Drug Disposition in the Maternal-Fetal Unit: Studies with Diphenhydramine and Valproic Acid in Pregnant Sheep

by

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Abstract

Diphenhydramine (DPHM, a high clearance amine) and valproic acid (VPA, a low clearance carboxylic acid) disposition was examined in chronically-instrumented pregnant sheep during late gestation (after 120 d, term 145 d) in order to: i) identify the important pharmacokinetic factors determining fetal drug exposure, and ii) examine the in utero fetal drug metabolism capacity for these two compounds.

Both drugs underwent rapid placental transfer after maternal dosing. Although the placental permeability for DPHM was higher, the steady-state fetal exposure relative to the mother was greater for VPA (70% vs. 20%). Significant fetal drug elimination capacity was detectable for both compounds, with DPHM having a much higher clearance. Moreover, there was significant (~44%) fetal hepatic first-pass uptake of the placentally transferred DPHM from the umbilical vein due to the unique geometrical position of the fetal liver. These factors were responsible for the observed lower fetal exposure to DPHM. Maternal and fetal placental and non-placental clearances of both drugs were highly dependent on their plasma protein binding. However, for DPHM, the importance of plasma protein binding in determining fetal drug exposure was overridden by the above more pronounced effect of the fetal liver.

Metabolism to diphenylmethoxyacetic acid (DPMA), and DPHM-N-oxide renal excretion accounted for only 1-2% of the DPHM dose in maternal and fetal sheep, as opposed to 50-80% in the monkey, dog and human. Also, DPMA was not secondarily metabolized in sheep. Approximately 95% of the maternal VPA dose was eliminated via glucuronidation and renal excretion, with β-oxidation and cytochrome P-450 pathways
accounting for the remainder. All these pathways appeared to be functional in the fetus; however, the low fetal VPA clearance could not be accurately measured. Studies conducted in 1 day old lambs revealed ~18 fold lower weight-normalized VPA elimination capacity relative to the mother, and this led to a lower newborn VPA clearance and longer half-life, as observed in human newborns. The reduced newborn lamb VPA elimination was mainly attributable to underdeveloped glucuronidation and renal excretion ability; this, combined with a high β-oxidation capacity at birth, resulted in a larger fraction of the VPA dose (~20%) being metabolized via β-oxidation and cytochrome P-450 pathways in lambs.
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<td>103</td>
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Figure 3.7 – Representative cumulative renal excretion profiles of the parent drug, the preformed metabolite and the in vivo generated metabolite in maternal and fetal urine of E303Y. Figures 3.7A and 3.7B are the data from separate maternal and fetal administration experiments, respectively. In both experiments, unlabeled DPMA was administered as the preformed metabolite in combination with $[^{2}\text{H}_{10}]$-DPHM. The $[^{2}\text{H}_{10}]$-DPMA is thus the in vivo generated metabolite. Figure 3.7B shows that even after fetal administration negligible amounts of preformed as well as in vivo generated metabolite are excreted in fetal urine compared to maternal urine.
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Figure 4.2 – Relationships between fetal and maternal DPHM clearances and corresponding plasma unbound fractions of the drug. A) CL_{ff} vs. F-UF; B) CL_{fm} vs. F-UF; C) CL_{fo} vs. F-UF; D) CL_{mm} vs. M-UF; E) CL_{mf} vs. M-UF; and F) CL_{mo} vs. M-UF. The CL_{fo}, CL_{mm} and CL_{mo} relationships with the corresponding plasma unbound fraction of the drug were analyzed according to the well-stirred model of organ drug clearance. UF: unbound fraction; F and M refer to mother and the fetus, respectively; Clearances are as defined in Table 4.1.

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Figure 4.5 – Influence of various clearance parameters of the 2-compartment model on different maternal-fetal plasma concentrations. A) CL_{mo} vs. C_{m}; B) CL_{mf} vs. C_{f}; C) CL_{fo} vs. C_{f}; D) CL_{fm} vs. C_{m}; E) CL_{fm} vs. C_{m}'; and F) CL_{mo} vs. C_{m}'. All relationships except B) and E) were analyzed according to the steady-state clearance model, CL=k_{o}/C_{ss}; the solid lines represent the best-fit lines determined by this model.

Figure 4.6 – Relationships between indices of placental drug transfer/fetal drug exposure and their determining factors. A) C_{f}/C_{m} vs. F-
Figure 4.7 - Relationship between fetal DPHM placental clearance of the unbound drug and gestational age. Actual experimental data (scatter points), linear regression line (solid) and 95% confidence interval are depicted.

Figure 4.8 - Relationship between fetal acetaminophen placental clearance and gestational age. Data were taken from Wang et al., 1986. Actual experimental data (scatter points), linear regression line (solid) and 95% confidence interval are shown.

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Figure 5.5 – Plasma protein binding characteristics of VPA in newborn lamb plasma; pooled data from 4 lambs. \(C_t\), \(C_b\) and \(C_u\) are the plasma concentrations of the total, bound and unbound VPA, respectively. A) Rosenthal plot of the data. B) Relationship between \(C_t\) and \(C_u\). C) Relationship between \(C_b\) and \(C_u\). Anomalous VPA binding characteristics in the newborn plasma are evident when compared to maternal and fetal data in Figure 5.2.

