Figure 2.5). This is different from our observations for VPA, where Cl^u_r was still significantly less than adult values at 2 months of age. It is difficult to assess the mechanism responsible for the observed difference, as we were unable to obtain estimates of unbound Cl_r of these two metabolites. One possibility is that the development of the urinary excretion of the mentioned metabolites is more rapid than for the parent compound. Another alternative is that the observed increase in Cl_r of 3-keto and 4-OH VPA may be merely due to an increase in the free fraction of these two metabolites in 2 month old lambs. Finally, the lower Cl_r of these metabolites in 10 d lambs may play a role in the significantly larger metabolite $AUC_{0-\infty}$ values observed for 3-keto and 4-OH VPA in the younger animals. Since metabolite $AUC_{0-\infty}$'s are similar for

10 d and 1 M lambs, our observations suggest that an alteration in the Cl_r of 3-keto and 4-OH VPA would most likely occur between 1 to 2 months of age. This, of course, is provided that Cl_r in fact plays a significant role in metabolite elimination.

2.3.4 Summary of VPA Study A

Age-related alterations in VPA clearance for unbound and total drug in developing lambs appears to follow a pattern similar to what is observed in humans. The mass balance urine data suggests that these changes in clearance may be largely related to post-natal development of enzymes involved in VPA glucuronidation. Further investigations are needed to fully understand the underlying mechanisms and impact of the age-related alterations in glucuronidation on VPA elimination.

Study B: Dose-Dependent VPA Pharmacokinetics in 10 Day Old Lambs and Adult Sheep

Valproic acid is mainly eliminated by hepatic metabolism and exhibits a low hepatic extraction ratio, (Levy and Shen, 1995). For a compound with these characteristics, systemic clearance is largely influenced by alterations in either plasma protein binding and/or metabolic capacity (Levy and Shen, 1995). Thus, knowledge of developmental alterations in these factors would benefit our understanding of age-related changes in VPA disposition. In study A, we observed both a higher unbound fraction and a lower unbound VPA clearance in 10 day old lambs when compared to adult sheep. Similar observations in VPA unbound fraction have been observed in both rats (Haberer and Pollack, 1994) and humans (Gal *et al.*, 1988) where unbound fractions were the highest for newborns. In addition, in newborn rats (Haberer and Pollack, 1994) and guinea pigs (Yu *et al.*, 1985; Yu *et al.*, 1987), and in human neonates (Levy and Shen, 1995) lower estimates of unbound VPA clearance have been observed suggesting a reduced intrinsic metabolic clearance in the young. Study B is a more detailed examination on the role of plasma protein binding and metabolic elimination (more specifically glucuronidation) in determining observed differences in VPA disposition in 10 day old lambs and adult sheep. The study involves dose-ranging experiments at the two age groups of interest. The use of lambs and adult sheep will allow us to determine plasma protein binding parameters *ex vivo* and enable us to examine differences in dose-dependent changes in VPA metabolism and renal excretion with age.

2.4 Methods

2.4.1 Animals and Surgical Preparation

Adult Sheep: Five non-pregnant Dorset Suffolk cross-bred ewes were surgically prepared at least three days prior to experimentation. Surgical preparation and pre-experimental procedures are as described for study A (section 2.1.1).

Newborn Lambs: A total of 21 Dorset Suffolk cross-bred lambs were employed in this study. Lambs were divided into a 10 mg/kg group (n=8), a 50 mg/kg group (n=5), a 100 mg/kg group (n=4), and a 250 mg/kg group (n=4). All lambs were surgically prepared and allowed to recover as detailed in study A (section 2.1.1)

2.4.2 Experimental Protocols

Adult Sheep: Adult experiments involved administration of an *iv* bolus of VPA (Sodium Valproate, Sigma Chemical Co., St. Louis, MO) equivalent to 10, 50, 100, or 250 mg VPA/kg body weight followed by a five day washout period after which the next dose was administered. This continued until each ewe received one of each dose (i.e. a total of four experiments were performed on each animal). Doses were administered in a randomized order over 1 min *via* the femoral vein catheter. Serial blood samples (~3 ml) were collected for adult sheep from the femoral artery at 5, 15, 30, 45, 60 min, and 2, 4, 6, 9, 12, 15, 24, 36, 48 60 72 h following drug administration. For the 10 and 50 mg/kg dose, the experiments continued for only 36 and 48 hours, respectively.

Cumulative urine was also collected for the adult sheep for the full duration of the experiment.

Newborn Lambs: Newborn Lamb experiments were initiated at approximately 10 days following birth. Lambs were administered an *i.v.* bolus dose of VPA equivalent to 10, 50, 100, or 250 mg VPA/kg body weight depending on which group the lamb had been assigned to. Drug administration in the lambs was *via* the jugular vein catheter over 1 min. For newborn lambs, serial blood samples (~2 ml) were collected from the carotid artery at 5, 15, 30, 45, 60 min, and 2, 4, 6, 9, 12, 24, 36, 48, 60, 72 h following drug administration. Cumulative urine samples from the lambs were also collected for the full duration of the experiment. The only exceptions were for four lambs in the 10 mg/kg group where urine collection was incomplete due to catheter failure; these were excluded from data analysis.

As in study A, all doses were prepared in sterile water for injection and were sterilized by filtering through a 0.22 μm nylon syringe filter (MSI, Westboro, MA) into a capped empty sterile injection vial. Collected blood samples were placed into heparinized Vacutainer® tubes (Becton-Dickinson, Rutherford, NJ) and centrifuged at 2000 × *g* for 10 min. The plasma supernatant was removed and placed into clean borosilicate test tubes with polytetrafluoroethylene-lined caps. Plasma and urine samples were stored at -20°C until the time of analysis.

Note: The data from the lamb and adult 10 mg/kg VPA *i.v.* bolus experiments from this study are the same experiments that are presented in study A.

2.4.3 Drug and Metabolite Assay and Determination of VPA Plasma Protein Binding

Concentrations of VPA and its metabolites in all biological fluids and plasma ultrafiltrate were measured as described in section 2.1.4. Unbound plasma concentrations of VPA were determined *ex vivo* in all adult sheep and post-natal lamb plasma samples using ultrafiltration as described in section 2.1.3.

2.4.4 Determination of Protein Concentrations in Adult and Lamb Plasma

Concentrations of total protein in adult sheep and 10 day old lamb plasma were determined using a hand-held refractometer (Model no. 5433, Fisher Scientific Instruments, Tustin, CA) as described by Kwan (1989).

2.4.5 Pharmacokinetic Analyses

Ex vivo protein binding data was analyzed by first calculating the bound VPA concentrations from the difference between the corresponding experimentally determined total and unbound concentrations. Rosenthal plots (bound/unbound concentration vs. bound concentration) were constructed in order for identification of the multiplicity of binding sites. Bound vs. unbound concentrations were then fitted to a two-site binding

model (a high affinity saturable and a low affinity linear site) using the nonlinear least squares regression program ADAPT II (D'Argenio and Schumitzky, 1997) in order to estimate plasma protein binding parameters (i.e. $B_{max,}$- maximal binding capacity; $K_{d,}$- dissociation constant for drug-plasma protein interaction). Adult sheep estimates of plasma protein binding parameters were obtained for individual animals and are presented as mean $\pm$ S.D. This was possible since a wide range of plasma VPA concentrations was achieved in each animal following the four dose-ranging experiments. For lambs, only a single estimate of the binding parameters could be estimated since each lamb only received one VPA dose, and therefore plasma concentration data from individual animals were insufficient to provide for individual estimates. Thus, VPA plasma concentration data for lambs were pooled together to generate estimates, and consequentially plasma protein binding parameters are presented for lambs as an estimate followed by its CV in parentheses.

In vivo estimates of apparent Michaelis–Menten parameters (V_{max}, K_m) for overall VPA elimination were obtained through simultaneous fitting of unbound concentration–time data from the 50, 100, and 250 mg/kg experiments of an individual adult animal (more details as to why only the three higher doses were modeled will be provided in section 2.5.7 of the results). A two-compartment model with Michaelis-Menten elimination provided for the best "fit" of the data from all adult animals. Briefly, the first step involved generating microconstant (i.e. k_{cp} and k_{pc} - refer to Figure 2.11) estimates for individual adult animals. This was accomplished by modeling of the unbound concentration–time data from their respective 10 mg/kg experiments to a standard two-compartment model. The resulting

microconstant estimates were fixed for subsequent modeling involving simultaneous fitting of unbound concentration–time data from multiple experiments (i.e. 50, 100, and 250 mg/kg experiments). Model selection was based upon lower AIC (Akaike Information Criterion) and Schwarz Criterion values generated when using a two-compartment model with Michaelis-Menten elimination as opposed to a simpler one-compartment model with similar elimination characteristics (Wagner, 1993; Bourne, 1995). In contrast to adult sheep, individual neonatal lambs received only a single dose of VPA, therefore, individual animal estimates of apparent V_{max} and K_m could not be obtained. Instead, pooled plasma profiles (i.e. unbound plasma VPA concentrations at each time point were averaged) were constructed for each dose and the resulting profiles were modeled as described for the adult above. Similar to adult sheep, a two-compartment model with nonlinear elimination provided the best fit for the pooled lamb data. CV's (coefficient of variation) for all estimates of apparent V_{max} and K_m were < 7% and < 20%, respectively. Adult estimates for both parameters are presented as a mean ± SD. The pooled estimate of V_{max} and K_m obtained for neonatal lambs, is presented as the parameter estimate followed by its respective CV in parentheses. As for plasma protein binding, all data was modeled using ADAPT II (D'Argenio and Schumitzky, 1997).

In vivo estimates of apparent V_{max} and K_m for VPA glucuronidation were determined by constructing plots of urinary excretion rate of the glucuronide metabolite *vs.* the unbound VPA concentration at the midpoint of the urine collection interval. Data was pooled from all experiments in adult sheep and in neonatal lambs resulting in one plot for adult sheep and one plot for lambs. As with the previous modelling exercises mentioned above, both

plots were fit to a standard Michaelis-Menten equation using ADAPT II (D'Argenio and Schumitzky, 1997). CV's generated for V_{max} and K_m were <8% and <20%, respectively. The pooled estimate of apparent V_{max} and K_m obtained for both adult sheep and neonatal lambs, is presented as the parameter estimate followed by its respective CV in parentheses.

All other pharmacokinetic parameters were calculated as detailed in section 2.1.5 of study A.

2.4.6 Statistical Analysis

All data are reported as mean ± S.D. Pharmacokinetic parameters were compared using either a t-test for comparison between two groups or an ANOVA followed by a Fischer's LSD multiple comparison test for multiple group comparisons. The significance level was $p < 0.05$ in all cases.

2.5 Results

2.5.1 Dose-Dependent Pharmacokinetics of VPA in Newborn Lambs and Adult Sheep

Average age of the newborn lamb groups on the start day of the experiment were 10.9 ± 1.4 days (10 mg/kg group), 10.4 ± 0.5 days (50 mg/kg group), 10.3 ± 1.0 days (100 mg/kg group), and 10.5 ± 1.0 days (250 mg/kg group). Mean adult ewe body weight was 61.9 ± 7.3 kg, and mean lamb body weights were 5.8 ± 1.4 kg (10 mg/kg group), 6.0 ± 1.0 kg (50 mg/kg group), 7.1 ± 1.6 kg (100 mg/kg group), and 6.8 ± 2.1 kg (250 mg/kg group). Figures 2.6 A-D are representative semilogarithmic plots of VPA (unbound and total) concentration *vs.* time for an adult sheep following *iv* administration of a 10, 50, 100, and 250 mg/kg VPA bolus. Figures 2.7 A-D are semilogarithmic plots of pooled VPA (unbound and total) concentration *vs.* time data from newborn lambs. Table 2.5 presents dose dependent changes in the f_p (area weighted unbound fraction), $AUC^u_{0-\infty}$ (AUC of unbound VPA), and Vd_{ss}' (steady-state volume of distribution term corrected for the effects of saturable protein binding) for newborn lambs and adult sheep. As expected, f_p increased with increasing dose for both age groups. At the three higher doses, f_p's for 10 day old lambs were similar to their corresponding adult f_p estimates. This contrasts to what is observed at the lowest dose.

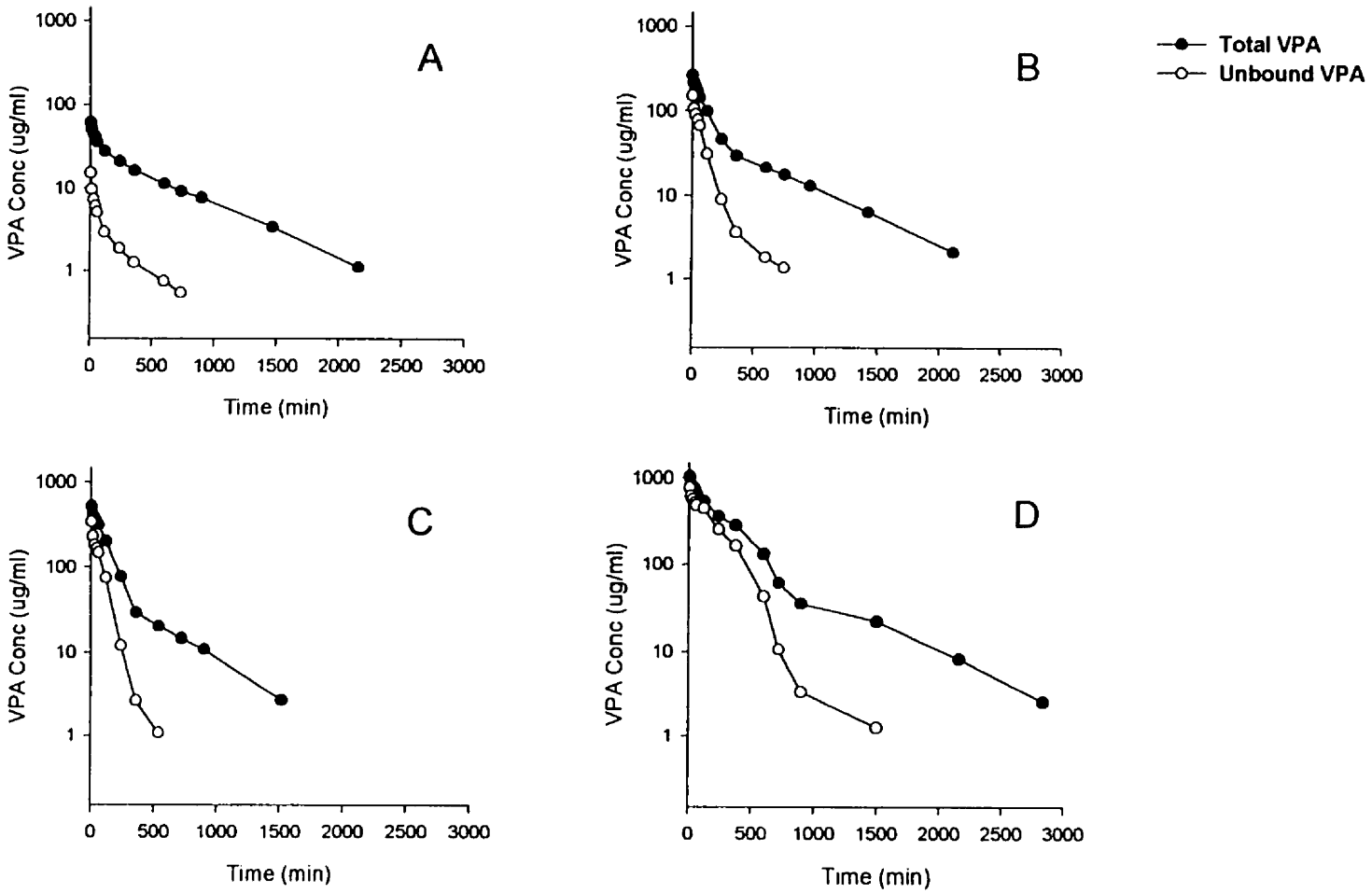

Figure 2.6 Representative VPA (total and unbound) plasma concentration *vs.* time profiles for an adult sheep (331Y) following *i.v.* bolus administration of a **(A)** 10 mg/kg, **(B)** 50 mg/kg, **(C)** 100 mg/kg, and **(D)** 250 mg/kg dose of VPA.

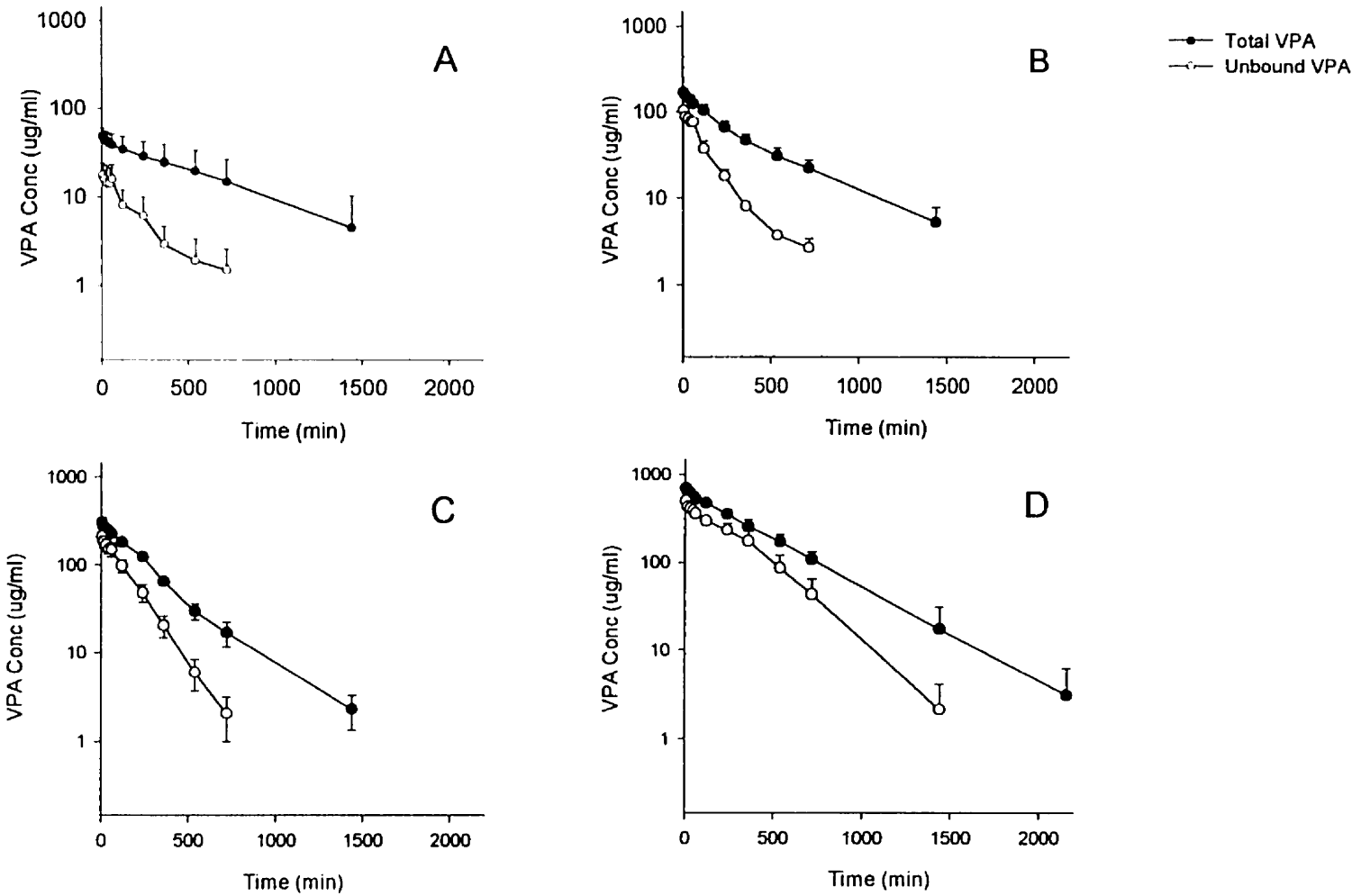

Figure 2.7 Average VPA (total and unbound) plasma concentration *vs.* time profiles for newborn lambs (10 days old) following *i.v.* bolus administration of a **(A)** 10 mg/kg, **(B)** 50 mg/kg, **(C)** 100 mg/kg, and **(D)** 250 mg/kg dose of VPA.

Table 2.5 Dose-dependent changes in f_p, $AUC^u_{0-\infty}$, and Vd_{ss}' in adult sheep and newborn lambs.

VPA DOSE (mg/kg)		f_p	$AUC^u_{0-\infty}$ (mg*min/ml)	Vd_{ss}' (L/kg)
Adult	10	0.09 ± 0.02^a	1.4 ± 0.4^a	0.13 ± 0.02^a
	50	0.27 ± 0.03^b	12.1 ± 1.9^b	0.13 ± 0.02^a
	100	0.41 ± 0.10^c	32.8 ± 6.3^c	0.13 ± 0.02^a
	250	0.61 ± 0.04^d	191.1 ± 43.8^d	0.18 ± 0.01^b
Newborn (10 day old)	10	$0.21 \pm 0.12^{a*}$	$4.5 \pm 1.8^{a*}$	0.14 ± 0.06^a
	50	0.29 ± 0.03^a	$16.3 \pm 1.5^{b*}$	$0.19 \pm 0.02^{a*}$
	100	0.44 ± 0.05^b	34.9 ± 3.8^c	$0.28 \pm 0.06^{b*}$
	250	0.56 ± 0.05^b	154.7 ± 32.4^d	$0.27 \pm 0.004^{b*}$

^{a,b,c,d} signifies different groups as determined by ANOVA followed by Fischer's LSD Multiple Comparison Test ($p<0.05$).
* denotes significant difference from the corresponding adult value as determined by t-test ($p<0.05$)

Dose-dependent changes in $AUC^u_{0-\infty}$ were examined as opposed to $AUC_{0-\infty}$ (AUC of total drug) since changes in $AUC^u_{0-\infty}$ are independent of changes in plasma protein binding. Increases in dose were associated with expected increases in $AUC^u_{0-\infty}$ for both age groups (Table 2.5). $AUC^u_{0-\infty}$ was significantly higher for 10 day old lambs at the 2 lower doses; however, for the 2 higher doses the adult and lamb estimates were similar. For adult sheep, the increase in $AUC^u_{0-\infty}$ with increasing dose is nonlinear suggestive of Michaelis-Menten elimination. In newborn lambs, $AUC^u_{0-\infty}$ increased linearly with dose until the 250 mg/kg dose where its increase was nonlinear. Additional evidence of nonlinear elimination can be observed in the unbound VPA plasma profiles in Figures 2.6

and 2.7 that displayed an increasing convex curvature with increasing dose. Although, the plasma profiles for total VPA concentrations also showed signs of convexity, the curvature of the total VPA profiles could be a result of saturable protein binding rather than nonlinear elimination.

For drugs exhibiting saturable protein binding, the volume terms Vd_{ss} and Vd^{u}_{ss} do not provide any information on possible shifts of drug into or out of the vascular space. However, this information is provided by the Vd_{ss}', therefore, this term has been presented in Table 2.5 to examine changes in drug distribution with dose. The Vd_{ss}' for both age groups increased with an increase in dose suggesting movement of drug out of the vascular space with increasing doses. In addition, for the three higher doses, Vd_{ss}' was significantly larger in lambs in comparison to adult sheep.

Figure 2.8 A depicts changes in VPA total body clearance (Cl_{tb}) with dose for newborn lambs and adult sheep. In both lambs and adult sheep, Cl_{tb} increases with dose to a maximum at the 100 mg/kg dose before decreasing at the highest dose. Figure 2.8 B illustrates changes in VPA unbound clearance (Cl^{u}_{tb}). For adult sheep, the Cl^{u}_{tb} decreases significantly with increasing dose consistent with metabolic saturation (Table 2.6). For neonatal lambs, Cl^{u}_{tb} decreases only following administration of the highest dose (Table 2.6). Adult Cl^{u}_{tb} is significantly higher than Cl^{u}_{tb} for newborn lambs at the two lower doses. However, at the two higher doses, Cl^{u}_{tb} is similar between the two age groups (Figure 2.8B).

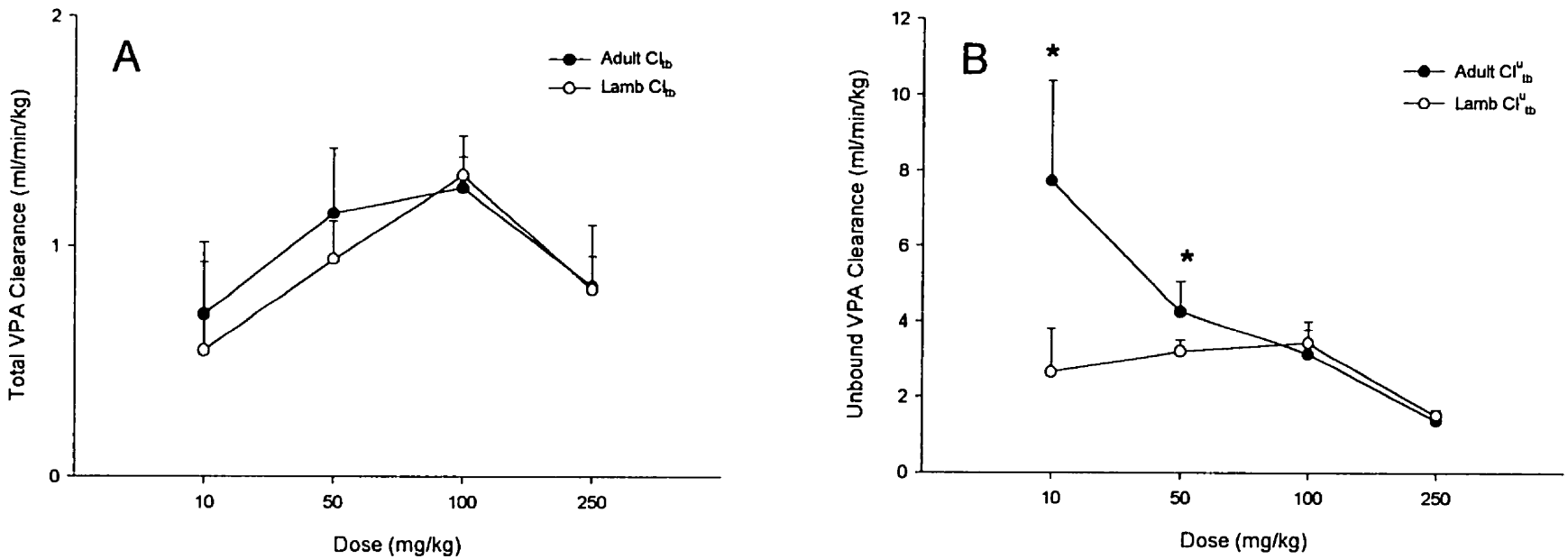

Figure 2.8 Changes in **(A)** total and **(B)** unbound drug clearances in adult sheep and 10 day old lambs for different doses of VPA. *denotes significant difference between the adult and lamb estimates ($p<0.05$).

Table 2.6 Changes in total and unbound VPA clearance in newborn lamb (10 day old) and adult sheep with increasing dose.

VPA DOSE (mg/kg)		Cl_{tb} (ml/min/kg)	Cl^{u}_{tb} (ml/min/kg)
Adult	10	0.70 ± 0.31	7.74 ± 2.63
	50	1.15 ± 0.29**	4.24 ± 0.82*
	100	1.25 ± 0.22**	3.15 ± 0.63*
	250	0.83 ± 0.13	1.37 ± 0.29*
Newborn	10	0.55 ± 0.38	2.65 ± 1.16
(10 day old)	50	0.94 ± 0.17**	3.21 ± 0.29
	100	1.31 ± 0.08**	3.42 ± 0.59
	250	0.81 ± 0.28	1.52 ± 0.12*

** significantly higher than the 10 mg/kg group ($p<0.05$).
* significantly lower than the 10 mg/kg group ($p<0.05$).

2.5.2 *Ex Vivo* Determination of Plasma Protein Binding Parameters

Rosenthal plots for a representative adult sheep and pooled lamb data are presented in Figure 2.9. Plots for both adult sheep and newborn lambs were biphasic in nature. The initial steep declining portion of the biphasic Rosenthal plots suggests the presence of a high affinity saturable binding site, whereas the relatively flat portion of the plots suggests the presence of a low affinity linear (non-saturable) site. Fitting the bound *vs.* unbound concentration data to a two-site binding model with a saturable and a non-saturable binding site (Equation 2.3) resulted in statistically better fits (lower AIC and Schwarz Criterion, smaller CV's for fitted parameters) when compared to a one-site binding model.

$$C_b = \frac{B_{max1} * C_u}{K_{d1} + C_u} + \frac{B_{max2}}{K_{d2}} * C_u \qquad \textbf{(Equation 2.3)}$$

where C_b and C_u are the corresponding bound and unbound concentrations, B_{max1} and B_{max2} are the maximal binding capacities of the first and second binding site, respectively. K_{d1} and K_{d2} are the equilibrium dissociation constants of VPA at the first and second binding site, respectively.

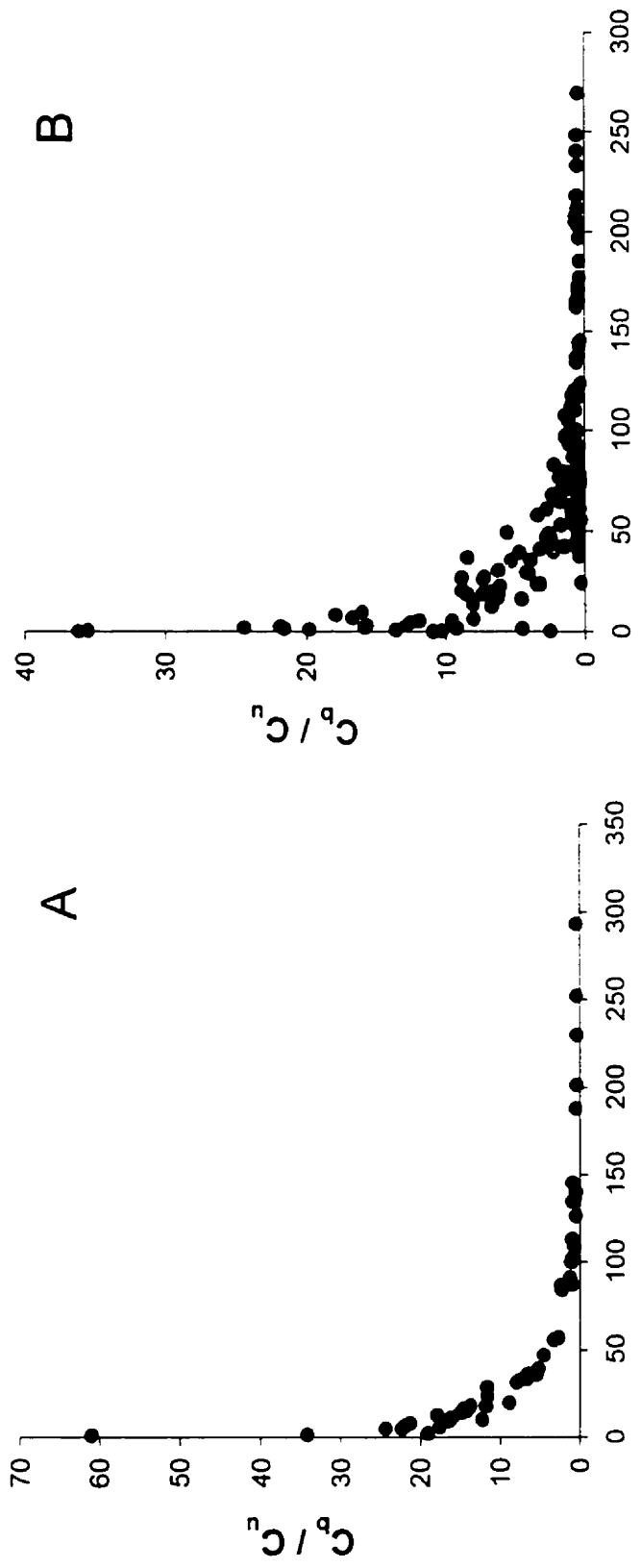

Figure 2.9 Rosenthal Plots for a representative adult sheep (E3216) **(A)** and neonatal lambs **(B)**. Graph A is plasma data pooled from all experiments (10, 50, 100, and 250 mg/kg) performed in E3216. Graph B is plasma data pooled from all 10 day old lamb experiments.

A scatter plot of bound *vs.* unbound VPA plasma concentration data for an individual adult sheep (i.e. plasma data is pooled from the 10, 50, 100, and 250 mg/kg experiments from a single animal) is presented in Figure 2.10 A. A similar plot was constructed for plasma data from all the newborn lamb experiments and is presented in Figure 2.10 B. The model-predicted lines based upon fitting to equation 2.3 are also depicted indicating a good fit of the data to the two-site binding model. Estimates of binding parameters for adult sheep and newborn lambs are presented in Table 2.7. From the data, it appears that binding capacity at the saturable binding site (B_{max1}) is higher in adult sheep. In contrast, the higher K_{d1} estimate in adult sheep suggests that binding affinity at the saturable site is lower when compared to lambs.

2.5.3 Total Protein Concentrations in Adult Sheep and Lamb Plasma

The total protein concentration in plasma from adult sheep (n=5) and 10 day old lambs (n=18) was determined to be 73.8 ± 6.4 mg/ml and 58.0 ± 4.6 mg/ml, respectively. Three lambs were excluded from total protein determination due to the lack of availability of plasma following drug and metabolite analysis. Total protein concentration was significantly higher in adult sheep in comparison to 10 day old lambs (unpaired t-test, $p<0.05$).

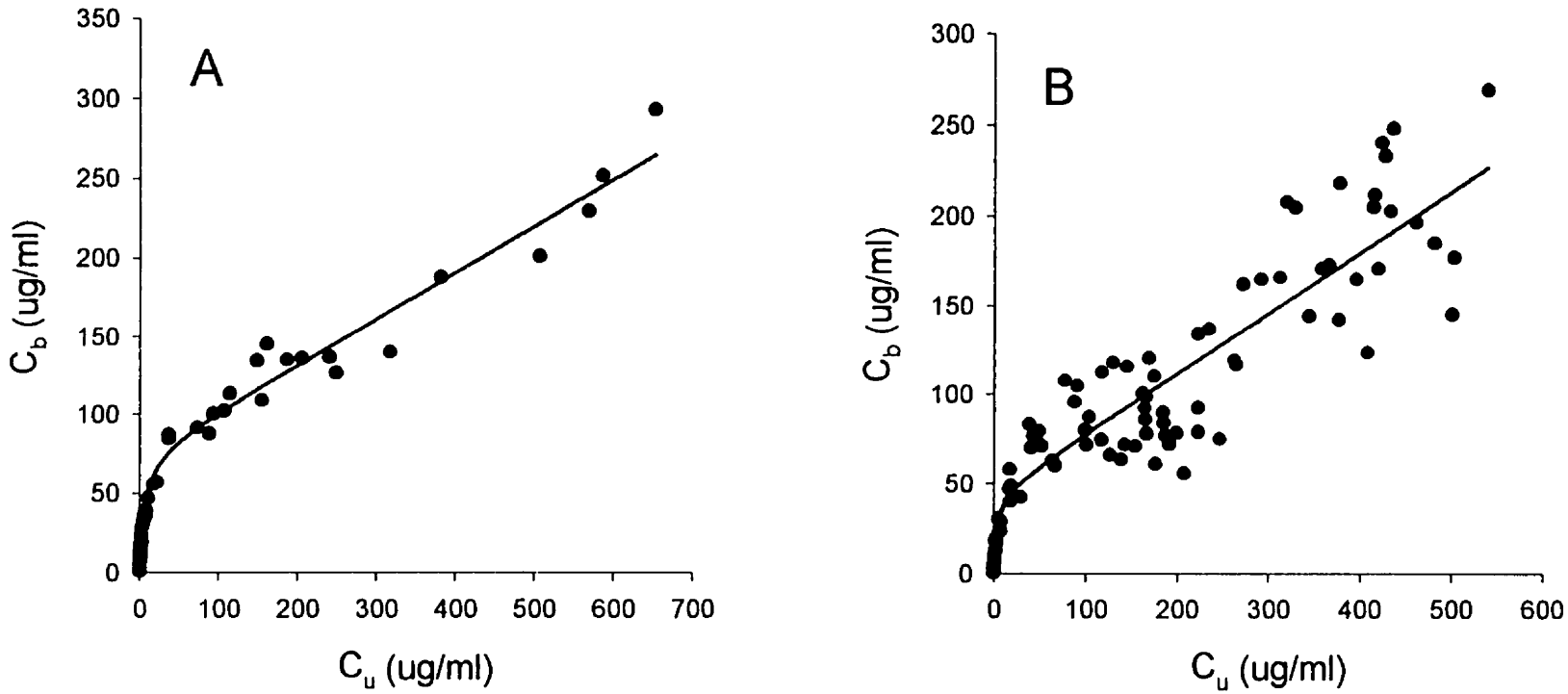

Figure 2.10 Relationship between bound drug (C_b) *vs.* unbound drug (C_u) in plasma from **(A)** a representative adult sheep (i.e. plasma data pooled from all experiments performed on E3216) and **(B)** all 10 day old lambs (i.e. plasma data pooled from all lamb experiments). In both cases, a model predicted line obtained from fit of data to a 2-site binding model is depicted.

Table 2.7 Plasma protein binding parameters for adult sheep and 10 day old lambs.

Parameter	Adult[a]	10 day old[b]
B_{max1} (μg/ml)	91.8 ± 24.3	44.9 (15.0)
K_{d1} (μg/ml)	9.6 ± 5.9	3.2 (8.3)
B_{max2}/K_{d2}	0.23 ± 0.06	0.33 (6.9)

[a] Adult estimates presented as mean ± S.D.
[b] Lamb estimates presented as the estimate followed by the CV in brackets.

2.5.4 VPA Metabolites in Adult and Neonatal Lamb Plasma

Tables 2.8 and 2.9 presents the C_{max} and t_{max} values for metabolites detected in adult and neonatal lamb plasma following the *i.v.* administration of different doses of VPA. As with previous sheep experiments (Kumar, 1998), the diunsaturated metabolites (see Figure 1.2) were not detected even at the highest dose. The t_{max} values for neonatal lambs were either the same or longer for all metabolites when compared to adult values. The only exception was the t_{max} for the 2-PGA metabolite at the 250 mg/kg dose where the t_{max} in lambs was 4 h as opposed to 6 h in adult sheep.

As expected, for virtually all of the metabolites, the C_{max} increased significantly with increasing dose. The only exception was for the (E)-2-ene metabolite in lambs (Table 2.9). An examination of the data reveals that aside from the 10 mg/kg dose, this metabolite appears to follow the same trend as the others (i.e. C_{max} increases with

increasing dose). The C_{max} for the 250 mg/kg lamb dosing group was significantly higher than C_{max}'s for both the 50, and 100 mg/kg dosing groups (ANOVA with Fisher's LSD, $p<0.05$). The observed discrepancy appears to be the result of the high variability of the (E)-2-ene C_{max} values observed in lambs from the 10 mg/kg dosing group.