Figure 5.6 – Partitioning of newborn lamb VPA plasma protein binding data into those obtained during day 1 of the experiment (A-C) and those obtained after day 1 (D-F). \(C_t\), \(C_b\) and \(C_u\) are the plasma concentrations of the total, bound and unbound VPA. A) and D) are the Rosenthal plots B) and E) are the relationships between \(C_t\) and \(C_u\). C) and F) are the relationships between \(C_b\) and \(C_u\). The data in F) were fitted to a 1-site binding model and the model-predicted line is also shown. Striking differences in VPA binding characteristics between the 2 groups of data are evident.

Figure 5.7 – In vitro glucuronidation of VPA in pooled maternal sheep liver microsomes. A) Saturation plot of reaction velocity vs. substrate concentration. B) Eadie-Hofstee plot showing monophasic nature of glucuronidation reaction in maternal liver microsomes. \(v\) and \([S]\) are the reaction velocity and substrate concentration, respectively.

Figure 5.8 – Representative pharmacokinetic fitting of newborn and maternal sheep unbound plasma concentration vs. time data to a one-compartment model with Michaelis-Menten elimination. Actual data (scatter) and model predicted profile (solid line) are shown. A) newborn lamb (NL4124), B) pregnant ewe (E5108).

Figure 5.9 – Representative cumulative amount excreted vs. time plots of VPA and VPA-glucuronide in a pregnant ewe (E4241, after maternal administration) and a newborn lamb [NL2243(1)]. Profound differences in the kinetics of urinary excretion of these two compounds relative to the duration of VPA infusion in the ewe and the newborn lamb are evident.

Figure 5.10 – Average maternal and fetal plasma concentration vs. time profiles of the (E)-2-ene (A and C) and 3-keto (B and D) VPA metabolites in 5 pregnant sheep during and after a 24 h steady-state VPA infusion. Upper panel: maternal VPA infusion; lower panel: fetal VPA infusion.

Figure 5.11 – Average maternal and fetal plasma concentration vs. time profiles of the (E)-3-ene (A and C) and 4-ene (B and D) VPA
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Figure 5.13 – A) and B) Average maternal and fetal plasma concentration vs. time profiles of 5-OH VPA in 5 pregnant sheep during maternal and fetal VPA infusions, respectively. C) Average maternal plasma concentration vs. time profile of 2-PGA during 24 h maternal steady-state VPA infusion. 2-PGA was below the LOQ in all fetal plasma samples and was measurable in maternal samples from only one ewe during fetal VPA infusion (see Tables 5.16 and 5.17 for details).

Figure 5.14 – Average plasma concentration vs. time profiles of the VPA metabolites formed via the β-oxidation pathway in 4 newborn lambs during and after a 6 h VPA infusion. A) (E)-2-ene VPA, B) 3-keto VPA, C) (E)-3-ene VPA.

Figure 5.15 – Average plasma concentration vs. time profiles of the VPA metabolites formed via microsomal oxidation pathways in 4 newborn lambs during and after a 6 h VPA infusion. A) 4-ene VPA, B) 4-keto VPA, C) 4-OH VPA, D) 5-OH VPA.

Figure 5.16 – Average concentrations of VPA before and after base-hydrolysis in fetal urine samples obtained from 4 pregnant sheep at different times during and after steady-state VPA infusion experiments. A) maternal infusion experiments, B) fetal infusion experiments.
List of Abbreviations

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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<tbody>
<tr>
<td>( \mu )</td>
<td>Micron</td>
</tr>
<tr>
<td>( \alpha )</td>
<td>Alpha, an exponential rate constant (apparent rate of distribution)</td>
</tr>
<tr>
<td>( \beta )</td>
<td>Beta, an exponential rate constant (apparent rate of elimination)</td>
</tr>
<tr>
<td>( \tau )</td>
<td>Duration of infusion</td>
</tr>
<tr>
<td>( \mu g )</td>
<td>Microgram</td>
</tr>
<tr>
<td>( \mu l )</td>
<td>Microliter</td>
</tr>
<tr>
<td>#</td>
<td>Ewe or lamb identification number</td>
</tr>
<tr>
<td>( \sim )</td>
<td>Approximately</td>
</tr>
<tr>
<td>( [^2H_{10}]\text{-DPHM} )</td>
<td>Stable isotope (deuterium) labeled diphenhydramine</td>
</tr>
<tr>
<td>( [^2H_{10}]\text{-DPMA} )</td>
<td>Stable isotope (deuterium) labeled diphenylmethoxyacetic acid</td>
</tr>
<tr>
<td>( [^2H_{10}]\text{-DPHMNOX} )</td>
<td>Stable isotope (deuterium) labeled diphenhydramine-N-oxide</td>
</tr>
<tr>
<td>( ^2H )</td>
<td>Deuterium</td>
</tr>
<tr>
<td>ACS</td>
<td>American Chemical Society</td>
</tr>
<tr>
<td>AMN</td>
<td>Amniotic</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the plasma concentration vs. time curve</td>
</tr>
<tr>
<td>AUMC</td>
<td>Area under the first moment curve</td>
</tr>
<tr>
<td>( B_{\text{max}} )</td>
<td>Maximal binding capacity</td>
</tr>
<tr>
<td>( ^\circ C )</td>
<td>Degree Celsius</td>
</tr>
<tr>
<td>ca.</td>
<td>Approximate</td>
</tr>
<tr>
<td>CA</td>
<td>Fetal carotid artery</td>
</tr>
<tr>
<td>( C_0 ) or ( C_{\text{bound}} )</td>
<td>Plasma concentration of the protein bound drug</td>
</tr>
<tr>
<td>( C_u ) or ( C_{\text{unbound}} )</td>
<td>Plasma concentrations of the unbound drug</td>
</tr>
<tr>
<td>( CL_{f(\text{net})} )</td>
<td>Net clearance from the fetal compartment based on total drug concentrations</td>
</tr>
<tr>
<td>( CL_f )</td>
<td>Total drug clearance from the fetal compartment based on total drug concentrations</td>
</tr>
<tr>
<td>( CL_{fm} )</td>
<td>Placental clearance of drug from the fetal to maternal compartment based on total drug concentrations</td>
</tr>
<tr>
<td>( CL_{fo} )</td>
<td>Non-placental clearance of drug from the fetal compartment based</td>
</tr>
</tbody>
</table>
on total drug concentrations

\( CL_{\text{m(net)}} \)  Net clearance from the maternal compartment based on total drug concentrations

\( CL_{\text{mm}} \)  Total clearance from the maternal compartment based on total drug concentrations

\( CL_{\text{mf}} \)  Placental clearance from the maternal to fetal compartment based on total drug concentrations

\( CL_{\text{mo}} \)  Non-placental clearance from the maternal compartment based on total drug concentrations

\( CL_{\text{f(net)}} \)  Net clearance from the fetal compartment based on unbound drug concentrations

\( CL_{\text{ff}} \)  Total clearance from the fetal compartment based on unbound drug concentrations

\( CL_{\text{fm}} \)  Placental clearance from the fetal to maternal compartment based on unbound drug concentrations

\( CL_{\text{fo}} \)  Non-placental clearance from the fetal compartment based on unbound drug concentrations

\( CL_{\text{m(net)}} \)  Net clearance from the maternal compartment based on unbound drug concentrations

\( CL_{\text{mm}} \)  Total clearance from the maternal compartment based on unbound drug concentrations

\( CL_{\text{mf}} \)  Placental clearance from the maternal to fetal compartment based on unbound drug concentrations

\( CL_{\text{mo}} \)  Non-placental clearance from the maternal compartment based on unbound drug concentrations

\( CL_{\text{int}} \)  Intrinsic clearance based on total drug concentrations

\( CL_{\text{int}} \)  Intrinsic clearance of the drug based on unbound drug concentrations