Comparison of C_{max} data from adult and neonatal sheep revealed that C_{max} values for β-oxidation metabolites (i.e. (E)-2-ene, (E)-3-ene, and 3-keto VPA) in neonatal lambs were significantly higher than observed in the adult for all doses (Table 2.8 and 2.9). The trends were not as clear for metabolites derived from P-450 metabolism (i.e. 4-ene, 4-keto, 4-OH, and 5-OH VPA and 2-PSA and 2-PGA). C_{max} values for 4-ene VPA were not different for the 10, 50, and 100 mg/kg doses between the age groups. However, at the highest dose, the C_{max} was significantly lower for newborn lambs in comparison to adult sheep. Both the 4-OH and 4-keto VPA metabolites exhibited C_{max} values that were similar at all doses except for the 50 mg/kg dose where the C_{max} of 4-OH VPA was significantly higher and the C_{max} of 4-keto VPA was significantly lower in lambs when compared to the adult. For 5-OH VPA, C_{max} was higher in the lambs only at the 250 mg/kg dose. For all other doses, C_{max} values were similar. 2-PSA plasma levels could only be quantitated at the two higher doses for the adult and only at the highest dose in lambs. The 2-PGA metabolite levels in lambs were significantly lower than observed for the adult only at the 50 and 100 mg/kg doses. As mentioned in the introduction, the formation of 3-OH VPA does not appear to be exclusive to either β-oxidation or cytochrome P-450 metabolism (Rettenmeier *et al.*, 1987). 3-OH VPA was detected only at the two higher doses for both age groups and was not significantly different between adult and neonatal sheep at these doses.

Table 2.8 VPA metabolite C_{max} and t_{max} following *i.v.* bolus administration of a 10, 50, 100, or 250 mg/kg dose of VPA in adult non-pregnant sheep.

VPA metabolites	VPA DOSE							
	10 mg/kg		50 mg/kg		100 mg/kg		250 mg/kg	
	C_{max} (μg/ml)	t_{max}[c] (h)	C_{max} (μg/ml)	t_{max}[c] (h)	C_{max} (μg/ml)	t_{max}[c] (h)	C_{max} (μg/ml)	t_{max}[c] (h)
(E)-2-ene	0.27 ± 0.08	12	0.34 ± 0.06	15	0.39 ± 0.08[a]	15	0.58 ± 0.06[a]	24
(E)-3-ene	0.07 ± 0.02	4	0.06 ± 0.03	4	0.08 ± 0.03	4	0.14 ± 0.02[a]	9
4-ene	0.04 ± 0.03	2	0.22 ± 0.11	2	0.40 ± 0.26[a]	2	1.17 ± 0.40[a]	6
3-keto	0.67 ± 0.26	6	1.54 ± 0.53	1	2.60 ± 0.58	2	5.99 ± 3.02[a]	4
4-keto	0.026 ± 0.004	1	0.27 ± 0.08	0.75	0.56 ± 0.16[a]	2	1.53 ± 0.40[a]	4
3-OH	BLOQ	BLOQ	BLOQ	BLOQ	0.70 ± 0.44	4	1.43 ± 0.33[b]	4
4-OH	0.40 ± 0.14	0.5	3.13 ± 1.00	1	6.85 ± 2.95	1	20.53 ± 13.58[a]	4
5-OH	0.08 ± 0.05	0.25	0.97 ± 0.45	0.75	1.86 ± 0.69[a]	1	4.74 ± 1.14[a]	4
2-PSA	BLOQ	BLOQ	BLOQ	BLOQ	0.06 ± 0.02	2	0.22 ± 0.23	9
2-PGA	0.02 ± 0.01	1	0.16 ± 0.07	2	0.30 ± 0.17	2	0.74 ± 0.36[a]	6

BLOQ below limit of quantitation.
[a] significantly higher than the 10 mg/kg value ($p<0.05$).
[b] significantly higher than the 100 mg/kg value ($p<0.05$).
[c] t_{max} presented as median.

Table 2.9 VPA metabolite C_{max} and t_{max} following *i.v.* bolus administration of a 10, 50, 100, or 250 mg/kg dose of VPA in 10 day old lambs.

VPA metabolites	VPA DOSE							
	10 mg/kg		50 mg/kg		100 mg/kg		250 mg/kg	
	C_{max} (μg/ml)	t_{max}[d] (h)	C_{max} (μg/ml)	t_{max}[d] (h)	C_{max} (μg/ml)	t_{max}[d] (h)	C_{max} (μg/ml)	t_{max}[d] (h)
(E)-2-ene	1.76 ± 1.45**	24	0.69 ± 0.14**	24	0.72 ± 0.07**	12	1.56 ± 0.17**	24
(E)-3-ene	0.13 ± 0.03**	9	0.14 ± 0.06**	12	0.16 ± 0.02**	12	0.28 ± 0.06**[a]	24
4-ene	0.02 ± 0.01	2	0.12 ± 0.08	2	0.25 ± 0.06[a]	4	0.77 ± 0.14**[a]	6
3-keto	6.83 ± 3.05**	12	6.78 ± 2.21**	12	11.50 ± 3.23**	9	18.35 ± 5.80**[a]	12
4-keto	0.03 ± 0.01	6	0.13 ± 0.05*	2	0.38 ± 0.13[a]	2	1.37 ± 0.54[a]	6
3-OH	BLOQ	BLOQ	BLOQ	BLOQ	0.79 ± 0.27	4	1.78 ± 0.79[c]	6
4-OH	0.34 ± 0.14	2	5.48 ± 1.91**[a]	2	6.44 ± 2.11[a]	2	16.09 ± 2.48[a]	6
5-OH	0.11 ± 0.10	9	0.80 ± 0.53	4	2.13 ± 0.82[a]	2	6.41 ± 1.11**[a]	6
2-PSA	BLOQ	BLOQ	BLOQ	BLOQ	BLOQ	BLOQ	0.05 ± 0.004	12
2-PGA	BLOQ	BLOQ	0.06 ± 0.05*	4	0.07 ± 0.05*	6	1.61 ± 0.99[b]	4

BLOQ below limit of quantitation.

* signifcantly lower than corresponding adult value ($p<0.05$).

** significantly higher than corresponding adult value ($p<0.05$).

[a] significantly higher than the 10 mg/kg value ($p<0.05$).

[b] significantly higher than the 50 mg/kg value ($p<0.05$).

[c] significantly higher than the 100 mg/kg value ($p<0.05$).

[d] t_{max} presented as median.

As a measure of relative exposure to VPA metabolites for a given dose of VPA, AUC ratios were calculated by normalizing the $AUC_{0-\infty}$ of each metabolite to the $AUC_{0-\infty}$ of the parent compound (i.e. $AUC_{0-\infty}$ of metabolite/$AUC_{0-\infty}$ of VPA). In some instances, especially at the lower doses, these AUC ratios were not calculated because accurate estimates of metabolite $AUC_{0-\infty}$'s could not be obtained (i.e. extrapolated portion of the metabolite $AUC_{0-\infty}$ > 20% of the total area). Metabolites AUC ratios for adult sheep and neonatal lambs were multiplied by 1000, for ease of data presentation, and are presented in Tables 2.10 and 2.11. The AUC ratios of the β-oxidation metabolites (i.e. (E)-2-ene, (E)-3-ene, and 3-keto VPA), decreased significantly with increasing dose for both age groups (Tables 2.10 and 2.11). In contrast, the AUC ratios of VPA derived from P-450 pathways generally increased with increasing dose. For both age groups examined, AUC ratios of 4-OH VPA increased significantly with increasing dose (Tables 2.10 and 2.11). However, AUC ratios of two other P-450 metabolites (i.e. 5-OH and 4-ene VPA) increased significantly with increasing dose only for neonatal lambs (Table 2.11). A similar trend exists for AUC ratios of these two metabolites in adult sheep, but the increase was not significant (Table 2.10).

In neonatal lambs, the AUC ratio of almost all the metabolites were either similar to or significantly higher than their corresponding estimate in adult sheep (Table 2.11). The only exception to this generalization was 4-ene VPA where AUC ratios of this metabolite were lower in lambs for all three doses that the AUC ratio was calculated (Table 2.11).

Table 2.10 Ratio of metabolite $AUC_{0-\infty}$ to parent compound $AUC_{0-\infty}$ following *i.v.* bolus administration of a 10, 50, 100, or 250 mg/kg dose of VPA in adult non-pregnant sheep.

VPA metabolites	($AUC_{0-\infty}$ metabolite / $AUC_{0-\infty}$ VPA) × 1000			
	10 mg/kg	50 mg/kg	100 mg/kg	250 mg/kg
(E)-2-ene	24.1 ± 3.8	12.5 ± 2.0[a]	7.8 ± 1.1[a]	4.3 ± 0.8[a]
(E)-3-ene	ND	1.42 ± 0.28	1.03 ± 0.12[b]	0.64 ± 0.13[b]
4-ene	ND	2.93 ± 1.58	2.41 ± 1.36	3.39 ± 1.11
3-keto	49.9 ± 14.4	27.0 ± 9.2[a]	19.5 ± 4.1[a]	12.3 ± 1.9[a]
4-keto	ND	1.67 ± 0.53	2.07 ± 0.84	2.78 ± 0.80[d]
3-OH	ND	ND	2.55 ± 1.25	2.64 ± 0.73
4-OH	5.7 ± 1.7	16.8 ± 6.3	21.5 ± 11.2	33.5 ± 23.1[c]
5-OH	ND	5.09 ± 2.35	5.32 ± 1.36	7.65 ± 2.52
2-PSA	ND	ND	0.65 ± 0.88	0.37 ± 0.42
2-PGA	ND	1.11 ± 0.58	1.80 ± 0.48	1.54 ± 0.42

ND not determined
[a] significantly lower than the 10 mg/kg value ($p<0.05$).
[b] significantly lower than the 50 mg/kg value ($p<0.05$).
[c] significantly higher than the 10 mg/kg value ($p<0.05$).
[d] significantly higher than the 50 mg/kg value ($p<0.05$).

Table 2.11 Ratio of metabolite $AUC_{0-\infty}$ to parent compound $AUC_{0-\infty}$ following *i.v.* bolus administration of a 10, 50, 100, or 250 mg/kg dose of VPA in 10 day old lambs.

VPA metabolites	$(AUC_{0-\infty}$ metabolite / $AUC_{0-\infty}$ VPA) × 1000			
	10 mg/kg	50 mg/kg	100 mg/kg	250 mg/kg
(E)-2-ene	72.4 ± 37.7**	20.4 ± 4.7**[a]	12.4 ± 0.5**[a]	9.5 ± 2.9**[a]
(E)-3-ene	ND	3.17 ± 0.74**	2.10 ± 0.41**[b]	1.75 ± 0.57**[b]
4-ene	ND	1.17 ± 0.76*	1.25 ± 0.29	2.32 ± 0.23*[d]
3-keto	336.6 ± 169.6**	192.9 ± 68.6**[a]	135.0 ± 50.9**[a]	115.3 ± 33.9**[a]
4-keto	ND	1.39 ± 0.25	2.63 ± 0.98	4.15 ± 1.75[d]
3-OH	ND	ND	11.5 ± 3.5**	11.5 ± 4.9**
4-OH	17.5 ± 8.6**	50.8 ± 18.5**[c]	37.8 ± 8.6**[c]	38.7 ± 9.7[c]
5-OH	ND	8.9 ± 5.9	18.0 ± 8.6**	22.6 ± 5.6**[d]
2-PSA	ND	ND	ND	ND
2-PGA	ND	ND	ND	2.13 ± 1.37

ND not determined.
* significantly lower than corresponding adult value ($p<0.05$).
** significantly higher than corresponding adult value ($p<0.05$).
[a] significantly lower than the 10 mg/kg value ($p<0.05$).
[b] significantly lower than the 50 mg/kg value ($p<0.05$).
[c] significantly higher than the 10 mg/kg value ($p<0.05$).
[d] significantly higher than the 50 mg/kg value ($p<0.05$).

2.5.5 Dose-Dependent Changes in VPA Renal Clearance

Table 2.12 displays dose dependent changes in VPA renal clearance. For both the adult sheep and neonatal lamb, there appears to be no significant changes in renal clearance of total VPA that occur with increases in dose. In contrast, Cl^u_r decreased significantly in adult sheep such that at the highest dose the Cl^u_r estimate was ~ 6.5 fold less than the estimate at the 10 mg/kg dose. Although it appeared that Cl^u_r decreased with dose in lambs (by ~ 2.5 fold), the change was not significant.

Table 2.12 Changes in total and unbound VPA renal clearance in newborn lamb (10 day old) and adult sheep with increasing dose.

VPA DOSE (mg/kg)		**Cl_r** (ml/min/kg)	**Cl^u_r** (ml/min/kg)
Adult	10	0.09 ± 0.05	0.97 ± 0.43
	50	0.09 ± 0.02	0.32 ± 0.04*
	100	0.09 ± 0.03	0.22 ± 0.05*
	250	0.09 ± 0.02	0.15 ± 0.02*
Newborn (10 day old)	10	0.05 ± 0.03	0.28 ± 0.22
	50	0.08 ± 0.03	0.26 ± 0.10
	100	0.08 ± 0.03	0.19 ± 0.06
	250	0.06 ± 0.01	0.11 ± 0.01

* significantly lower than the 10 mg/kg group ($p<0.05$).

2.5.6 Dose-Dependent Changes in Urinary Excretion of VPA and its Metabolites

Tables 2.13 and 2.14 present dose dependent changes in the percentage of the total VPA dose recovered in urine as unchanged VPA and its metabolites in adult sheep (n=5) and newborn lambs (10 mg/kg, n=4; 50 mg/kg, n=5; 100mg/kg, n=4; 250 mg/kg n=4). Aside from the group of lambs receiving the 10 mg/kg dose, almost the entire dose (>90%) was recovered as either unchanged VPA or one of its metabolites (Table 2.13 and 2.14). The major metabolite in both age groups and at all doses was VPA-glucuronide. In adult sheep, ~70-80% of the dose appeared in urine as the glucuronide metabolite, and there was no significant changes in the recovery of this metabolite with dose (Table 2.13). In neonatal lambs, recovery of VPA-glucuronide in urine increased significantly from ~30% at the lowest dose to ~ 66-69% at the higher doses (Table 2.14). Recovery of the administered dose in urine as the unchanged drug appeared to follow no pattern with increasing dose. In adults, recovery of VPA in urine was significantly less at the 50 and 100 mg/kg doses than at the 10 mg/kg dose (Table 2.13). No such changes occurred with increasing dose in neonatal lambs (Table 2.14).

The major β-oxidation metabolite recovered in urine in both lambs and adult sheep was 3-keto VPA. For both age groups, the recovery of this metabolite in urine decreased significantly with increasing dose suggestive of metabolic saturation of the β-oxidation pathway (Table 2.13 and 2.14). At the 50 and 100 mg/kg doses, the percentage of the dose recovered as this metabolite in neonatal lambs was significantly higher than observed in adult sheep. The renal excretion of the other β-oxidation metabolites (E)-2-

ene and (E)-3-ene VPA were limited in both adult sheep and lambs accounting for no more than 0.08% of the dose. Similar to 3-keto VPA, the recovery of 3-OH VPA appeared to decrease with increasing dose in neonates (Table 2.14). In adults the mass balance of this metabolite appeared to follow no trend (Table 2.13).

The prominent metabolite formed by cytochrome P-450 pathways that was recovered in urine in both age groups appeared to be 4-OH VPA. In both newborn lambs and adult sheep, the urinary recovery 4-OH VPA significantly increased with increasing dose going as high as ~11% of the dose in lambs (Table 2.14) and ~7% of the dose in adults (Table 2.13). Similarly, in both age groups, 2-PGA increased significantly with increasing dose. All other P-450 metabolites (i.e. 5-OH, 4-ene and 4-keto VPA, and 2-PSA) accounted for less than 1% of the VPA dose at all dosing levels. Similar to (E)-2-ene and (E)-3-ene VPA, the recovery of 4-ene VPA in urine appeared to be particularly low accounting for no more than ~0.05% of the dose in any of the adult sheep or lamb groups.

Table 2.13 Recovery of VPA and its metabolites following *i.v.* bolus administration of a 10, 50, 100, or 250 mg/kg dose of VPA in adult non-pregnant sheep.

VPA or metabolites	% of VPA DOSE RECOVERED			
	10 mg/kg	50 mg/kg	100 mg/kg	250 mg/kg
VPA	12.2 ± 2.2	7.6 ± 1.3[a]	7.0 ± 1.6[a]	11.3 ± 3.3
VPA-glu	73.8 ± 5.3	77.9 ± 5.9	78.7 ± 5.6	68.3 ± 6.5
(E)-2-ene	< 0.01 %	< 0.01 %	< 0.01 %	< 0.01 %
(E)-3-ene	< 0.01%	< 0.01 %	< 0.01 %	< 0.01 %
4-ene	< 0.01 %	< 0.01 %	0.012 ± 0.002	0.029 ± 0.015[c]
3-keto	11.3 ± 6.5	3.5 ± 0.9[a]	2.7 ± 0.4[a]	1.8 ± 0.3[a]
4-keto	0.28 ± 0.08	0.25 ± 0.04	0.25 ± 0.07	0.32 ± 0.07
3-OH	0.53 ± 0.23	0.21 ± 0.06[a]	0.32 ± 0.16	0.54 ± 0.34
4-OH	2.0 ± 0.6	3.7 ± 1.5	4.5 ± 2.6	7.1 ± 3.6[b]
5-OH	0.76 ± 0.30	0.52 ± 0.24	0.44 ± 0.11[a]	0.51 ± 0.17
2-PSA	0.05 ± 0.01	0.05 ± 0.01	0.07 ± 0.01	0.14 ± 0.08[b]
2-PGA	0.6 ± 0.4	0.7 ± 0.3	1.1 ± 0.5	1.6 ± 0.6[b]
Total	101.5 ± 8.5	94.5 ± 6.3	95.0 ± 6.7	91.7 ± 2.1

[a] significantly lower than the 10 mg/kg value ($p<0.05$).
[b] significantly higher than the 10 mg/kg value ($p<0.05$).
[c] significantly higher than the 100 mg/kg value ($p<0.05$).

Table 2.14 Recovery of VPA and its metabolites following *i.v.* bolus administration of a 10, 50, 100, or 250 mg/kg dose of VPA in 10 day old lambs.

VPA or metabolites	% of VPA DOSE RECOVERED			
	10 mg/kg	50 mg/kg	100 mg/kg	250 mg/kg
VPA	5.6 ± 3.1*	8.6 ± 2.8	6.4 ± 1.9	7.0 ± 1.5*
VPA-glu	29.2 ± 16.0*	65.7 ± 8.5*[b]	69.6 ± 1.1*[b]	68.6 ± 2.9[b]
(E)-2-ene	0.071 ± 0.076	0.019 ± 0.012	0.011± 0.004[a]	0.010 ± 0.003[a]
(E)-3-ene	0.01 ± 0.02	< 0.01 %	< 0.01 %	< 0.01 %
4-ene	< 0.01 %	< 0.01 %	< 0.01 %	0.010 ± 0.002*
3-keto	12.6 ± 9.8	11.9 ± 3.5**	5.7 ± 2.1**	2.0 ± 0.8[a]
4-keto	0.04 ± 0.04*	0.16 ± 0.06*[b]	0.07 ± 0.02*	0.05 ± 0.01*
3-OH	0.34 ± 0.05	0.26 ± 0.11	0.20 ± 0.08[a]	0.18 ± 0.06*[a]
4-OH	0.6 ± 0.4*	4.1 ± 1.6	8.6 ± 7.2[b]	11.3 ± 5.5[b]
5-OH	0.17 ± 0.15*	0.56 ± 0.55	0.59 ± 0.38	0.65 ± 0.32
2-PSA	0.07 ± 0.05	0.05 ± 0.02	0.05 ± 0.04	0.08 ± 0.04
2-PGA	0.46 ± 0.33	0.89 ± 0.50	0.73 ± 0.32	1.19 ± 0.31[b]
Total	49.2 ± 25.7	92.3 ± 7.3	91.9 ± 7.1	91.0 ± 6.1

* significantly lower than corresponding adult value ($p<0.05$).
** significantly higher than corresponding adult value ($p<0.05$).
[a] significantly lower than the 10 mg/kg value ($p<0.05$).
[b] significantly higher than the 10 mg/kg value ($p<0.05$).

2.5.7 *In Vivo* Estimation of Apparent V_{max} and K_m of Overall VPA Elimination

Unbound concentration *vs.* time profiles from the 50, 100, and 250 mg/kg bolus experiments were modeled simultaneously in order to obtain *in vivo* estimates of apparent V_{max} (maximum turnover number of VPA elimination) and K_m (Michaelis-Menten constant) of overall VPA elimination. The 10 mg/kg experiments from both age groups were excluded from this modeling exercise due to our inability to recover the majority of the 10 mg/kg VPA dose administered to neonatal lambs (Table 2.14). For the three larger doses we were able to recover the majority of the dose as known VPA metabolites derived from hepatic metabolism (Table 2.13 and 2.14). Thus, the apparent V_{max} and K_m estimated from the modeling of these doses are hybrid constants largely reflective of overall metabolic elimination. The unbound concentration *vs.* time data from both age groups was modeled using a two-compartment model with Michaelis-Menten elimination (Figure 2.11) based upon a better fit (lower AIC and Schwarz Criterion, smaller CV's for fitted parameters) with this model when compared to fitting to a one-compartment model with nonlinear elimination. Unbound concentration *vs.* time profiles from adult sheep and neonatal lambs are presented in Figures 2.12 and 2.13 along with their model-predicted plasma profile lines. Adult animals were individually modeled; however, for lambs the mean unbound plasma concentration *vs.* time profiles at each dose were modeled. Estimates of apparent V_{max} and K_m obtained from modeling are presented in Table 2.15. From the estimates, it appears that V_{max} is similar between the two age groups. However, K_m appears to be higher for lambs.

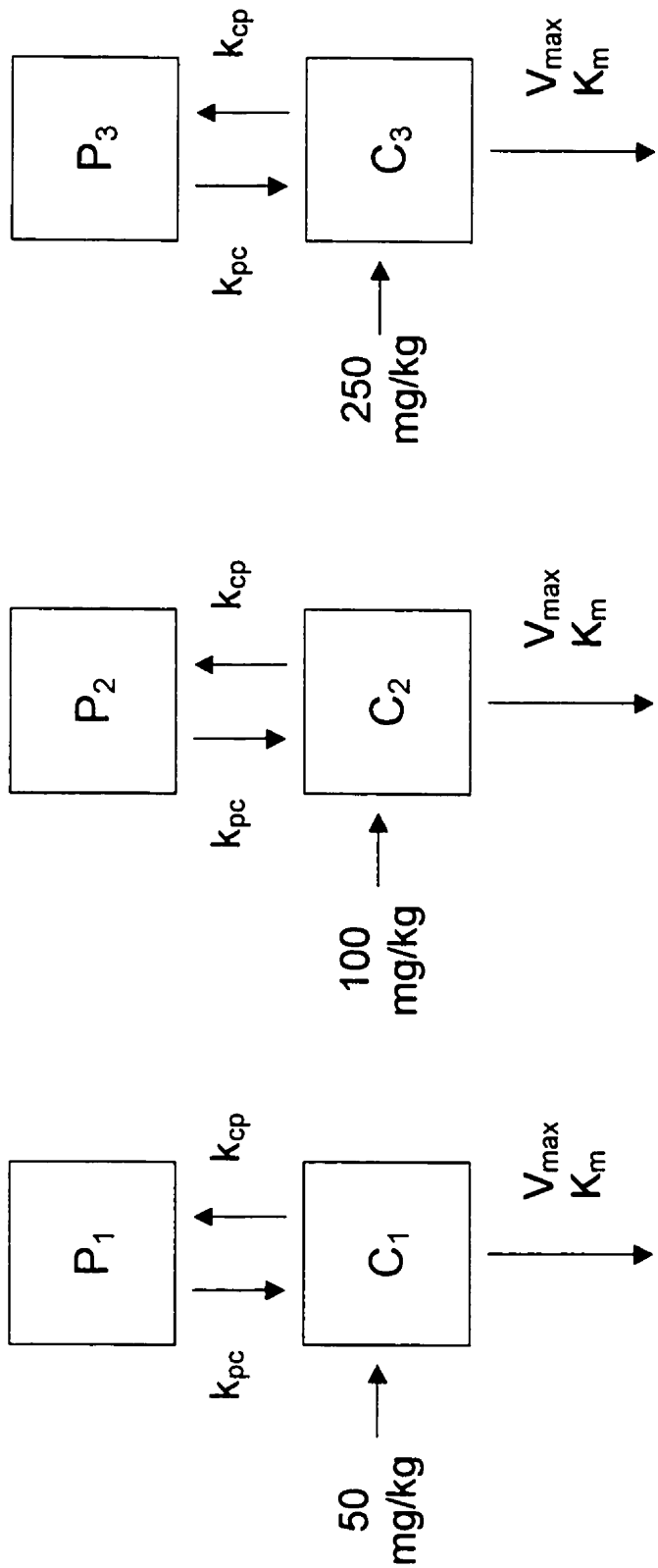

Figure 2.11 Diagrammatic representation of simultaneous modeling of unbound VPA plasma concentrations to a two compartment model with Michaelis-Menten elimination. V_{max} and K_m parameters for all three dosing situations are equivalent. C_1, C_2, and C_3 represent the central compartments for the different dosing situations. P_1, P_2, and P_3 represent the peripheral compartments for the different dosing situations. k_{pc} and k_{cp} represent the inter-compartmental transfer rate constants between the peripheral and central, and central and peripheral compartments, respectively.

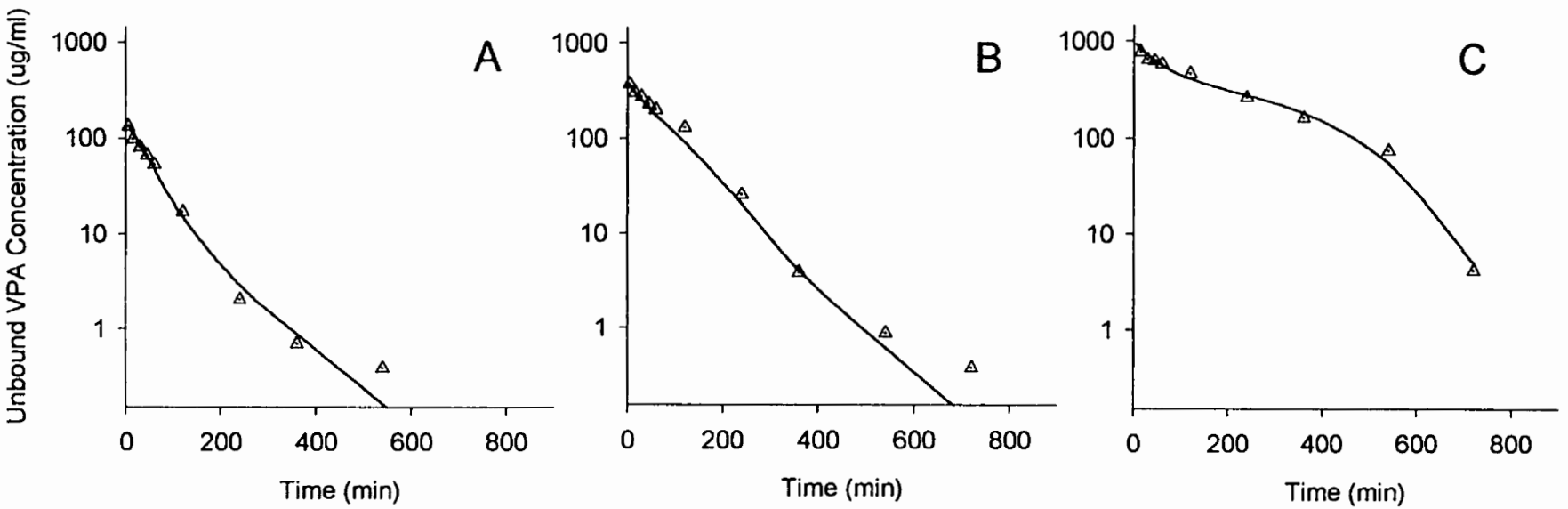

Figure 2.12 Representative unbound VPA plasma concentration *vs.* time profiles from an adult sheep (E6208) receiving **(A)** a 50 mg/kg bolus **(B)** a 100 mg/kg bolus and **(C)** a 250 mg/kg bolus. In all three cases, a model predicted line obtained from fit of the data to a two-compartment model with Michaelis-Menten elimination is depicted.

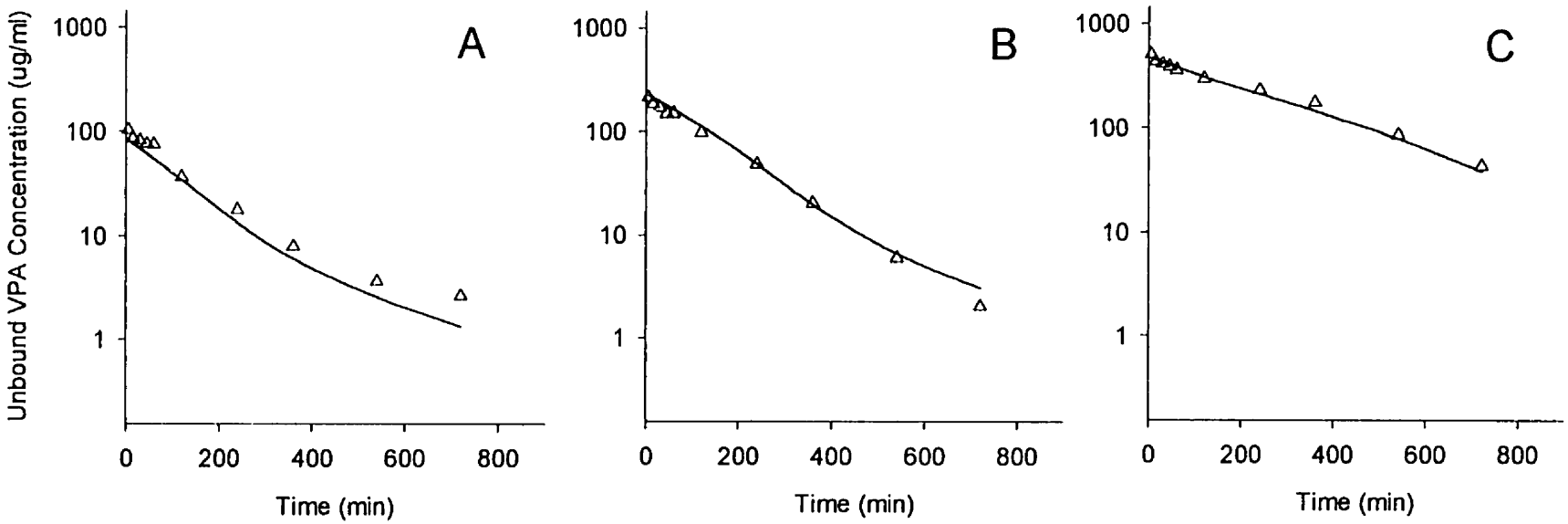

Figure 2.13 Mean unbound VPA plasma concentration *vs.* time profiles from 10 day old lambs receiving **(A)** a 50 mg/kg bolus **(B)** a 100 mg/kg bolus and **(C)** a 250 mg/kg bolus. In all three cases, a model predicted line obtained from fit of the data to a two-compartment model with Michaelis-Menten elimination is depicted.

Table 2.15 *In vivo* estimation of apparent V_{max} and K_m of overall VPA elimination in adult sheep and 10 day old lambs *via* modeling of unbound VPA concentrations in plasma.

Parameter	**Adult**[a]	**10 day old**[b]
V_{max} (μg/min/kg)	445.0 ± 91.2	429.9 (2)
K_m (μg/ml)	30.0 ± 12.6	69.6 (4)

[a] Adult estimates presented as mean ± S.D.
[b] Lamb estimates presented as the estimate followed by the CV in brackets.

2.5.8 *In Vivo* Estimation of Apparent V_{max} and K_m of VPA Glucuronidation

A condition for estimating the apparent V_{max} and K_m of VPA glucuronidation using urine data is that the appearance of VPA-glucuronide in urine is formation rate-limited as opposed to elimination rate-limited (Gilbaldi and Perrier, 1982). In previous investigations with VPA using pregnant sheep, the glucuronide metabolite did not appear to accumulate substantially in plasma consistent with formation rate-limited urinary excretion (Sanjeev Kumar, unpublished results). Metabolites demonstrating formation rate-limited urinary excretion exhibit plasma profiles that decline in parallel to the plasma profile of parent compound (Houston, 1986). Thus, apparent plasma half-lives determined from the terminal slopes should be similar for both the parent compound and its metabolite. Due to the lack of plasma following drug (unbound and total) and metabolite analysis, we could

not determine VPA-glucuronide levels in plasma. As an alternative, a plot of urinary excretion rate *vs.* t_{mid} (i.e. time at the midpoint of the urine collection interval) was constructed (Gilbaldi and Perrier, 1982). The apparent plasma half-life of the metabolite can be obtained from the terminal slopes of the semilogarithmic form of these plots (Gilbaldi and Perrier, 1982). Since elimination rate-limited urinary excretion of the metabolite is likely to occur at the highest dose of VPA, we constructed semilogarithmic plots of excretion rate *vs.* t_{mid} for the 250 mg/kg experiments. Figure 2.14 shows representative semilogarithmic plots of VPA-glucuronide (VPA-Glu) excretion rate *vs* t_{mid} for an adult sheep and a neonatal lamb. VPA-glucuronide half-lives of 4.70 ± 1.03 h (adult sheep) and 3.88 ± 0.62 h (10 day lambs) were obtained from the terminal slopes and were not significantly different from unbound VPA half lives determined from plasma data (adult, 4.86 ± 2.50 h; 10 day old lamb, 3.28 ± 0.34 h) (unpaired t-test, $p<0.05$). Thus, our assumption of formation rate-limited urinary excretion of the glucuronide metabolite appears to be reasonable.

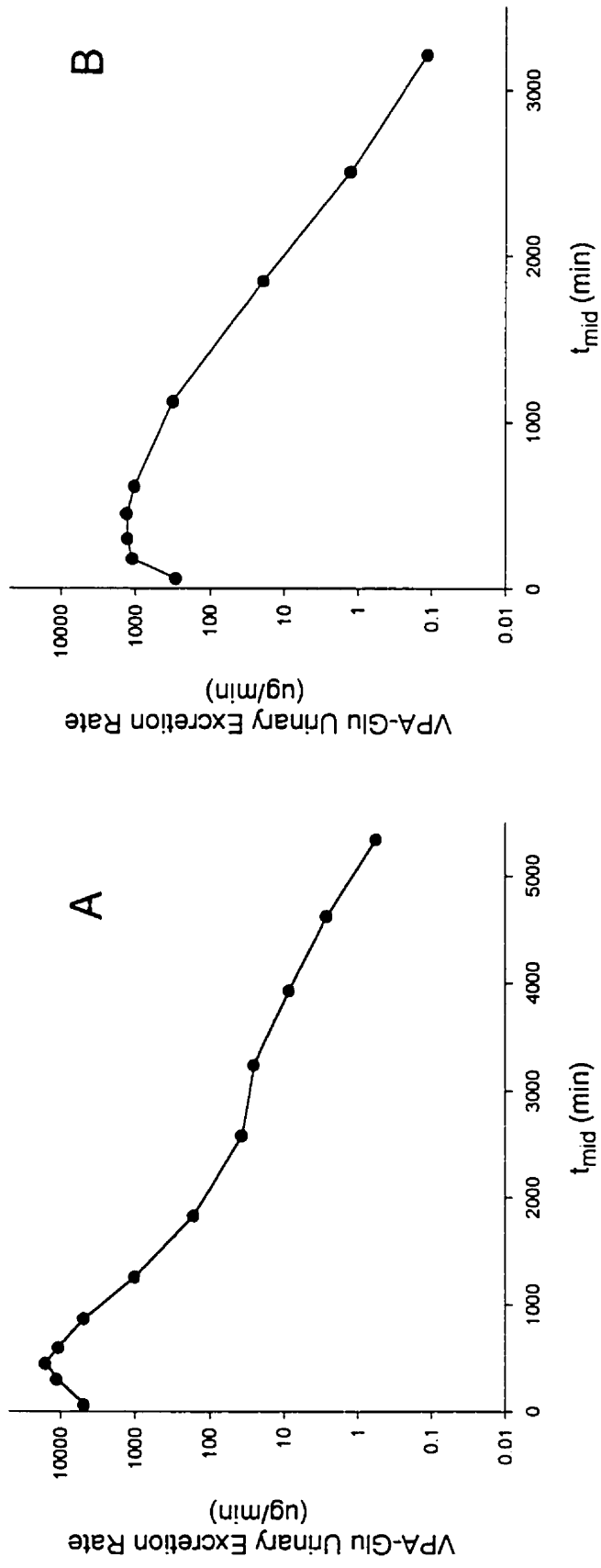

Figure 2.14 Representative plots of VPA-glucuronide urinary excretion rate *vs.* time at the midpoint of the urine collection interval for **(A)** an adult sheep (EB220X) and **(B)** a neonatal lamb (L8215) following administration of a 250 mg/kg VPA *i.v.* bolus.

Plots of V (urinary excretion rate of VPA-glucuronide) *vs.* C^{u}_{mid} (the unbound VPA plasma concentration at the midpoint of the urine collection interval) along with the model fitted line for adult sheep and neonatal lambs are presented Figure 2.15. The data was fit to a standard Michaelis-Menten equation as follows:

$$V = \frac{V_{max}}{K_m + C^{u}_{mid}} \times C^{u}_{mid} \qquad \textbf{(Equation 2.4)}$$

where V and C^{u}_{mid} are as defined above, V_{max} is the maximal formation rate of VPA-glucuronide and K_m is the Michaelis-Menten constant (Gilbaldi and Perrier, 1982). The resulting estimates of V_{max} and K_m of VPA-glucuronidation are presented in Table 2.16. As with V_{max} estimates of overall VPA elimination, the V_{max} estimates of VPA-glucuronidation were similar between the two age groups. However, K_m estimates appeared to be higher for neonatal lambs. In fact, K_m estimates of VPA glucuronidation for both adult and neonatal sheep are very similar to K_m estimates of overall VPA elimination (Table 2.15).

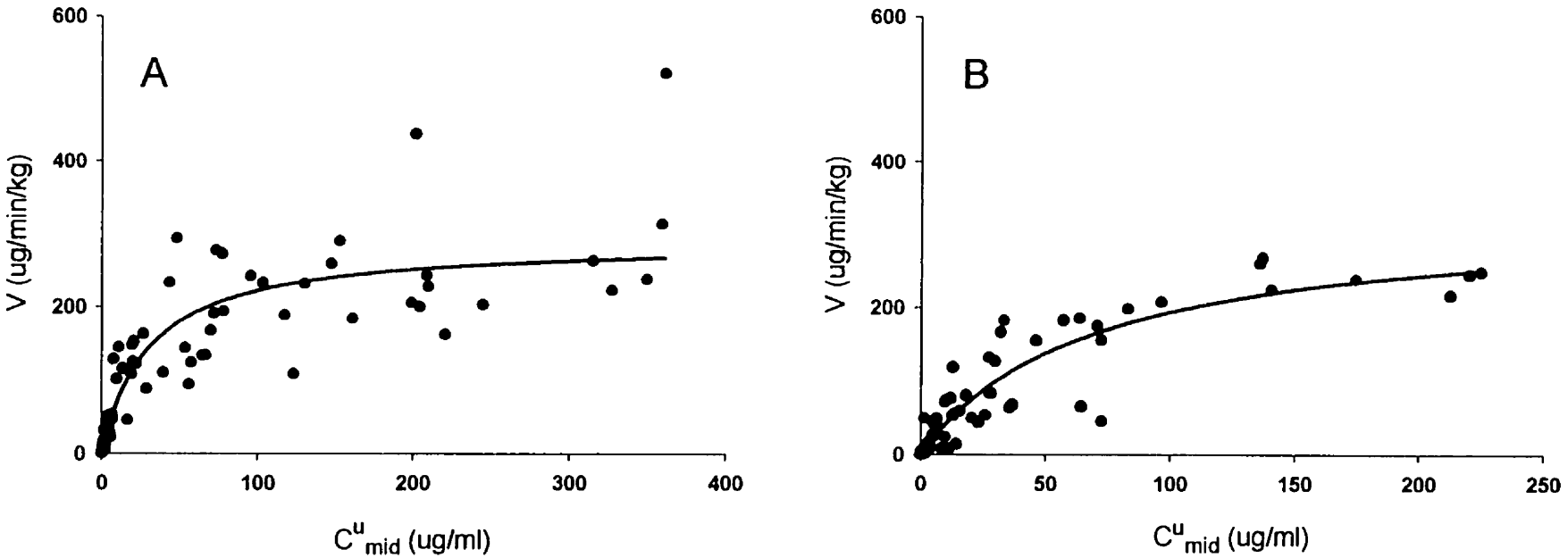

Figure 2.15 Relationship between the rate of urinary excretion of VPA-glucuronide (V) *vs.* the unbound VPA plasma concentration at the midpoint of the urine collection interval (C^u_{mid}) for **(A)** adult sheep and **(B)** 10 day old lambs. In both cases, a model predicted line obtained from fit of data to a standard Michaelis-Menten equation is depicted.