\( CL_r \)  Renal Clearance of the total drug

\( CL_{r} \)  Renal Clearance of the unbound drug

\( CL_{tb} \)  Total body clearance based on total drug concentrations

\( CL_{tb} \)  Total body clearance based on unbound drug concentrations

\( C_{\text{last}} \)  Plasma concentration at the last sampling time point

\( C_{\text{m}} \)  Maternal plasma steady-state total drug concentration after maternal administration
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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<tbody>
<tr>
<td>C_m'</td>
<td>Maternal plasma steady-state total drug concentration after fetal administration</td>
</tr>
<tr>
<td>C_f</td>
<td>Fetal plasma steady-state total drug concentration after maternal administration</td>
</tr>
<tr>
<td>C_f'</td>
<td>Fetal plasma steady-state total drug concentration after fetal administration</td>
</tr>
<tr>
<td>C_t</td>
<td>Total (bound + unbound) plasma concentration of the drug</td>
</tr>
<tr>
<td>C_m</td>
<td>Maternal plasma steady-state unbound drug concentration after maternal administration</td>
</tr>
<tr>
<td>C_m'</td>
<td>Maternal plasma steady-state unbound drug concentration after fetal administration</td>
</tr>
<tr>
<td>C_f'</td>
<td>Fetal plasma steady-state unbound drug concentration after fetal administration</td>
</tr>
<tr>
<td>cm</td>
<td>Centimeter</td>
</tr>
<tr>
<td>C_max</td>
<td>Maximal plasma concentration</td>
</tr>
<tr>
<td>C_p</td>
<td>Plasma concentration</td>
</tr>
<tr>
<td>C_ss</td>
<td>Steady state plasma concentration</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>d</td>
<td>Day of gestation or gestational age in days</td>
</tr>
<tr>
<td>D_bolus</td>
<td>Bolus loading dose</td>
</tr>
<tr>
<td>dl</td>
<td>Deciliter</td>
</tr>
<tr>
<td>DOS</td>
<td>Disc Operating System</td>
</tr>
<tr>
<td>DPAA</td>
<td>Diphenylacetic acid</td>
</tr>
<tr>
<td>DPHM</td>
<td>Diphenhydramine</td>
</tr>
<tr>
<td>DPMA</td>
<td>Diphenylmethoxyacetic acid</td>
</tr>
<tr>
<td>DPHMNOX</td>
<td>Diphenhydramine-N-oxide</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>E_H</td>
<td>Hepatic extraction ratio</td>
</tr>
<tr>
<td>E_g</td>
<td>Gut extraction ratio</td>
</tr>
<tr>
<td>EI</td>
<td>Electron impact</td>
</tr>
<tr>
<td>Symbol</td>
<td>Definition</td>
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<tr>
<td>--------</td>
<td>------------</td>
</tr>
<tr>
<td>eV</td>
<td>Electron volts</td>
</tr>
<tr>
<td>F</td>
<td>Bioavailability</td>
</tr>
<tr>
<td>FA</td>
<td>Fetal femoral arterial plasma</td>
</tr>
<tr>
<td>fH</td>
<td>Fraction of intravenous dose metabolized in liver</td>
</tr>
<tr>
<td>Fm</td>
<td>Fraction of drug converted to metabolite or formation clearance of the metabolite as a fraction of total body clearance of the drug</td>
</tr>
<tr>
<td>Fm'</td>
<td>Formation clearance of the metabolite as a fraction of non-placental clearance of the drug</td>
</tr>
<tr>
<td>fp</td>
<td>Area weighted free fraction of the drug (=AUC_{unbound}/AUC_{total})</td>
</tr>
<tr>
<td>Fr</td>
<td>Fraction of total dose excreted unchanged in urine</td>
</tr>
<tr>
<td>F-UF</td>
<td>Unbound fraction of the drug in fetal plasma at steady-state</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>GA</td>
<td>Gestational age</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>GFR</td>
<td>Glomerular filtration rate</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>H₂</td>
<td>Hydrogen gas</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HP</td>
<td>Hewlett Packard</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatograph</td>
</tr>
<tr>
<td>i.d.</td>
<td>Internal diameter</td>
</tr>
<tr>
<td>i.e.,</td>
<td><em>id est</em>; that is</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>IVC</td>
<td>Inferior vena cava</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium chloride</td>
</tr>
<tr>
<td>K_d</td>
<td>Dissociation constant for drug - plasma protein interaction</td>
</tr>
<tr>
<td>K_f</td>
<td>Apparent first-order rate constant describing the formation of metabolite from parent drug</td>
</tr>
<tr>
<td>kg</td>
<td>Kilogram</td>
</tr>
<tr>
<td>K_m</td>
<td>A Michaelis-Menten parameter for enzymatic reactions; substrate concentration at which the reaction velocity is at half-maximal.</td>
</tr>
</tbody>
</table>
\( k_0 \) Drug infusion rate to the mother
\( k_0' \) Drug infusion rate to the fetus
kPa Kilopascals
LC Liquid chromatograph
LOQ Limit of quantitation of the assay
M Molar (moles/litre)
m Meter
\( m/z \) Mass to charge ratio
MA Maternal arterial plasma
mg Milligram
MHz Megahertz
min Minute
mm Millimeter
mM Millimolar
MRM Multiple reaction monitoring
MRT Mean residence time of the total drug
\( \text{MRT}' \) Mean residence time of the unbound drug
MS Mass spectrometry
MSD Mass selective detector
MS/MS Tandem mass spectrometry
msec Millisecond
MTBSTFA N-Methyl-N-(tert-butyldimethylsilyl)trifluoroacetamide
M-UF Unbound fraction of drug in maternal plasma at steady-state
MV Maternal femoral vein
n Number of subjects or animals
NADPH Reduced \( \beta \)-nicotinamide-adenine dinucleotide tetrasodium salt
NaOH Sodium hydroxide
NCI Negative chemical ionization
ng Nanogram
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>NPD</td>
<td>Nitrogen-phosphorous detector</td>
</tr>
<tr>
<td>o.d.</td>
<td>Outer diameter</td>
</tr>
<tr>
<td>pcg</td>
<td>Picogram</td>
</tr>
<tr>
<td>Po₂</td>
<td>Partial pressure of oxygen in the blood</td>
</tr>
<tr>
<td>PcO₂</td>
<td>Partial pressure of carbon dioxide in blood</td>
</tr>
<tr>
<td>pH</td>
<td>Negative logarithm of hydrogen ion concentration</td>
</tr>
<tr>
<td>P.S.I.</td>
<td>Pounds per square inch</td>
</tr>
<tr>
<td>PTFE</td>
<td>Polytetrafluoroethylene</td>
</tr>
<tr>
<td>p.v.</td>
<td>Portal venous</td>
</tr>
<tr>
<td>Qₜₕ</td>
<td>Liver blood flow</td>
</tr>
<tr>
<td>Qum</td>
<td>Umbilical blood flow</td>
</tr>
<tr>
<td>r</td>
<td>Pearson correlation coefficient</td>
</tr>
<tr>
<td>r²</td>
<td>Coefficient of determination</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cells</td>
</tr>
<tr>
<td>S.D.</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SIM</td>
<td>Selected ion monitoring</td>
</tr>
<tr>
<td>SIR</td>
<td>Single ion recording</td>
</tr>
<tr>
<td>t</td>
<td>Time</td>
</tr>
<tr>
<td>t₁/₂</td>
<td>Half-life in a 1-compartment model based on total drug concentrations</td>
</tr>
<tr>
<td>t₁/₂ᵤ</td>
<td>Half-life in a 1-compartment model based on unbound drug concentrations</td>
</tr>
<tr>
<td>t₁/₂β</td>
<td>Terminal elimination half-life in a 2-compartment model based on total drug concentrations</td>
</tr>
<tr>
<td>t₁/₂ᵤβ</td>
<td>Terminal elimination half-life in a 2-compartment model based on unbound drug concentrations</td>
</tr>
<tr>
<td>tₙₐₙₜ</td>
<td>Time of the last sample</td>
</tr>
<tr>
<td>tₘₜₓ</td>
<td>Time of occurrence of maximal plasma concentration</td>
</tr>
<tr>
<td>TEA</td>
<td>Triethylamine</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>TR</td>
<td>Tracheal fluid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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</tr>
<tr>
<td>TRIS</td>
<td>Tris(hydroxymethyl) aminomethane</td>
</tr>
<tr>
<td>U</td>
<td>International units (insulin dosages)</td>
</tr>
<tr>
<td>USP</td>
<td>United States Pharmacopeia</td>
</tr>
<tr>
<td>UV</td>
<td>Umbilical vein</td>
</tr>
<tr>
<td>V</td>
<td>Volts</td>
</tr>
<tr>
<td>$V_c$</td>
<td>Apparent volume of distribution of the central compartment</td>
</tr>
<tr>
<td>$V_d$</td>
<td>Volume of distribution</td>
</tr>
<tr>
<td>$V_{dss}$</td>
<td>Apparent steady-state volume of distribution of the total drug</td>
</tr>
<tr>
<td>$V_{dss}'$</td>
<td>Apparent steady-state volume of distribution of the total drug corrected using area weighed free fraction</td>
</tr>
<tr>
<td>$V_{dss}^u$</td>
<td>Apparent steady-state volume of distribution of the unbound drug</td>
</tr>
<tr>
<td>VPA</td>
<td>Valproic Acid</td>
</tr>
<tr>
<td>x g</td>
<td>Times gravity (centrifugal force)</td>
</tr>
<tr>
<td>UDPGA</td>
<td>Uridine-5'-diphosphoglucuronic acid</td>
</tr>
<tr>
<td>$V_{max}$</td>
<td>Maximal velocity of an enzymatic reaction; a Michaelis-Menten parameter</td>
</tr>
</tbody>
</table>
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Chapter 1
Introduction

1.1 Drug Use in Pregnancy

Although drug use in pregnancy is generally not recommended, many surveys have shown that it does occur in a majority of pregnancies as a result of legitimate medical problems. Rurak et al., (1991) reviewed the available epidemiological data on drug use during pregnancy prior to 1990 and concluded that the incidence of drug use in pregnant women reported in various surveys ranged from 35-100%, with an average of ~2-4 drugs taken by each woman during gestation (Rurak et al., 1991). More recent surveys of drug use in pregnancy as well as reviews by other investigators also present similar statistics (Bonati et al., 1990; Collaborative Group on Drug Use in Pregnancy, 1992; de Jong-van den Berg et al., 1993; Irl et al., 1997). These epidemiological data come from all parts of the world indicating that a huge proportion of human fetuses are exposed to drug(s) at some point during gestation. The most commonly used agents include vitamin and mineral supplements, antibiotics, vaginal and urinary tract antiinfectives/antiseptics, analgesics, cough and cold remedies, laxatives, drugs for the control of nausea and vomiting, and antiallergic medications. In addition, drugs are also used to treat certain pregnancy-associated complications (e.g., preeclampsia, Kyle and Redman, 1992; Hauth et al., 1993; preterm labour, Viamontes, 1996) and other existing medical conditions (e.g., epilepsy, Yerby et al., 1992; heart disease, Mitani et al., 1987). Recently, there has also been increased interest in maternal drug administration for the treatment of fetal disorders via transplacental therapy (Ward, 1992). Finally, there is illicit drug use during pregnancy that exposes the fetus to agents, which may have profound effects on fetal neurological, cardiovascular and metabolic functions (Rurak, 1992; Chiriboga, 1993). Hence, in spite
of societal efforts to limit drug consumption during pregnancy, the human fetus will likely continue to be exposed to a wide range of non-prescription, prescription and illicit drugs. Continued study of fetal toxicology and adverse effects of drugs is thus warranted, and since these are in large part determined by the extent of fetal drug exposure, investigation of the factors affecting fetal drug disposition is also important.

1.2 Factors Affecting Placental Drug Transfer

It has long been recognized that most drugs can cross the placenta from the mother to the fetus and vice versa. Hence, there has been extensive study of the factors affecting placental transfer of the drugs and the resulting fetal drug exposure. Although significant anatomical differences exist among the placentas of different species, the limiting barrier to the movement of drugs and other compounds is essentially a single or multi-layered lipid membrane in all species. Thus, substances can move across the placenta by the usual mechanisms of biological membrane transport, i.e., passive diffusion, active transport, carrier-mediated transfer or facilitated diffusion, pinocytosis, and via passage through the paracellular aqueous pores (Reynolds and Knott, 1989). However, it appears that the major mechanism of placental transfer for most drugs is passive diffusion and other processes are relatively unimportant (Rurak et al., 1991). Thus, it has been suggested that the rate and extent of placental drug transfer is dependent on the physicochemical properties of the drug and is governed by the principles of simple lipid diffusion (Reynolds and Knott, 1989). In agreement with this, low molecular weight lipid soluble compounds (molecular weight < 600) exhibit almost unimpeded placental transfer, whereas there is a significant barrier to the transport of hydrophilic molecules (Reynolds and Knott, 1989; Rurak et al., 1991). Factors
influencing placental transfer of drugs have been extensively reviewed (Mirkin, 1973; Levy and Hayton, 1973; Green et al., 1979; Waddell and Mariowe, 1981; Szeto, 1982; Mihaly and Morgan, 1984; Reynolds and Knott, 1989; Rurak et al., 1991; Nau, 1992). These include: 1) physicochemical properties of the drug such as its molecular weight, lipophilicity, and degree of ionization at physiological pH, 2) plasma protein binding of the drug in maternal and fetal circulation, 3) maternal and fetal placental blood flows, 4) stage of placental development, 5) concentration gradient of the free drug across the placenta, and 6) maternal-fetal blood pH gradient (Rurak et al., 1991).