Table 2.16 *In vivo* estimation of apparent V_{max} and K_m of VPA glucuronidation in adult sheep and 10 day old lambs *via* modeling of urine data.

Parameter	**Adult**[a]	**10 day old**[a]
V_{max} (μg/min/kg)	288.5 (5)	326.5 (8)
K_m (μg/ml)	30.0 (17)	68.2 (17)

[a] Estimates presented as the parameter estimate followed by the CV in brackets.

2.6 Discussion

2.6.1 Dose-Dependent Pharmacokinetics in Adult Sheep and Neonatal Lambs

The pharmacokinetics of VPA are unique exhibiting both saturable/nonlinear plasma protein binding and saturable/capacity-limited metabolism at clinically relevant plasma concentrations. These characteristics are largely due to the high therapeutic doses of VPA in comparison with other drugs. Since VPA exhibits a low hepatic extraction and is mainly eliminated *via* hepatic metabolism (Levy and Shen, 1995), according to the well-stirred model (Wilkinson and Shand, 1975) the Cl_{tb} of VPA can be described as:

$$Cl_{tb} = f_u Cl_{int} \qquad \textbf{(Equation 2.5)}$$

where f_u is the unbound fraction and Cl_{int} is the hepatic intrinsic clearance. Thus, saturation of plasma protein binding and metabolism influence VPA Cl_{tb} in opposite directions. Specifically, saturation of plasma protein binding increases drug free fraction resulting in an increase in Cl_{tb}. In contrast, metabolic saturation acts to decrease Cl_{tb} by decreasing intrinsic clearance. Significant alterations in plasma protein binding occurred in our dose-ranging experiments as evidenced by the observed changes in f_p (a measure of overall drug free fraction) (Table 2.5). In addition, the nonlinear increases in $AUC^u_{0-\infty}$ in both adult sheep and neonatal lambs, especially at the highest dose, provide evidence of metabolic saturation. Thus, dose-dependent alterations in VPA Cl_{tb} will be

the result of relative changes in plasma protein binding and metabolism that occur with increasing dose.

For both adult sheep and neonatal lambs, we observed significant increases in VPA Cl_{tb} up to the 100 mg/kg dose (Table 2.6). At the highest dose (250 mg/kg), Cl_{tb} for both age groups fell to a level similar to that observed at the 10 mg/kg dose (Table 2.6). The observed increases in Cl_{tb} are a result of the observed increases in overall drug free fraction (i.e. f_p) with increasing dose (Table 2.5). The influence of metabolic saturation on VPA Cl_{tb} is only evident at the 250 mg/kg dose for both age groups. This is also the dose at which we observed the most substantial nonlinear increases in $AUC^u_{0-\infty}$. (i.e. a 2.5 fold increase in dose resulted in an ~6 fold and an ~4.5 fold increase in $AUC^u_{0-\infty}$ in adult sheep and neonatal lambs, respectively). In previous studies examining the dose-dependent pharmacokinetics of VPA in adult guinea pigs (Yu *et al.*, 1987; Yu *et al.*, 1993), rats (Liu *et al.*, 1990; Liu and Pollack, 1993) and humans (Bowdle *et al.*, 1980; Anderson *et al.*, 1992; Gomez Bellver *et al.*, 1993), Cl_{tb} either increased with dose, decreased with dose or exhibited no changes with dose. This discrepancy in alterations in Cl_{tb} with dose is largely related to differences in the range of doses utilized in these studies. Species and individual differences in VPA plasma protein binding and metabolic capacity may also play a role.

Unlike VPA Cl_{tb}, Cl^u_{tb} appeared to follow different trends in adult and 10 day old sheep. In adults, Cl^u_{tb} decreased significantly with increasing dose. Similar decreases in Cl^u_{tb} were observed in dose ranging studies conducted in adult guinea (Yu *et al.*, 1987; Yu *et*

al., 1993) and humans (Bowdle *et al.*, 1980; Anderson *et al.*, 1992; Gomez Bellver *et al.*, 1993). The observed decreases in Cl^{u}_{tb} in these studies are indicative of metabolic saturation as the unbound clearance of VPA approximates hepatic intrinsic clearance (See equation 2.5). Surprisingly, Cl^{u}_{tb} in neonatal lambs did not decrease significantly until the highest dose (Table 2.6). In contrast, a decrease in Cl^{u}_{tb} was observed with increasing dose in both 3 day old and 21 day old guinea pigs (Yu *et al.*, 1987). However, in these experiments two of the three doses administered (i.e. 20, 200, and 600 mg/kg) were either similar or substantially larger than the highest dose (i.e. 250 mg/kg) used in our studies. The use of similar doses in lambs would likely result in a similar trend in Cl^{u}_{tb}.

As expected, Vd_{ss}' increased with increasing dose in both adult sheep and neonatal lamb (Table 2.5). The modest increase in Vd_{ss}' is consistent with the VPA's low tissue binding (Davis *et al.*, 1994). Interestingly, the Vd_{ss}' in lambs is significantly larger than in adults at the 50, 100, and 250 mg/kg doses. The larger Vd_{ss}' observed in lambs may be related to the larger total body water content in the young (Moreselli *et al.*, 1980). A similar phenomenon is observed in human neonates who exhibit higher volumes of distribution (i.e. 0.28-0.43 L/kg) in comparison to adults (i.e. 0.13-0.20 L/kg) (Levy and Shen, 1995).

2.6.2 Age-Related Differences in VPA Plasma Protein Binding

As mentioned in Study A, plasma protein binding exhibits nonlinear characteristics in adult and neonatal lambs even at the lowest dose (i.e. 10 mg/kg). One of our goals of the dose-ranging study was to saturate plasma protein binding to such an extent that we would have a wide enough range of bound (C_b) and unbound (C_u) drug concentrations required for appropriate characterization of plasma protein binding parameters. Examination of our Rosenthal (Figure 2.9) and our C_b vs C_u plots (Figure 2.10) provide obvious evidence that a 2-site binding model is required to describe VPA plasma protein binding. This was not surprising since a similar model was used previously to characterize VPA plasma protein binding in pregnant sheep (Kumar, 1998). The presence of a high affinity saturable binding site and a low affinity non-saturable site has also been demonstrated in rats (Semmes and Shen, 1990; Haberer and Pollack, 1994; Slattum *et al.*, 1996), guinea pigs (Yu and Shen, 1992), and humans (Riva *et al.*, 1984; Scheyer *et al.*, 1990). Our estimates of binding parameters for adult sheep (Table 2.7) appear to be reasonably similar to estimates obtained from humans (i.e. B_{max1} ~ 169 μg/ml and K_{d1} ~ 6-13 μg/ml; Riva *et al.*, 1984; Scheyer *et al.*, 1990). The K_{d1} (9.6 ± 5.9 μg/ml) estimate obtained for adult sheep is consistent with observable saturation of VPA plasma protein binding at therapeutic concentrations (i.e. 50-100 μg/ml).

A comparison of VPA binding parameters obtained from previous experiments in pregnant sheep and current estimates in non-pregnant sheep revealed a subtle difference in binding capacity. Kumar (1998) estimated a B_{max1} of ~ 63 μg/ml in

pregnant sheep as opposed to B_{max1} estimates of 91.8 ± 24.3 μg/ml in non-pregnant animals. A reduction of albumin (the primary protein involved in VPA plasma protein binding) concentrations that occurs with pregnancy has been suggested as one of mechanisms involved in reduced VPA binding in pregnant women (Riva *et al.*, 1984; Nau *et al.*, 1984; Nau and Krauer, 1986). A similar phenomenon may occur in pregnant sheep and result in the lower observed B_{max1} estimate. K_{d1} estimates were similar in pregnant and non-pregnant sheep being ~8 μg/ml and ~10 μg/ml, respectively.

Estimates of the linear component (i.e. B_{max2}/K_{d2}) of VPA plasma protein binding for adult sheep and neonatal lambs were similar (Table 2.7). However, both the binding capacity (B_{max1}) and affinity (K_{d1}) of the high affinity saturable binding site of the two age groups appeared to be different. B_{max1} estimates from adult sheep were 2 fold higher than the corresponding estimate in 10 day lambs (44.9 μg/ml). The lower binding capacity may play a role in the higher f_p observed in study A for 10 day old lambs. Similar apparent reductions in plasma protein binding have been observed in neonates for several anticonvulsants (including VPA), antibiotics and analgesics (Kearns *et al.*, 1989; Reed *et al.*, 1989; Levy and Shen 1995). Causes for differences in binding include lower plasma levels of total protein, albumin, and α_1-glycoprotein in neonates compared to adults (Kearns *et al.*, 1989; Reed *et al.*, 1989). As well neonates exhibit an increased level of unconjugated bilirubin and free fatty acids which can act to displace albumin bound drugs from their binding sites (Kearns *et al.*, 1989; Reed *et al.*, 1989). Since B_{max1} is a measure of binding capacity the presence of binding inhibitors (i.e. unconjugated bilirubin and free fatty acids) would have no effect on this parameter.

Thus, it is likely that the lower B_{max1} observed in neonatal lambs is a result of lower total protein and albumin concentrations. Our measurements of total protein in adult and neonatal sheep plasma support this explanation since we observed significantly lower concentrations of total protein in neonatal plasma in comparison to the adult (See section 2.5.3). Similar increases in binding capacity of VPA with age were observed developmental studies in rats (Haberer and Pollack, 1994) and guinea pigs (Yu *et al.*, 1985).

Surprisingly, the binding affinity of VPA appeared to be higher in neonatal lambs (Table 2.7). As mentioned, in the young there is generally a higher concentrations of substances such as unconjugated bilirubin and free fatty acids which can act as inhibitors of VPA binding (Kearns *et al.*, 1989; Reed *et al.*, 1989). The presence of such inhibitors influence binding by decreasing binding affinity (i.e. increase K_{d1}). In addition, the lingering presence of fetal albumin in neonates usually results in a decrease in binding affinity (Moreselli, 1976). Consistent with the expected situation, binding affinity was observed to increase with age in rats (Haberer and Pollack, 1994). An explanation for the unexpected results observed in sheep may be due to the presence of a rumen in these animals. In newborn ruminants, blood glucose and fatty acid concentrations are similar to monogastric animals. As the animal ages and the rumen develops, glucose levels in the blood fall to half of what is observed in non-ruminants. In contrast, volatile fatty acid concentration in blood increases substantially due to their production in the rumen and subsequent absorption (Annison and Lewis, 1959). The presence of higher concentrations of fatty acids in adult sheep plasma may act as binding inhibitors

resulting in the observed reduction in VPA binding affinity (i.e. increases K_{d1}) in adult sheep.

2.6.3 Dose-Dependent Alterations in VPA Metabolites in Plasma

Detailed information on VPA metabolism is unavailable in the human neonatal population. However, as mentioned in study A, we observed similarities in our plasma metabolite data with information gathered from human epileptic children (Nau *et al.*, 1991; Siemes *et al.*, 1993). In these studies, plasma levels of VPA metabolites were determined for epileptic children on VPA therapy for at least four weeks. One of the variables examined was the effect of age on the VPA metabolic profile. In general, higher concentrations of β-oxidation metabolites (i.e. 3-keto, 2-ene, 2,3'-diene and 3-ene VPA) were observed in children < 2 years old in comparison to children >2 years of age. Furthermore, higher concentrations of P-450 derived metabolites such as 4-OH, 5-OH, and 4-keto VPA were observed in the younger children (Siemes *et al.*, 1993).

Aside from a few cases, a similar or a significantly higher C_{max} was observed for VPA metabolites in 10 day old lambs in comparison to the corresponding metabolite C_{max} in adult sheep at all doses in the current dose ranging study (Tables 2.8 and 2.9). This statement appears to be true for the majority of the metabolites regardless of whether they were derived from β-oxidation or P-450 catalyzed pathways. The only exceptions were C_{max} values for 2-PGA and 4-keto VPA at the 50 mg/kg dose and 2-PGA at the 100 mg/kg dose. In addition, the t_{max} for all metabolites were either similar or longer in

neonatal lambs in comparison to adult sheep. Thus, it appears that in general, VPA metabolites persist longer in neonatal plasma. Although we had previously observed a similar phenomenon at the 10 mg/kg dose, the situation appears to remain the same at higher doses of VPA. Again, the higher concentrations generally observed in the younger animals is similar to the situation that is observed in epileptic children < 2 years of age (Nau *et al.*, 1991; Siemes *et al.*, 1993). Since metabolite concentrations are a function of both the formation and elimination rate, the observed results are likely related to either an increased formation and/or a reduced elimination of the metabolites. In study A we observed a comparatively lower Cl_r of 4-OH and 3-keto VPA for 10 day old lambs suggesting that the a reduced renal elimination of VPA metabolites may be involved in their higher plasma concentrations when compared to the adult.

As a measure of relative exposure to VPA metabolites in comparison to the parent compound, $AUC_{0-\infty}$ ratios (AR) were calculated by dividing $AUC_{0-\infty}$ of the metabolite of interest by the $AUC_{0-\infty}$ of the parent compound. In line with the C_{max} data discussed above, the AR's of the majority of the metabolites were higher in neonatal lambs compared to adult sheep. The AR's β-oxidation metabolites, 3-keto, (E)-2-ene and (E)-3-ene VPA, decreased with dose for both adult sheep and neonatal lambs. In contrast, the overall trend for the AR's of the P-450 derived metabolites from both age groups was an increase with increasing dose. Studies in healthy adults (Granneman *et al.*, 1984) and epileptic patients (Dickinson *et al.*, 1989), revealed reductions in the percentage of the VPA dose recovered as β-oxidation metabolites. Granneman *et al.* (1984) attributed this phenomenon to saturation or product inhibition of the β-oxidation

pathway. However, recovery of 4-OH and 5-OH VPA in urine showed no dose dependency (Granneman *et al.*, 1984). A later study by Anderson *et al.* (1992) showed similar results demonstrating a reduction in the formation clearance of the β-oxidation pathway with increasing dose. In the same study, formation clearances for P-450 derived metabolites were shown to either increase (4-ene pathway) or exhibit no change (i.e. 4-OH and 5-OH pathways). The trend observed in the AR's of the β-oxidation metabolites in both age groups is consistent with a decrease in VPA β-oxidation with increasing dose. In contrast, our data is consistent with increased formation of metabolites resulting from P-450 metabolism. Although no apparent dose dependency was observed for the 4-OH (ω-1 oxidation) and 5-OH VPA (ω oxidation) metabolic pathways in humans, the range of doses used in these studies were noticeably smaller with a maximum dose of 17 mg/kg (Granneman *et al.*, 1984; Anderson *et al.*, 1992). The higher doses used in our study may have resulted in a more substantial saturation of the primary pathways responsible for VPA elimination (i.e. β-oxidation and glucuronidation), thus diverting more of the dose to P-450 metabolism. Examination of dose-dependent changes in VPA urine mass balance data is discussed below and will help to clarify the situation.

2.6.4 Dose-Dependent Changes in Urinary Excretion of VPA

In study A, we observed a significantly lower VPA Cl^{u}_{r} in 10 day old lambs when compared to adult. This was attributed to differences in the ability of the lambs and adult sheep to excrete organic acids. The organic acid secretory pathway in the renal

tubules is an active transport process, thus it is subject to saturation at high drug concentrations (Aranda, 1992). In the current study, we examined changes in the Cl_r of total and unbound VPA with increasing dose for the two age groups of interest. A significant decrease was observed in the Cl^u_r for adult sheep. Although a slight decrease was observed in neonatal lambs, this decrease was neither statistically significant nor of the same magnitude as observed in adult sheep (Table 2.12). Our data is consistent with the presence of a saturable process involved in the renal excretion of VPA in adult sheep, and perhaps to a lesser degree in neonatal lambs. Although our results do not provide conclusive evidence that tubular secretion accounts for the observed differences in VPA urinary excretion noted in study A, our data is consistent with this notion. A similar decrease in VPA renal clearance with dose was also observed in dose-ranging experiments in rats (Liu and Pollack, 1993).

Interestingly, the changes in the urinary recovery of unchanged VPA did not appear to follow any consistent pattern of increase or decrease in adult sheep, and did not appear to change with dose in neonatal lambs (Tables 2.13 and 2.14). With the observed decreases in Cl^u_r for both age groups, an expected decrease in the recovery of unchanged VPA in urine was expected at least in adult sheep. Likely, saturation of other available routes of elimination at higher doses resulted in more of the drug being cleared *via* renal excretion than would be expected from examining Cl^u_r data.

2.6.5 Dose-Dependent Changes in VPA Metabolism

The interpretation of VPA metabolite mass balance data from the dose-dependent studies is complex, as the contribution to overall elimination of the different metabolic pathways are not entirely independent of each other. At high concentrations, saturation of primary pathways of elimination results in the "shunting" of the administered dose to normally minor routes of elimination. Our mass balance data is consistent with metabolic saturation of β-oxidation. For both age groups, we observed an overall decrease in the percentage of the administered dose recovered as β-oxidation metabolites (i.e. (E)-2-ene, (E)-3-ene, 3-OH, and 3-keto VPA) with 3-keto VPA being the primary metabolite at all doses (Table 2.13 and 2.14). As mentioned above (Section 2.6.3), a similar phenomenon has been previously observed in dose ranging studies in man (Granneman *et al.*, 1984; Dickinson *et al.*, 1989). In contrast, the contribution of ω oxidation (5-OH VPA and 2-PGA) and ω-1 oxidation (4-OH and 4-keto VPA, and 2-PSA) to VPA elimination increased with increasing dose (Table 2.13 and 2.14). Of the metabolites derived from P-450 metabolism, the increase in the percentage of the dose recovered as the ω-1 oxidation metabolite, 4-OH VPA, was the greatest. This mass balance data is consistent with the plasma AR data described above (Section 2.6.3) suggesting increased formation of P-450 metabolites. As mentioned, dose-dependency has not been previously observed for ω and ω-1 oxidation in man; however; the doses used in these experiments were substantially lower than the doses use in our sheep studies. The urinary excretion of 4-ene VPA was minor for both adult and neonatal sheep and appeared only at the higher doses.