1.3 Study of Placental Drug Transfer and Determinants of Fetal Drug Exposure

Due to the obvious implications of the extent of fetal drug exposure in fetal adverse drug effects, the quantitative study of fetal drug exposure has been the subject of numerous scientific investigations in animals as well as humans. Because of practical and ethical restraints, detailed placental transfer and fetal exposure studies cannot be conducted in humans during pregnancy. Hence, most human data on placental drug transfer and fetal exposure are based on the measurement of relative drug concentrations in maternal and umbilical cord blood at birth. Intuitively, these data, although clinically relevant in terms of providing evidence of fetal drug exposure in utero in humans, are highly dependent upon the time of sampling after the last dose of the drug. In addition, at the time of birth, the placenta undergoes considerable trauma and there are alterations in uterine and umbilical blood flow patterns (Hamshaw-Thomas et al., 1984). These factors may influence the maternal-fetal drug concentration relationships at birth and hence the data obtained may not be quantitatively representative of the situation during pregnancy. With the advent of ultrasound assisted fetal blood sampling
techniques, maternal-fetal drug concentration ratios have also been determined during pregnancy in humans (Brown et al., 1990; Moise Jr. et al., 1990; Pons et al., 1991). However, these techniques can also provide maternal and fetal drug concentration data only at a single time point, and unless the drug is at steady-state, the quantitative interpretation of these concentrations with respect to overall fetal drug exposure is difficult. Because of the difficulties in obtaining reliable and detailed in utero placental drug transfer data in humans, the in vitro perfused term human placental preparation has been extensively utilized to obtain such data (Krishna et al., 1993; Johnson et al., 1995; Bassily et al., 1995; Bourget et al., 1995; Bloom et al., 1996). The perfused human term placenta has a number of advantages. These include: 1) ease of availability, 2) this is likely the only way to obtain detailed placental transfer data in humans (Rurak et al., 1991), 3) it can be used to examine the placental transfer of toxic substances such as environmental toxins, dioxins etc. (Bourget et al., 1995), 4) the effects of variables such as changes in plasma protein binding and placental perfusion rate on placental transfer can be studied (Krishna et al., 1993; Bassily et al., 1995). However, this model has a number of serious limitations as well. These include: 1) difficulty in extrapolating the results to the in vivo situation, 2) it provides no information on the extent of fetal exposure resulting from placental transfer of the drug, 3) it lacks maternal and fetal physiological and metabolic components which could themselves have significant influence on the extent of fetal drug exposure (see below), 4) the viability and integrity of the placental membranes may be questionable because of trauma during delivery and deterioration of the tissue after a few hours.

Due to the fact that limited information can be obtained from human studies, a number of pregnant animal models have been utilized to examine placental transfer and fetal
drug exposure. Small animals such as rats, guinea-pigs and rabbits possess hemochorial placentas, similar to the human. Due to the small size of their fetuses, studies in these species are conducted by measuring drug concentrations in whole conceptuses after maternal drug administration (DeVane and Simpson, 1985; Laishley et al., 1989; Huang et al., 1996). A fetal drug concentration vs. time profile can be constructed by removing different fetuses from the same animal at different time points or by conducting studies in different animals and sampling at different times (Laishley et al., 1989; Huang et al., 1996). The potential limitations of studies in these species include the inability to perform serial sampling of maternal and fetal blood and hence clearly define maternal and fetal pharmacokinetic relationships. In addition, the majority of these studies are conducted in acute anesthetized animal preparations; this could affect maternal and fetal drug disposition and the degree of placental drug transfer (Rurak et al., 1991). Similar to the human, perfused placental preparations from these species have also been utilized to examine placental transfer of drugs and these studies share the advantages and limitations of the human placental perfusion model.

Perhaps the most detailed studies on placental drug transfer and fetal drug exposure have been conducted in two large chronically-catheterized animal models, pregnant sheep and the pregnant mecaque (Macaca nemestrina and M. mulatta). Such animal models allow serial sampling of maternal and fetal blood and also of other fluid compartments (e.g., amniotic and allantoic fluids, fetal tracheal fluid) so that detailed maternal-fetal drug disposition studies can be conducted. Fetal drug exposure indices after single dose administration (fetal-to-maternal plasma AUC ratio) as well as those at steady-state (fetal-to-maternal steady-state plasma concentration ratio) can be determined. In terms of the validity of these data to the human situation, the mecaque
model has a hemochorial placenta similar to the human, and also being a primate, it is likely to be more similar to the human in terms of its physiologic and drug metabolism characteristics. On the other hand, the epitheliochorial placenta of sheep has a significantly lower permeability to hydrophilic endogenous compounds as well as polar drugs and their metabolites (Faber and Thornburg, 1983; Olsen et al., 1988; Rurak et al., 1991). Thus, placental transfer of such compounds in sheep may not correlate with that in the human (Reynolds and Knott, 1989). However, it can be safely said that placental transfer of most compounds in the human will be at least equal to or greater than that in sheep, and in the case of lipophilic compounds, the sheep and the human may be fairly similar. Examples of drugs studied in sheep include acetylsalicylic acid (Anderson et al., 1980b), indomethacin (Anderson et al., 1980a), meperidine (Szeto et al., 1978), morphine (Szeto et al., 1982b; Olsen et al., 1988), methadone (Szeto et al., 1982b), acetaminophen (Wang et al., 1986a), omeprazole (Ching et al., 1986), propranolol (Czuba et al., 1988; Morgan et al., 1988), cimetidine (Mihaly et al., 1983), ranitidine (Mihaly et al., 1982a), metoclopramide (Riggs et al., 1988), diphenhydramine (Yoo et al., 1986a), quinine and quinidine (Czuba et al., 1991), nifedipine (Nugent et al., 1991), labetalol (Yeleswaram et al., 1992), ritodrine (Wright, 1992) and valproic acid (Gordon et al., 1995). The pregnant mecaque models have been essentially utilized by two groups of investigators for the study of anti-HIV nucleosides or cocaine (Binienda et al., 1993; Pereira et al., 1994; Pereira et al., 1995; Sandberg et al., 1995; Odinecs et al., 1996a; Odinecs et al., 1996b; Odinecs et al., 1996c; Tuntland et al., 1996; Patterson et al., 1997; Tuntland et al., 1998). Thus, it appears that until now, the chronically-catheterized pregnant sheep has been by far the most commonly used model in the study of placental transfer and fetal exposure of drugs. This is likely related to its generally lower cost and ease of handling as compared to non-human primate models.
Sheep have also been the most widely used species for the study of fetal physiology during late gestation. This is primarily because of the fact that the physiologic and behavioral parameters in the fetal lamb during the last 3rd of gestation are similar to those of the human fetus under normal conditions and also in response to cardiovascular and CNS drugs (Rurak, 1992; Thornburg and Morton, 1994; Nijhuis and van de Pas, 1992; Szeto, 1992).