By far the main pathway of VPA elimination is glucuronidation. In adult sheep, no significant change was observed in the recovery of VPA with increasing dose. In fact, VPA-glucuronide accounted for ~70-80% of the administered VPA at all doses. A different situation was observed in lambs. At the 10 mg/kg dose, ~30% of the administered VPA was recovered as the glucuronide metabolite. This percentage significantly increased to ~65-70% at the three higher doses (Table 2.14). Previous dose ranging experiments in adult rats (Dickinson *et al.*, 1979a) and humans (Granneman *et al.*, 1984; Dickinson *et al.*, 1989; Anderson *et al.*, 1992) showed a similar pattern as neonatal lambs where VPA-glucuronidation appeared to increase with dose. The observed differences in changes in VPA-glucuronidation with dose in adult rat and humans in comparison to adult sheep could be a result of species differences in VPA-glucuronidation. Species differences in the relative contribution of other routes of elimination (i.e. especially β-oxidation) may also play a role since mass balance data for each metabolic pathway is not independent of other routes of elimination.

2.6.6 *In Vivo* Estimation of Apparent V_{max} and K_m of Overall VPA Elimination and VPA Glucuronidation.

The mass balance data presented in the preceding section clearly shows that VPA glucuronidation is the main pathway responsible for VPA elimination in both adult sheep and neonatal lambs. In fact at the 50, 100 and 250 mg/kg doses, the majority of the administered dose was recovered as this metabolite in both neonatal lambs (~65-70%)

and adult sheep (~70-80%). Thus, the estimated apparent *in vivo* V_{max} and K_m for overall VPA metabolic elimination is largely reflective of VPA-glucuronidation. This would explain the similar information obtained from the V_{max} and K_m estimates using plasma data and urine data. Estimates from both methods suggest that V_{max} is similar in both age groups (Table 2.15 and 2.16). The higher estimates of V_{max} obtained from plasma data (adult sheep ~445 μg/min/kg and lambs ~430 μg/min/kg) in comparison to the urine data (adult sheep ~290 μg/min/kg and lambs ~330 μg/min/kg) may be a consequence of the fact that VPA-glucuronidation does not account for elimination of the entire dose. The presence of other routes of elimination will also influence the parameter estimates obtained from plasma data. However, urine data directly monitors the production of the specific metabolite of interest. Apparent K_m estimates from the two methods were surprising similar with both suggesting that K_m is higher in lambs. A similar phenomenon was observed in a study examining acetaminophen glucuronidation in adult and fetal sheep microsomes (Wang *et al.*, 1986). In this study, estimates of K_m obtained from microsomes were higher for the fetus than for the adult. This difference in K_m was attributed to either a different form of UDP-glucuronosyltransferase (UDP-GT) being expressed in fetal microsomes and/or developmental changes in enzyme function resulting from alterations in the phospholipids in the immediate environment of the UDP-GT's within the microsomal membrane (Wang *et al.*, 1986). It is possible that the higher K_m estimate observed for VPA glucuronidation in neonatal lambs is a result of similar reasons since the neonatal lamb is an early phase in the transition from the fetal to the adult situation.

The Michaelis-Menten parameter, V_{max} is defined as the maximum enzyme capacity and is related to the total concentration of enzyme. The other Michaelis-Menten parameter, K_m, is the Michaelis-Menten constant and has an inverse relationship to enzyme affinity. (Rowland and Tozer, 1980). Therefore, the apparent V_{max} and K_m from our dose-ranging studies suggest that metabolic capacity is similar in the two age groups of interest. However, since K_m was estimated *in vivo*, it is not possible to determine if a difference in K_m is related to developmental changes in enzyme affinity and/or enzyme function. A higher apparent K_m in 10 day old lambs explains the relative changes in Cl^u_{tb} with increasing dose in both age groups. Earlier we stated that VPA Cl^u_{tb} approximates intrinsic clearance. Intrinsic clearance is related to V_{max} and K_m by the following equation:

$$Cl_{int} = \frac{V_{max}}{K_m + C_u} \qquad \textbf{(Equation 2.6)}$$

where Cl_{int} is the intrinsic clearance and C_u is the unbound drug concentration (Rowland and Tozer, 1980; Gilbadi and Perrier, 1982). At lower drug concentrations, the denominator approximates K_m and thus $Cl_{int} \approx V_{max}/K_m$. Since K_m is smaller in adult sheep, the adult VPA Cl^u_{tb} estimates should be higher than for lambs in this first scenario. At higher concentrations, the denominator of equation 2.6 approximates C_u and $Cl_{int} \approx V_{max}/C_u$. Therefore, as concentrations increase, the Cl^u_{tb} in adult and neonatal sheep should become increasingly similar since V_{max} is similar for both age groups. Our Cl^u_{tb} data follows this exact pattern with adult Cl^u_{tb} values being

significantly higher at the two lower doses. By the 100 mg/kg dose, the adult Cl^{u}_{tb} estimates have decreased to the point where they are no longer different than the lamb values. The Cl^{u}_{tb} estimates from both age groups remain similar at the highest dose (Figure 2.8).

Differences observed in the recovery of VPA-glucuronide in adult (~74% of the dose) and neonatal lambs (~30% of the dose) following the 10 mg/kg VPA dose are also consistent with a higher K_m in neonatal lambs. VPA metabolism is essentially a "competition" between various metabolic pathways. Therefore, a decrease in the ability of one pathway to eliminate VPA will result in the elimination of the compound by one of many alternate metabolic routes. Thus, the higher apparent K_m of VPA glucuronidation in lambs would allow a larger portion dose to be eliminated by other processes than would occur in adult sheep. With increases in dose, elimination pathways tend to saturate which would allow a larger portion of VPA to be glucuronidated. This theory is consistent with our data as VPA glucuronidation in neonatal lambs increase substantially with an increase in dose. In fact, at the 50 mg/kg dose, almost the entire dose is recovered (~90%) with the majority of this being VPA-glucuronide (~65% of the dose). We have yet to recover ~50% of the dose in 10 day old lambs following the administration of the lowest dose (i.e. 10 mg/kg). Thus, the presence of a high affinity, low capacity VPA elimination process would explain our results. The nature of this process remains to be identified.

V_{max} and K_m of VPA glucuronidation have been previously estimated in adult guinea pigs using both *in vitro* (i.e. 5% liver homogenate and microsomes) and *in vivo* methodologies (Yu *et al.*, 1993; Yu and Shen, 1996). In these studies, *in vitro* V_{max} estimates of ~260 μg/min/kg and ~170 μg/min/kg were obtained using liver homogenate and microsomes, respectively. A similar apparent V_{max} estimate of ~220 μg/min/kg was obtained *in vivo* in dose-ranging drug infusion experiments. K_m estimates obtained for guinea pigs from these studies were similar being ~45 μg/ml (5% liver homogenate), ~23 μg/ml (microsomes) and ~22 μg/ml (*in vivo*) (Yu *et al.*, 1993; Yu and Shen, 1996). These adult guinea pig estimates are comparable to our estimates obtained for adult sheep using urine data (V_{max} ~288 μg/min/kg and K_m ~30 μg/ml). K_m estimates from both adult sheep and guinea pigs are within the clinical range of unbound drug (Yu, 1984) suggesting that VPA glucuronidation is nonlinear at therapeutic doses in these two species.

Estimation of *in vivo* apparent V_{max} and K_m of overall VPA elimination has also been assessed using plasma data from developing rats (Haberer and Pollack, 1994). The V_{max} (~302 μg/min/kg) and K_m (~100 μg/ml) for 10 day old rats in this study are comparable to our own estimates obtained from lamb plasma data (V_{max} ~430 μg/min/kg, K_m ~70 μg/ml). However, V_{max} estimates in slightly older rats (i.e. 20 days and 60 days) were substantially larger (i.e. 975 μg/min/kg (20 day old) and 4460 μg/min/kg (60 day old)) than even our adult estimates. It must be mentioned that these estimates were obtained following single dose bolus experiments. Reliable estimation of *in vivo* V_{max} and K_m requires dose-ranging experiments at 3-4 dose levels with at

least some doses where saturation kinetics is evident (Metzler and Tong, 1981; Gilbaldi and Perrier, 1982). Although not nearly as reliable, estimates can still be obtained from single dose studies exhibiting some degree of saturation kinetics. However, signs of saturation kinetics (i.e. nonlinear terminal slope of semilogarithmic plasma concentration *vs.* time plot) were only present in the plasma profiles of the 5 and 10 day old rats in these studies. Thus, it is difficult to assess the validity of the estimates obtained from the older rats (I.e. 20 and 60 day old) and make comparisons to our own adult data.

2.6.7 Summary for Study B

Developmental differences exist in both plasma protein binding and metabolism of valproic acid. VPA plasma protein binding capacity was found to be higher in adult sheep, whereas binding affinity appeared to be lower. The unexpected lower binding affinity in adult sheep may be attributed to higher volatile fatty acid levels in plasma of adult ruminants in comparison to lambs and monogastric animals. Differences in apparent K_m rather than the metabolic capacity of the glucuronidation pathway appeared to be primarily responsible for the differences in Cl^u_{tb} observed in study A between adult and neonatal sheep. Since mass balance data from study A suggests that rapid changes in VPA glucuronidation occur during the time period between 10 days and 2 months of age, future dose-ranging studies in 1 and 2 month old lambs would provide further insight on developmental changes in VPA-glucuronidation.

Chapter 3

Developmental Disposition of DPHM in Post-natal Lambs

In our previous studies using chronically instrumented pregnant sheep we investigated the disposition of DPHM and its metabolites (DPMA, DPHMNO) in both the mother and the fetus. As mentioned in section 1.2.5 of the introduction, our estimates of weight normalized fetal non-placental clearance (Cl_{fo}) (i.e. the component of fetal total body drug clearance that is not due to drug eliminated *via* the placenta) were ~ 3 times that of the ewe (Kumar *et al.*, 1997). Also, DPHM renal clearance was higher in the fetus while DPMA renal clearance was negligible. As a continuation of these studies, this chapter of my thesis describes a study investigating changes in DPHM disposition in the post-natal period in lambs. Such a developmental study allows us to determine if the high fetal Cl_{fo} values persist into the post-natal period. Also, the study provides us with a unique opportunity to examine differences in the developmental pattern of the renal excretion of acidic (i.e. DPMA) and basic (i.e. DPHM) compounds. Furthermore, the developmental disposition of DPMA and DPHMNO will be investigated.

3.1 Methods

3.1.1 Animals and Surgical Preparation

A total of 11 Dorset-Suffolk cross-bred lambs were employed in this study. The study was approved by the University of British Columbia Animal Care Committee, and the procedures performed on sheep conformed to the guidelines of the Canadian Council on Animal Care. The 11 lambs were of two different age groups: a 15 day old group (15 d, n=5) and a 2 month old group (2 M, n=6). Mean 15 d and 2 M lamb body weights were 6.3 ± 1.5 kg and 12.7 ± 2.9 kg, respectively. Since procedures and methodologies were already in place for *in vivo* experiments on lambs for the VPA study, similar ages were chosen for the DPHM lamb study. It has been shown that maturation of drug-metabolizing enzymes in sheep occurs during the first 11 months of life (Kaddouri *et al.*, 1990; Kawalek and El Said, 1990). Although the two ages chosen are in the early part of this period, it is usually early on (< 7 months) that some of the most rapid changes in drug metabolizing enzyme activity occurs (Kaddouri *et al.*, 1990).

All lambs were surgically prepared 7-8 days prior to the experiment under isoflurane (1%) anesthesia (Ayerst Laboratories, Montreal, Canada). As with the lambs in the VPA study, catheters were implanted in a carotid artery, a jugular vein, and the urinary bladder. Surgical and pre-experimental procedures for the lambs in this study are as described for the VPA studies (see Chapter 2 section 2.1.1).

3.1.2 Experimental Protocols

15 day old (15 d) Group: Experiments were performed at approximately 15 days of age (mean = 15.6 ± 1.3 days; range = 15-18 days old). A 10 mg *i.v.* DPHM bolus (Sigma Chemical Co., St. Louis, MO) was administered *via* the jugular vein catheter over 30 seconds. Serial blood samples (2 ml) were collected from the carotid artery catheter at 2.5, 5, 10, 20, 30, 45, 60, 90, 120, 180 min, and 4, 6, 9, 12, 24, 36, 48, 72, 96 h following drug administration. Cumulative urine samples were also collected for 96 h.

2 month old (2 M) Group: Experiments were performed at approximately 2 months of age (mean = 61.3 ± 1.5 days; range = 60-64 days old). 2 M lambs were administered a 20 mg *i.v.* DPHM bolus (Sigma Chemical Co., St. Louis, MO) *via* the jugular vein catheter over 30 seconds. Serial blood samples and cumulative urine were collected as for 15 d lambs, except sampling of both blood and urine ended at 72 h following drug administration. Cumulative urine was not collected in two lambs from this group due to failure of the urinary bladder catheter.

DPHM doses were prepared in sterile water for injection and were sterilized by filtering through a 0.22 μm nylon syringe filter (MSI, Westboro, MA) into a capped empty sterile injection vial.

All blood samples were placed into heparinized Vacutainer® tubes (Becton-Dickinson, Rutherford, NJ) and centrifuged at 2000 × *g* for 10 min. The plasma supernatant was removed and placed into clean borosilicate test tubes with polytetrafluoroethylene-lined

caps. Aliquots of urine (~10 ml) were collected at intervals and stored similarly in borosilicate test tubes with polytetrafluoroethylene-lined caps. All collected samples were stored frozen at -20°C until the time of analysis.

3.1.3 Determination of DPHM Plasma Protein Binding

DPHM unbound fraction was determined *ex vivo* in pooled plasma samples using the equilibrium dialysis procedure described by Yoo *et al.* (1993). Briefly, dialysis was carried out at 39°C in Plexiglass® dialysis cells. Plasma and isotonic phosphate buffer were separated by cellophane dialysis membrane (molecular cutoff = 12,000 Da; Sigma Chemical Co., St. Louis, MO) which prior to use was boiled in distilled water (for 1 h) and soaked in isotonic phosphate buffer (for 1 h). Equilibration time was 3 h after which the plasma and buffer were analyzed for DPHM. DPHM plasma unbound fraction was calculated by dividing the DPHM concentration of the buffer by the concentration of the plasma following dialysis.

3.1.4 Drug and Metabolite Assay

DPHM and DPHMNO concentrations in all biological fluids were measured using a liquid chromatographic tandem mass spectrometric (LC-MS/MS) analytical method previously developed in our laboratory (Kumar *et al.*, 1998). Briefly, appropriate volumes of biological fluids (with appropriate internal standards added) were alkalinized (pH 11.5) by the addition of 0.5 ml of saturated sodium carbonate buffer and extracted for 20 min with 6 ml of ethyl acetate (Caledon Laboratories, Georgetown, Canada) containing 0.05

M triethylamine on a rotary mixer. The organic extract was separated and dried under a gentle stream of nitrogen at 25°C. Next, the residual was reconstituted in 200 µl of acetonitrile-water (9:1) and 10 µl was injected into the HPLC system (HP 1090 II; Hewlett-Packard; Avondale, PA). Chromatographic separation was achieved using a YMC propyl amino column (100 × 2.0 mm i.d.,5 µm; YMC, Inc., Wilmington, NC) employing normal phase chromatography. The chromatographic conditions are as follows. Mobile phase flow rate was 0.4 ml/min with a 50:50 split between the mass spectrometer and waste. The chromatographic run began with acetonitrile-2mM ammonium acetate buffer (95:5) with 1% glacial acetic acid (pH 3.0). The aqueous buffer proportion was increased to 25% in a 6 min linear gradient, held for 0.5 min, brought back to the initial conditions at 7.0 min, and held there for 3 min. This resulted in a total run time of 10 min. The tandem mass spectrometer (Fisons VG Quattro I Triple Quad Tandem-Mass Spectrometer; Micromass, Cheshire, UK) was operated in multiple reaction monitoring mode. Compounds were ionized in positive ion electrospray mode using nitogen as both the nebulizing and bath gas. The ion transitions monitored were m/z 256-167 (DPHM), m/z 272-167 (DPHMNO), and m/z 270-181 (orphenadrine, internal standard). Argon gas at a pressure of 3×10^{-4} mbar was used to achieve collision induced dissociation. Collision energy, ion source temperature and cone voltage were optimized at 70 eV, 110°C, and 30 V, respectively. The calibration curve ranged from 0.2-250 ng/ml for DPHM and 0.4-100 ng/ml for DPHMNO. Inter- and intra-day variability and bias of this assay were $\leq$15% at analyte concentrations below 2.0 ng/ml and <10% at all other concentrations (Kumar *et al.*,

1998). DPHMNO was generously provided by the Parke-Davis Pharmaceutical Research Division (Ann Arbor, MI).

DPMA concentrations in plasma and urine were measured using a previously developed gas chromatographic mass spectrometric analytical method (Tonn *et al.*, 1995). Briefly, appropriate volumes of biological fluids (with appropriate internal standard added) were acidified with 0.4 ml of 1*M* HCl, and extracted for 20 min with 5 ml of toluene (Caledon Laboratories, Georgetown, Canada) on a rotary mixer. The toluene extract was separated and dried under a gentle stream of nitrogen at 40°C. Next, the residue was reconstituted in 200 µl of dry toluene. The reconstituted residue was derivatized with 25 µl MTBSTFA (Pierce Chemical Co., Rockville, IL) at 60°C for 1 h in order to form tert-butyldimethylsilyl (t-BDMS) derivatives of DPMA. A 1 µl aliquot of the derivatized extract was injected into the GC-MS using the splitless mode of sample introduction (purge time 1.5 min). Chromatographic separation was achieved using a HP Ultra-2 (25 m x 0.25 mm i.d., 0.25 µm film thickness) fused silica capillary column (Hewlett Packard, Avondale, PA) with helium as the carrier gas at a 15 psi column head pressure. The GC operating conditions were as follows. The injection port temperature was 280°C. The oven temperature program consisted of an initial temperature of 125°C for 1 min, and a 12.5°C/min ramp to 280°C where it was held for 4.0 min. This resulted in a total run time of 17.4 min. The mass spectrometer was operated in electron impact ionization mode (electron ionization energy 70eV) with selected ion monitoring (EI-SIM) at transfer line and ion source temperatures of 285°C and 180°C, respectively. Ion fragments *m/z* 165 and 183 were used to monitor diphenylacetic acid (DPAA; internal

standard) (Sigma Chemical Co., St. Louis, MO), and DPMA, respectively. The DPMA calibration curve concentration range for this assay is 2.5–250.0 ng/ml. Inter- and intra-day variability and bias of this assay were <20% at the LOQ and <10% at all other concentrations (Tonn *et al.*, 1995). The DPMA metabolite was synthesized as previously described (Tonn *et al.*, 1995).

3.1.5 Pharmacokinetic Analyses

Pharmacokinetic parameters were calculated by standard methods as described in Gibaldi and Perrier (1982) (see section 2.1.5). The apparent elimination $t_{1/2}$'s of DPMA and DPHMNO were estimated from their respective terminal phases. DPHM terminal elimination half-life ($t_{1/2\beta}$), $AUC_{0-\infty}$, and $AUMC_{0-\infty}$ was obtained from a 2-compartment model fitting of the data using the nonlinear least-squares regression software, WinNonlin (Scientific Consulting, Inc., Apex, NC). All model fittings were carried out using a weighting factor of 1/predicted y^2.

Renal Clearances for DPHM and DPHMNO were calculated by dividing the total amount of each compound excreted in urine divided by their respective plasma $AUC_{0-\infty}$. DPHM and DPHMNO plasma $AUC_{0-\infty}$'s were calculated by the linear trapezoidal rule. Because of the long apparent $t_{1/2}$ of DPMA, the $AUC_{0-\infty}$ could not be estimated accurately. Consequently, the mean DPMA renal clearance was estimated by dividing the average urinary excretion rate by the plasma drug concentration at the time corresponding to the

midpoint of the collection interval. The resulting DPMA Cl_r values for all urine collection intervals were averaged to get the mean DPMA Cl_r for the lamb.

3.1.6 Statistical Analysis

All data are reported as mean ± S.D. Pharmacokinetic parameters were compared using an unpaired t-test (for two groups) or ANOVA (Analysis of Variance) followed by Fischer's LSD multiple comparison test (for more than two groups). The significance level was $p < 0.05$ in all cases. Linear regression analysis was performed using SigmaPlot Version 5.0 (SPSS Inc., Chicago, IL).

3.2 Results

3.2.1 Comparative Pharmacokinetics of DPHM in Fetal, Post-Natal and Adult Sheep

Figure 3.1. is a semilogarithmic plot of mean DPHM concentration *vs.* time for 15 d and 2 M lambs plasma following *i.v.* administration. With the exception of two animals from the 15 d group, the plasma profile for both lamb groups is best described by a biexponential equation; a rapid DPHM distribution in the two animals resulted in an apparent monoexponential plasma profile. Pharmacokinetic parameters for 15 d and 2 M lambs are presented in Table 3.1. DPHM weight normalized total body clearance (Cl_{tb}) and other pharmacokinetic parameters in Table 3.1 were not significantly different between the two post-natal lamb groups. When compared to our previous adult and fetal estimates (Kumar *et al.*, 1999a), the mean Cl_{tb} for both post-natal lamb groups were similar to fetal Cl_{fo}, but significantly higher than adult Cl_{mo}. No significant differences were found when comparing steady-state volume of distribution (Vd_{ss}) between the post-natal lambs and adult sheep; however; Vd_{ss} was significantly larger for fetal lambs (Table 3.1). Elimination half-life ($t_{1/2\beta}$) and mean residence time (MRT) for DPHM in post-natal lambs were significantly shorter when compared to the corresponding adult values.

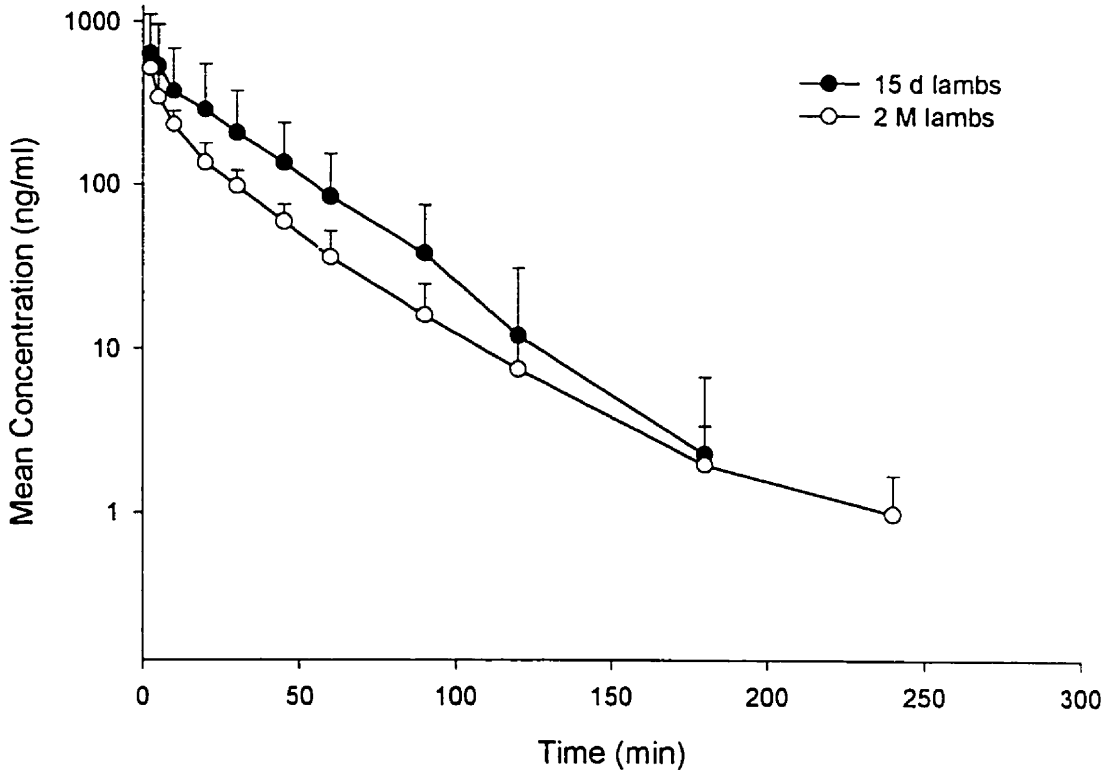

Figure 3.1 Mean DPHM plasma concentration *vs.* time profiles in 15 d and 2 M lambs following *i.v.* bolus administation.

Table 3.1 Pharmacokinetic parameters for fetal, 15 day old, 2 month old, and adult sheep.

Parameter	AGE GROUP			
	Fetus[a]	15 day (15 d)	2 month (2 M)	Adult[a]
C_{ltb}(ml/min/kg)	116.3 ± 49.6[b]*	138.7 ± 80.5*	165.7 ± 51.3*	38.5 ± 12.3[c]**
Vd_{ss} (l/kg)	13.1 ± 3.1*	4.8 ± 2.9**	4.4 ± 2.4**	2.1 ± 1.1**
$t_{1/2\beta}$ (min)	33.1 ± 21.6*	27.6 ± 9.7[d]*	22.4 ± 5.1*	57.2 ± 18.2**
MRT (min)	51.3 ± 18.9**	35.4 ± 9.4*	25.5 ± 5.9*	60.8 ± 19.1**

[a] data from Kumar *et al.*, 1999a
[b] fetal non-placental clearance value
[c] maternal non-placental clearance value
[d] $t_{1/2}$ from 2 animals in group estimated from 1 compartment model. $t_{1/2}$ from remaining 3 animals estimated using 2 compartment model.
Values with different numbers of asterisks (* or **) are significantly different as determined by Fischer's LSD Multiple-Comparison Test ($p<0.05$).

DPHM plasma unbound fractions ranged between 0.05-0.29 for the 15 d group and 0.07-0.26 for the 2 M group. The mean DPHM plasma unbound fraction (f_u) for 15 d (0.15 ± 0.10), 2 M (0.15 ± 0.06) and adult sheep (0.12 ± 0.07) (Kumar, 1998) were not significantly different. However, DPHM f_u for both groups of post-natal lambs and adult sheep were significantly lower than values previously observed for fetal lambs (0.30 ± 0.09) (Kumar, 1998). Figure 3.2 shows a highly significant relationship between Cl_{tb} and DPHM plasma unbound fraction (pooled data).

3.2.2 Plasma Disposition of DPHM Metabolites

Representative profiles for DPMA and DPHMNO concentrations after DPHM *i.v.* administration are presented in Figure 3.3. As observed in Figure 3.3, DPMA elimination from plasma is extremely slow in comparison to DPHM and DPHMNO, especially in 15 d lambs. Figure 3.4 compares the apparent elimination $t_{1/2}$ of DPMA in fetal, post-natal, and adult sheep. DPMA apparent elimination $t_{1/2}$ is significantly longer for 15 d lambs when compared to all other groups. No differences existed for DPHMNO apparent elimination $t_{1/2}$'s in 15 day old (0.88 ± 0.51 h) and 2 month old (1.56 ± 0.82 h) lambs (unpaired t-test, $p<0.05$). DPHMNO apparent elimination $t_{1/2}$'s for fetal and maternal sheep were not calculated in previous studies (Kumar, 1998), and thus comparisons to the post-natal lamb data are not possible.

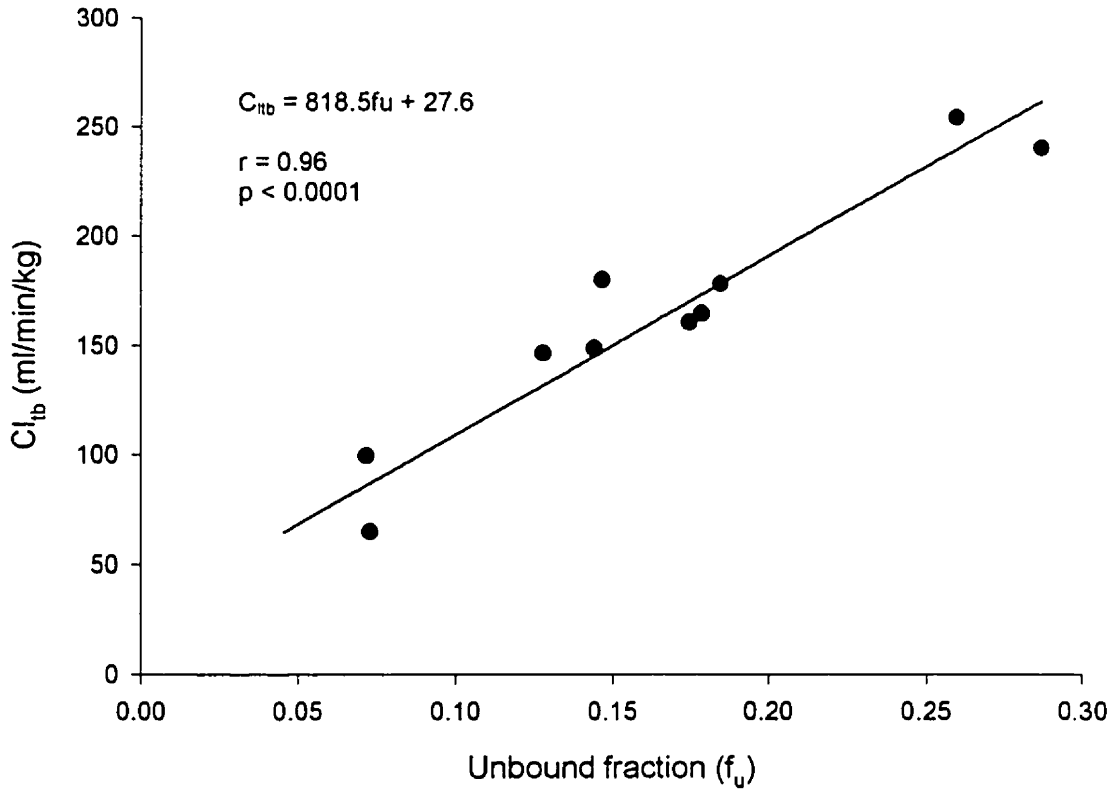

Figure 3.2 DPHM total body clearance *vs.* unbound fraction for post-natal lambs (15 d and 2 M lambs). Regression line shows relationship between total body clearance and DPHM unbound fraction.

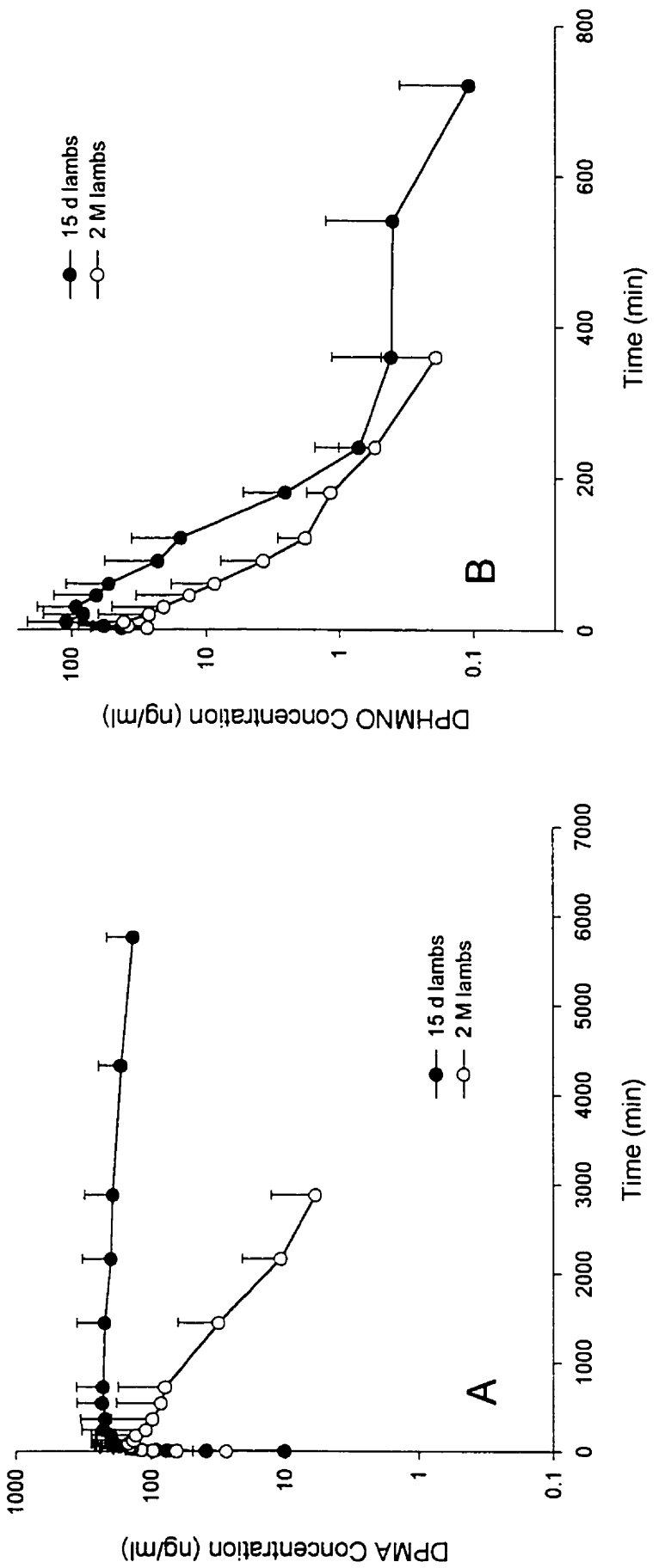

Figure 3.3 **(A)** Mean DPMA plasma concentration *vs.* time profiles for 15 d and 2 M lambs. **(B)** Mean DPHMNO plasma concentration *vs.* time profiles for 15 d and 2 M lambs.

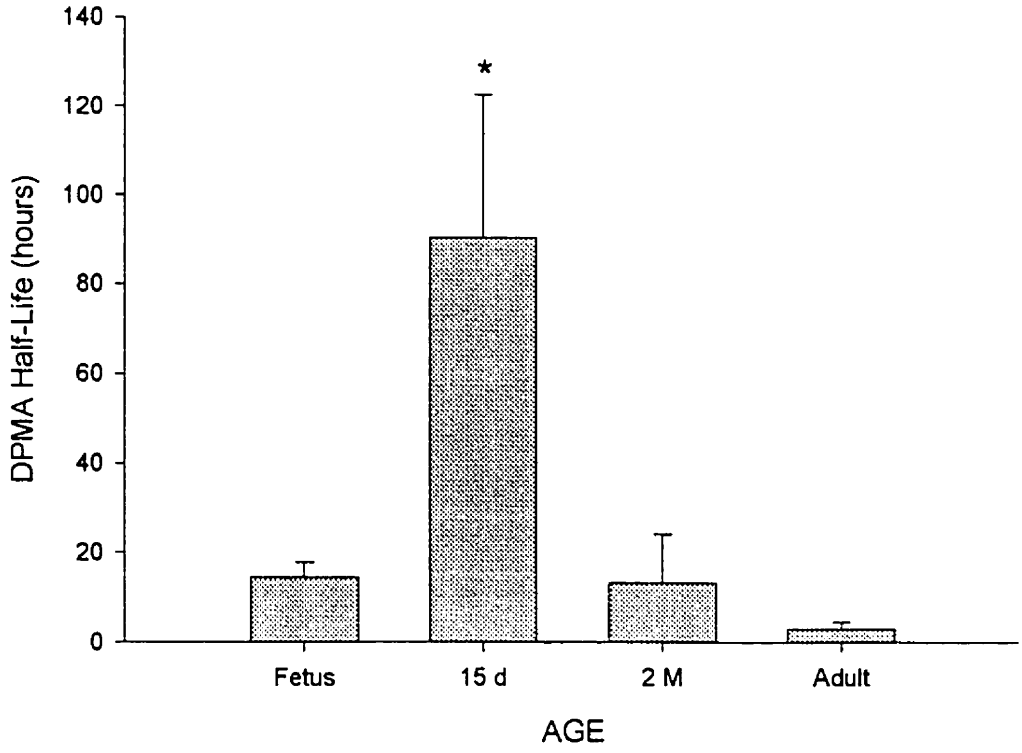

Figure 3.4 DPMA plasma half-life in fetal, 15 d, 2 M, and adult sheep. Fetal and adult data are from Kumar *et al.* 1999a. *denotes significant difference from the fetus, 2 M lambs and adult values ($p<0.05$).

3.2.3 Urinary Excretion of DPHM, DPMA, and DPHMNO

After birth, the renal clearance (Cl_r) of DPHM decreases with age (Figure 3.5A). DPHM Cl_r for fetal (2.06 ± 0.24 ml/min/kg, n=5; Kumar *et al.*, 1997) and 15 d lambs (1.80 ± 1.24 ml/min/kg, n=5) were not significantly different. However, DPHM Cl_r for both fetal and 15 d lambs were significantly higher than for 2 M lambs (0.26 ± 0.17 ml/min/kg, n=4). The adult DPHM Cl_r of 0.012 ± 0.005 ml/min/kg (n=4; Kumar *et al.*, 1997) was the lowest in comparison to all other age groups (Figure 3.5A). As expected from the DPHM Cl_r data, the percentage of the dose excreted in urine as the parent compound was significantly higher for the 15 d group (1.32 ± 0.73%, n=5) in comparison to the 2 M group (0.14 ± 0.08%, n=4).

In contrast to DPHM, DPMA Cl_r increased progressively with age (Figure 3.5B). Mean DPMA Cl_r was the lowest in the fetus (0.007 ± 0.006 ml/min/kg, n=3; Kumar *et al.*, 1999a) followed by 15 d lambs (0.02 ± 0.02ml/min/kg, n=5), 2 M lambs (0.05 ± 0.01, n=4), and finally adult sheep (0.53 ± 0.19 ml/min/kg, n=5; Kumar *et al.*, 1999a). DPMA Cl_r was found to be significantly lower in all groups of lambs when compared to adult values (Figure 3.5B). Mass balance data was not compared due to incomplete collection of this metabolite in lambs during the experimental period.

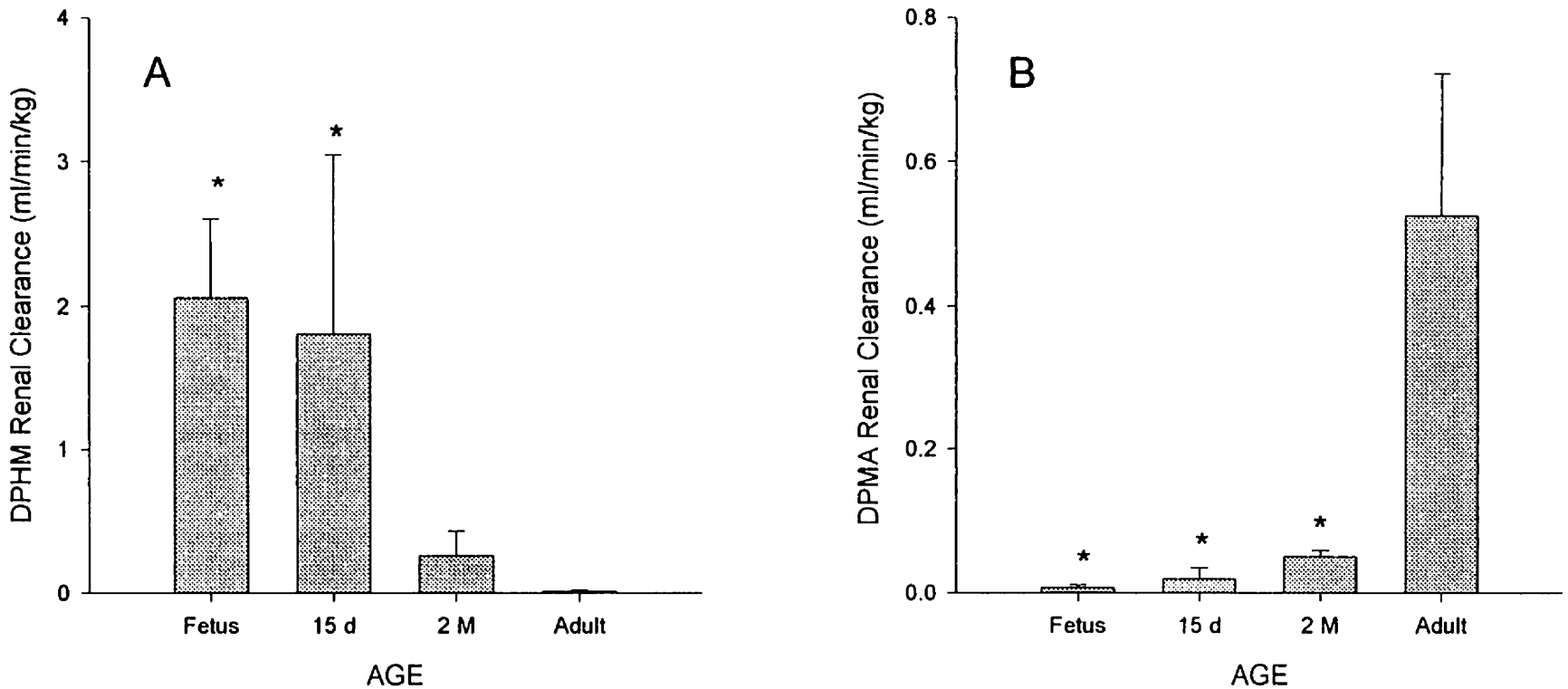

Figure 3.5 (A). Changes in DPHM renal clearance with age. Fetal and adult data are from Kumar *et al.* 1999a. *denotes significant difference from the adult value ($p<0.05$).
(B). Changes in DPMA renal clearance with age. Fetal and adult data are from Kumar *et al.* 1999a. *denotes significant difference from the adult value ($p<0.05$).