Many of the above referenced studies in pregnant sheep and the mecaque model have quantified the extent of fetal drug exposure relative to the mother by measuring the fetal-to-maternal plasma AUC or steady-state concentration ratios after maternal drug administration. However, in some of these studies, separate maternal and fetal administration of the drug has been carried out and maternal and fetal placental and non-placental clearances of the drug have been determined using a 2-compartment pharmacokinetic model of the maternal-fetal unit (Figure 1.1) (Szeto et al., 1982a). Drugs studied in pregnant sheep in this manner include morphine (Szeto et al., 1982b), methadone (Szeto et al., 1982b), acetaminophen (Wang et al., 1986a), metoclopramide (Riggs et al., 1990), diphenhydramine (Yoo et al., 1993) and labetalol (Yeleswaram et al., 1993). Similarly, in the mecaque model, maternal and fetal placental and non-placental elimination of the drug has been studied for dideoxyinosine (Pereira et al., 1994), zalcitabine (2',3'-dideoxycytidine, Tuntland et al., 1996), stavudine (2',3'-didehydro-3'deoxythymidine, Odinecs et al., 1996a) and zidovudine (Tuntland et al., 1998). This approach, in addition to providing information on the bi-directional placental transfer rates of the drug, also provides evidence of possible fetal drug elimination in the form of a fetal non-placental clearance parameter. The relative magnitudes of fetal and maternal non-placental clearance can be used as a measure of the extent of
development of fetal drug elimination capacity via routes other than the placenta (e.g., metabolism, renal excretion etc.) as compared to the mother.

Figure 1.1 - A representation of various placental and non-placental drug clearances in the 2-compartment pharmacokinetic model of the maternal-fetal unit (CL\textsubscript{mo} - maternal non-placental clearance; CL\textsubscript{fo} - fetal non-placental clearance; CL\textsubscript{mf} - placental clearance from the mother to the fetus; CL\textsubscript{fm} - placental clearance from the fetus to the mother).

Although the kinetics of placental transfer of most drugs appear to follow the principles of simple diffusion across biological membranes, the extent and kinetics of fetal drug exposure are not related solely to the ease of placental drug transfer. Instead these are the result of a complex interplay between the kinetics of placental drug transfer as well as many other factors related to maternal and fetal components of the pregnant system. These include the relative extent of maternal and fetal plasma protein binding of the drug, the efficiency of maternal and fetal drug elimination via metabolism or renal excretion, and recirculation of the drug between amniotic and allantoic fluid compartments and the fetal circulation. The measurement of fetal plasma AUC or
steady-state concentration after maternal drug administration, although a clinically useful index of the extent of fetal drug exposure, does not provide any information about the role of different factors in determining its magnitude. The computation of maternal and fetal placental and non-placental clearances partitions the complex array of these pharmacokinetic factors into 3 main categories, i.e., factors related to the placenta (maternal and fetal placental clearance), the mother (maternal non-placental clearance) and the fetus (fetal non-placental clearance). Thus, it is possible to separately examine the effect of various physicochemical (e.g., drug lipophilicity and pKa etc.), and maternal and fetal biological variables (e.g., plasma protein binding, placental blood flows, drug metabolism capacity) on these 3 classes of pharmacokinetic factors and the resultant effects on fetal drug exposure. This makes it feasible to determine the relative importance of each pharmacokinetic variable in determining fetal exposure to a particular drug, and to make comparisons among different drugs in terms of the most important factor(s).

In recent years there has been an increased interest in in utero fetal drug therapy via maternal, intraamniotic, fetal intraperitoneal or intravascular drug administration for the treatment of a number of medical conditions such as arrhythmias, congestive heart failure, pulmonary immaturity, bacterial infections and maternal-fetal HIV transmission (Hamamoto et al., 1990; Ward, 1995; Gilbert et al., 1995; Rayburn, 1997). The detailed studies of drug disposition in the maternal-fetal unit and surrounding fluid compartments (amniotic and allantoic fluids), that are possible with large chronically-catheterized animals, may provide important data for pharmacokinetic rationalization of fetal drug therapy in humans. The study of fetal drug elimination capacity yields important information regarding the ontogenetic development of drug metabolism pathways and
renal drug excretion capacity. Such information also has obvious importance in
devising better therapeutic strategies for pregnant women and also in the immediate
newborn and infant period.