No significant changes in DPHMNO renal clearance were observed with age (15 d lambs = 7.7 ± 6.0 ml/min/kg, 2 M lambs = 3.0 ± 2.5 ml/min/kg and Adult = 2.6 ± 1.7 ml/min/kg (Kumar, 1998)) (ANOVA, $p<0.05$). Fetal data was not included for DPHMNO because of the low number of animals (i.e. n=2). A significantly higher percentage of the DPHM dose was excreted as DPHMNO in 15 d lambs (2.4 ± 1.3%) when compared to the 2 M lambs and adult sheep (< 0.4%).

3.3 Discussion

3.3.1 DPHM Pharmacokinetics in Post-Natal Lambs

The results of the present study indicate that DPHM Cl_{tb} in post-natal lambs up to 2 months of age is similar to previously calculated fetal Cl_{fo} and is ~3-4 fold higher compared to that in adult sheep. A similar situation seems to occur in humans where DPHM Cl_{tb} in children was ~2 fold higher than adult values (Simons *et al.*, 1990). The observed DPHM Cl_{tb} in 15 d lambs exceeds estimated values of hepatic blood flow (~58 ml/min/kg) (Gleason *et al.*, 1985) suggesting the presence of an extrahepatic clearance component. A similar situation exists for 2 month old lamb where DPHM Cl_{tb} approximates the total cardiac output (~160 ml/min/kg) (Rudolph, 1985). Since the lung receives the entire cardiac output, significant DPHM lung uptake (*via* either metabolism or strong tissue binding) in post-natal lambs would explain our observations. This is a possibility since the accumulation of high concentrations of DPHM in the lung have been observed previously in rat (Glazko and Dill, 1949; Okumura *et al.*, 1978), guinea pig (Glazko and Dill, 1949), and humans (Hausmann *et al.*, 1983). DPHM volume of distribution estimates for both groups of post-natal lambs and adult sheep were not significantly different. However, post-natal lamb Vd_{ss} was significantly lower than fetal values. A decrease in Vd_{ss} following birth is expected since drug administered post-natally cannot distribute to the maternal compartment that is available to the fetus. DPHM half-life and MRT for adult sheep were ~2 fold higher than was observed for both 15 d and 2 M lambs (Table 3.1). A similar situation occurs in humans where $t_{1/2}$ in

adults is ~2 fold higher than is observed in children (Simons *et. al* 1990). Thus, it appears that the pharmacokinetics of DPHM in plasma does not change significantly during the first 2 months of life, and aside from expected differences in Vd_{ss}, are similar to those in the fetus. Since the development of drug metabolizing enzymes occurs largely in the first 11 months of life (Kaddouri *et al.*, 1990; Kawalek and El Said, 1990), a study in slightly older lambs (i.e. ~5-6 month old) may perhaps be beneficial. However, since we are still unclear as to what enzymes are responsible for DPHM metabolism, it is difficult to chose an appropriate age to study since the onset of activity for different enzymes vary (Kaddouri *et al.*, 1990). Furthermore, the assessment of possible extrahepatic clearance components in lambs, and their changes with development needs to be investigated for a better understanding of DPHM disposition in post-natal lambs.

A linear relationship was observed between Cl_{tb} and unbound drug fraction for post-natal lambs from both groups. We have also observed a similar close relationship between plasma protein binding and clearance in adult and fetal sheep suggesting that plasma unbound fraction is an important determinant of DPHM systemic clearance (Kumar *et al.*, 1999b).

3.3.2 Developmental Disposition of DPMA and DPHMNO in Post-Natal Lambs

As part of this study, we examined the developmental disposition of the DPHM metabolites, DPMA and DPHMNO, in post-natal lambs. No age-related changes were observed in the apparent plasma elimination $t_{1/2}$ of DPHMNO of the two post-natal lamb groups. On the other hand, DPMA apparent elimination $t_{1/2}$ increased dramatically after birth and then decreased with age. Previously, we have shown that DPMA administered to the fetus is almost entirely eliminated *via* the placenta and excreted in maternal urine (Kumar *et al.*, 1999a). Therefore, the long apparent elimination $t_{1/2}$ after birth is expected since the main source of DPMA elimination for the fetus is *via* the placenta. Following this increase, DPMA $t_{1/2}$ decreases by ~30 fold by adulthood. DPMA is not sequentially metabolized in sheep and is eliminated almost entirely by renal excretion (Kumar *et al.*, 1999a). Thus, changes in DPMA apparent elimination $t_{1/2}$ after birth may be attributed to developmental changes in renal clearance of the compound, as discussed below.

3.3.3 Developmental Changes in Urinary Excretion of DPHM and its Metabolites

Renal clearance of DPHM decreased with age after birth. Previously, we found significant differences in the ability of fetal and adult sheep to excrete DPHM (Kumar *et al.*, 1997). DPHM Cl_r in fetal lamb (~2 ml/min/kg) was found to exceed reported values of GFR (~1 ml/min/kg) (Hill and Lumbers, 1988) suggesting the involvement of tubular secretion. The ability of the fetal lamb in late gestation to secrete organic cations such

as tetraethylammonium (Elbourne *et al.*, 1990), meperidine (Szeto *et al.*, 1980), cimetidine (Mihaly *et al.*, 1983; Czuba *et al.*, 1990), and ranitidine (Czuba *et al.*, 1990) has also been observed by others. In fact, for ranitidine and cimetidine, the processes involved in their secretion were operating at full efficiency by 80 days of gestation (Czuba *et al.*, 1990). In contrast, DPHM Cl_r in adult sheep (~0.01 ml/min/kg) (Kumar *et al.*, 1997) was substantially less than the reported GFR (~2.4 ml/min/kg) (Hill and Lumbers, 1988). This suggests reabsorption in the proximal tubule of a portion of the DPHM load that is filtered and/or secreted. The data in the current study, indicates that significant reabsorption likely develops after the first two weeks of life as the DPHM Cl_r values of 15 d and fetal lambs are similar (Figure 3.5A). A similar delayed development of tubular reabsorption in comparison to other kidney processes (i.e. tubular secretion and glomerular filtration) has also been observed in human infants for various basic compounds (i.e. lidocaine and mepivacaine; Moreselli *et al.*, 1980). This change in DPHM reabsorption may be in part attributable to age-related increases in urine pH (Moreselli, 1976) and increases in urine concentrating capacity (Lindshaw, 1992). Differences in DPHM Cl_r between 15 d and 2 M lambs are reflected in the significantly higher percentage of the administered dose excreted as the unchanged drug in the 15 d group ($1.32 \pm 0.73\%$) in comparison to the 2 M group ($0.14 \pm 0.08\%$).

In contrast to DPHM Cl_r, DPMA Cl_r increased with age. The mechanisms responsible for the tubular secretion of organic acids are not fully functional in the late gestational fetal lamb (Jones and Stapleton, 1992). Thus, the urinary secretion of acidic compounds, such as para-aminohippurate (Elbourne *et al.*, 1990), valproic acid (Kumar,

1998), and indomethacin (Krishna *et al.*, 1995), by the fetal lamb is limited. Our data for post-natal lamb shows a slow increase in mean DPMA Cl_r during the first 2 months of life (Figure 3.5B). This is similar to our observations with VPA in post-natal lambs (Figure 2.4). As mentioned in Chapter 2, increases in organic acid excretion capacity with age are mainly attributable to increases in intrinsic renal tubular transport capacity (Jones and Stapleton, 1992). In addition, age–related increases in renal blood flow and glomerular filtration rate may also play a role (Jones and Stapleton, 1992). Thus, the dramatic decreases in DPMA apparent elimination $t_{1/2}$ observed post-natally reflect the changes in DPMA Cl_r since the elimination of DPMA in sheep is almost entirely *via* renal excretion.

No significant differences were detectable in the Cl_r of DPHMNO among different age groups (i.e. 15 d and 2 M lambs, and adult sheep). The higher percentage of the dose excreted as DPHMNO in 15 d lambs suggests that either a larger fraction of the dose is metabolized *via* the N-oxidation pathway and/or there is reduced sequential clearance of this metabolite. However, the urinary excretion of DPHMNO appears to be of minor significance (<3%) to overall DPHM elimination in sheep in spite of this observed difference.

3.3.4 Summary of DPHM Post-Natal Lamb Study

In summary, DPHM Cl_{tb} in lambs at 15 day and 2 months of age is much higher than in adult sheep, and is similar to fetal Cl_{fo} estimates. Thus, the decrease in DPHM Cl_{tb} to adult values likely occurs after the first two months of life. The age-related alterations in renal clearance of the basic compound, DPHM, and its carboxylic acid metabolite, DPMA, appear to follow opposite trends (i.e. DPHM Cl_r decreases and DPMA Cl_r increases with age). The opposing trends are likely related to differences in the rate of development of kidney mechanisms (i.e. tubular secretion of organic acids and bases, tubular reabsorption, and GFR) involved in drug excretion.

Chapter 4

Overall Summary and Conclusions

Studies were conducted with VPA, a broad spectrum anticonvulsant, and DPHM, a common antihistamine, in order to investigate the developmental disposition of these two compounds. VPA and DPHM were chosen as compounds for investigation due to their contrasting pharmacokinetic characteristics (i.e. low clearance (VPA) *vs.* high clearance (DPHM)), and physicochemical properties (carboxylic acid (VPA) *vs.* basic amine (DPHM)). As there is limited detailed information on neonatal pharmacokinetics, developmental studies utilizing drugs with contrasting features such as VPA and DPHM will provide a better understanding of the ontogenetic development of various factors affecting drug disposition (i.e. metabolism, plasma protein binding, renal elimination).

Our initial investigation on the developmental disposition of VPA involved administration of a 10 mg/kg *i.v.* bolus to 10 day, 1 month and 2 month lambs, and adult sheep. Plasma protein binding was found to be nonlinear in all ages examined with area weighted unbound fraction being significantly higher in 10 day old lambs in comparison to all other age groups. Both unbound and total VPA clearance increased significantly up to 2 months of age before decreasing to adult levels. A comparison between sheep and humans of similar age revealed that VPA clearance estimates in human were lower than observed in sheep (Irvine-Meek *et al.*, 1982; Gal *et al.*, 1988; Davis *et al.*, 1994; Levy and

Shen, 1995). However, a similar pattern of change in clearance was observed in humans where metabolic clearance is lowest in newborns, reaches a maximum from 2-36 months of age, and then decreases to adult values (Levy and Shen, 1995).

In terms of metabolites in plasma, it appeared that the major metabolites were the β-oxidation products 3-keto and (E)-2-ene VPA followed by the P-450 mediated hydroxylation metabolite, 4-OH VPA. Similar to previous investigations in sheep (Kumar, 1998), no di-unsaturated metabolites were observed in plasma in the current studies. All metabolites appeared to persist longer (i.e. larger $AUC_{0-\infty}$) in the younger lambs (10 day old and 1 month old) in comparison to 2 month old lambs and adult sheep. This was attributed to a higher formation and/or reduced elimination of these metabolites. A reduced renal clearance of 3-keto and 4-OH VPA in younger animals appeared to play a role in this phenomenon at least for these two metabolites. Similarities were drawn between our observations in sheep and metabolite levels observed in children< 2 years of age and children > 2 years of age (Nau *et al.* 1991, Siemes *et al.*, 1993).

Cumulative urine was only collected for 10 day and 2 month old lambs, and adult sheep due to catheter failure in all the 1 month old lambs. Analysis of cumulative urine revealed that a significantly larger portion of the VPA dose was excreted in the adult (~12%) urine in comparison to 10 day (~6%) and 2 month old (~3%) lambs. The contribution of β-oxidation appeared to be similar in all age groups

as the percent of the dose recovered as the main β-oxidation metabolite, 3-keto VPA, was similar (~11-13%). The main VPA metabolite in all age groups was VPA-glucuronide accounting for ~30% of the dose in 10 day old lambs, and ~75% of the dose in 2 month old lambs and adult sheep. The substantial (~2.5 fold) increase in urinary recovery of VPA-glucuronide in 2 month old lambs in comparison to 10 day old lambs suggested that changes in clearance may be largely related to post-natal development of enzymes involved in VPA glucuronidation. Finally, similar to other organic acids, VPA renal clearance of unbound drug was initially low and increased with age. This is consistent with the observed differences in the portion of the dose excreted as the unchanged drug with age that is mentioned above.

A unique characteristic of VPA is that it exhibits both saturable protein binding and saturable metabolism at clinically relevant concentrations (Levy and Shen, 1995). A subsequent dose-ranging study in 10 day old lambs and adult sheep was conducted to provide a detailed examination of differences in plasma protein binding and intrinsic clearance with age. The doses administered caused both metabolic saturation and significant changes in the area weighted unbound fraction in both age groups. Characterization of VPA plasma protein binding revealed that lambs appeared to have a lower binding capacity, but a higher binding affinity than adult sheep. The unexpected lower binding affinity in adult sheep may be due to higher volatile fatty acid levels in the plasma of adult ruminants in comparison to lambs and monograstric animals (Annison and Lewis,

1959). Metabolic capacity was similar in 10 day old and adult sheep, however, K_m was higher in the lambs. Differences in K_m appeared to account for the observed dose-dependent changes in the intrinsic or unbound clearance for the two age groups. Estimates of apparent *in vivo* V_{max} and K_m of overall VPA elimination appeared to largely reflect developmental differences in VPA glucuronidation.

The second drug investigated in my thesis was DPHM, a basic amine with high clearance properties. The developmental disposition of DPHM was investigated in 15 day and 2 month old lambs, and compared to previous data collected from fetal and maternal sheep (Kumar, 1998). Similar to observations in fetal and adult sheep, the extent of plasma protein binding appeared to be an important determinant of DPHM systemic clearance in post-natal lambs. In contrast to VPA, DPHM total body clearance in both groups of lambs appeared to be similar to non-placental clearance estimates in fetal lamb, and significantly higher than observed for adult sheep. The high clearance estimates in the lambs exceeded hepatic blood flow suggesting the presence of an extrahepatic clearance component. The renal clearance of DPHM and its acidic metabolite (DPMA) followed different and opposite trends with one (DPMA) increasing with age and the other (DPHM) decreasing with age.

Data on developmental changes in renal clearance from our current studies is consistent with what is known about the ontogenetic development of urinary

excretion of organic acids and bases. The late gestational fetal lamb has been shown to have a limited ability to excrete organic acids such as indomethacin (Krishna *et al.*, 1995), VPA (Kumar, 1998,) DPMA (Kumar, 1998), and para-aminohippurate (Elbourne *et al.*, 1990). Studies in newborn humans (Calcagno and Rubin, 1963), dogs (Kleinman and Lubbe, 1972) and rats (Horster and Lewy, 1970) with para-aminohippurate have demonstrated a gradual post-natal increase in the renal tubular extraction of this organic acid with age. Thus, our observations with VPA and DPMA renal clearance are consistent with increases in renal tubular secretion following birth. In contrast, the ability to secrete organic bases has been demonstrated in late gestation fetal lamb for a number compounds such as tetraethylammonium (Elbourne *et al.*, 1990), meperidine (Szeto *et al.*, 1980), cimetidine (Mihaly *et al.*, 1983; Czuba *et al.*, 1990), and ranitidine (Czuba *et al.*, 1990). In fact for ranitidine and cimetidine, it was demonstrated that the processes involved in their secretion were operating at full efficiency by 80 days of gestation (Czuba *et al.*, 1990). The post-natal ontogenetic development of the renal handling of basic compounds has not been extensively studied. Previously in adult sheep, we have observed low DPHM renal clearance estimates in relation to GFR suggesting that a portion of the drug that is filtered and/or secreted is reabsorbed (Kumar *et al.*, 1997). The present data on DPHM renal clearance in lambs suggests that a significant change in tubular reabsorption of DPHM occurs between 2 weeks to 2 months of age. Similar higher estimates of renal clearance have been observed in human newborns for the lidocaine and mepivacaine (Morselli *et al.*, 1980). As

mentioned in Chapter 3, reabsorption of bases is promoted by increased concentrating capacity of the kidney as well as changes in urine pH (Moreselli, 1976; Lindshaw, 1992). Thus it appears that certain generalizations can be made regarding developmental aspects of renal clearance, which appear to be related to physicochemical characteristics (i.e. acid *vs.* base) of the compounds investigated.

In contrast, it appears that it is very difficult to make generalizations about the ontogenetic development of other clearance processes. In the past, the general belief was that newborns exhibit a reduced ability to eliminate drugs in comparison to the adult (Moreselli, 1976; Moreselli *et al.*, 1980). However, our own studies have suggested that the development of drug elimination appears to be compound specific as the pattern of changes in VPA and DPHM clearance appeared very different. The data that was acquired from our *in vivo* studies suggest that developmental differences in VPA clearance can be largely attributable to age-related differences in plasma protein binding, and more importantly in VPA glucuronidation. It appeared that the apparent K_m of VPA glucuronidation was different between the two age groups examined. We noted that in a previous study with acetaminophen (Wang *et al.*, 1986), a similar phenomenon (i.e. a higher K_m) was observed in fetal microsomes in comparison to the adult. A similar study utilizing *in vitro* methodologies with neonatal lamb hepatocytes or microsomes may provide additional support for our *in vivo* findings. Ultimately, the identification of UDP-GT isozymes in sheep, and their

expression at different ages would provide a clearer explanation of our observations. The cause of developmental alterations in DPHM clearance remains less clear. From our estimates of post-natal lamb total body clearance, it is obvious that an extrahepatic clearance component is present. A preliminary qualitative assessment of metabolic capacity using microsomes suggests that adult and lamb (1.5 months old) liver microsomes are equally efficient in DPHM metabolism (Harvey Wong, K. Wayne Riggs, and Dan W. Rurak, unpublished data). However, we have yet to identify the metabolic pathways of DPHM in sheep. Identification of these pathways and their metabolites would allow for a better understanding of developmental changes in DPHM disposition. More importantly, assessment of DPHM lung uptake requires examination in neonatal lambs and adult sheep as it appears to be the likely explanation for the high clearance estimates observed in lambs. Thus, many areas remain to be investigated in order to obtain a clearer understanding of the developmental disposition of DPHM.

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