1.4 Diphenhydramine

Diphenhydramine [2-(diphenylmethoxy)-N,N-dimethylethylamine, DPHM] is a classical
first-generation H₁-receptor antagonist of the ethanolamine class (Garrison, 1991). The
drug is a low molecular weight (255.4 Da) lipophilic (octanol/water partition coefficient
1862) weakly basic amine with a pKₐ of 9.0 (Figure 1.2) (de Roose et al., 1970) and is
marketed as its hydrochloride salt.

![Chemical Structure of DPHM]

Figure 1.2- Chemical Structure of DPHM

1.4.1 Pharmacology and Therapeutic Use
The pharmacology of DPHM has been extensively reviewed (Melville, 1973; Hahn, 1978; Drouin, 1985; Garrison, 1991). It is a competitive $H_1$-histamine receptor antagonist with little or no effect on $H_2$-receptors (Cooper et al., 1990). Thus, DPHM can inhibit the action of histamine mediated allergic and anaphylactic responses such as smooth muscle contraction, wheal formation, edema and increased capillary permeability (Hahn, 1978). It is used therapeutically for the relief of allergic symptoms such as hay fever, allergic rhinitis, cough, urticaria, dermatoses and pruritis (Garrison, 1991). It is also effective in the management of motion sickness, post-operative nausea and vomiting, emesis due to antineoplastic drug use and as a hypnotic (Garrison, 1991). The most common side effects with normal DPHM therapy include sedation or drowsiness and anticholinergic effects, and at higher doses it may result in convulsions and death (Garrison, 1991; Koppel et al., 1987). The newer second generation antihistamines (e.g., astemizole, terfenadine, loratidine) lack the undesirable drowsiness and impaired performance caused by first-generation $H_1$-antagonists primarily because of their much reduced blood-brain barrier permeability (Estelle et al., 1991).

1.4.2 Basic Pharmacokinetics

In humans, DPHM is rapidly absorbed after oral administration with peak plasma concentrations occurring 2-4 h after administration (Carruthers et al., 1978; Blyden et al., 1986; Luna et al., 1989; Simons et al., 1990). In healthy adult humans, the peak plasma concentration after a 50 mg therapeutic dose is 40-100 ng/ml (Albert et al., 1975; Carruthers et al., 1978; Blyden et al., 1986; Luna et al., 1989). The drug undergoes substantial first-pass metabolism after oral administration and systemic
bioavailability ranges from 40-70% (Albert et al., 1975; Carruthers et al., 1978; Berlinger et al., 1982; Blyden et al., 1986).

In human plasma, DPHM is ~70-85% protein bound, with unbound fractions being significantly higher in orientals compared to caucasians (Spector et al., 1980; Meredith et al., 1984; Zhou et al., 1990). The degree of DPHM binding to human serum albumin is low (Drach et al., 1970), and therefore as with other basic amine drugs, DPHM may bind to $\alpha_1$-acid glycoprotein in plasma. The apparent volume of distribution of DPHM is large (~3-7 L/kg) and suggests extensive distribution within the body (Carruthers et al., 1978; Spector et al., 1980; Berlinger et al., 1982; Blyden et al., 1986). The classical tissue distribution studies of Glazko and Dill (1949) in rats and guinea pigs showed that the highest concentrations of drug, in decreasing order, were present in lung, spleen, brain, liver and kidney after oral, subcutaneous, intraperitoneal or intravenous administration.

The human systemic total body clearance of DPHM is in the range of 6-15 ml/min/kg (Carruthers et al., 1978; Spector et al., 1980; Meredith et al., 1984; Blyden et al., 1986). DPHM clearance appears to show ethnic variation, likely due to inter-racial differences in plasma protein binding (Spector et al., 1980; Zhou et al., 1990). The adult human oral clearance of the drug is considerably higher (~20-30 ml/min/kg) due to a substantial first-pass effect (Luna et al., 1989; Simons et al., 1990). DPHM clearance also exhibits age-dependency, being ~2 fold lower in the elderly (~70 years old) and ~2 fold higher in children (~10 year old) as compared to adult humans (~30 years old) (Simons et al., 1990). The terminal elimination half-life of DPHM in humans ranges from 3-9 h (Albert et al., 1975; Carruthers et al., 1978; Spector et al., 1980; Berlinger et al., 1982; Blyden...
et al., 1986; Luna et al., 1989). The excretion pattern of the drug shows considerable species variation. In rats, after the administration of a 10 mg/kg subcutaneous dose, ~4-6% is excreted unchanged in the urine (Glazko and Dill, 1949), whereas in rabbits this figure is ~21% (Hald, 1947). In man, urinary DPHM excretion accounts for only 2-4% of the administered dose (Hald, 1947; Albert et al., 1975). The sum of urinary excretion of unchanged DPHM and its metabolites identified thus far can account for ~50-60% of the oral dose in humans (Glazko et al., 1974). In contrast, only ~33% of the total administered radioactivity was excreted in urine following subcutaneous administration of $^{14}$C-DPHM to rats, with the remainder being recovered in feces, suggesting the possibility of biliary excretion (Glazko et al., 1949).

1.4.3 Metabolism

The liver of the rat, guinea pig, and rabbit is highly active in metabolizing DPHM and appears to be the primary site for DPHM biotransformation, although the lung and kidney also have some metabolic activity (Glazko et al., 1949). A reduced clearance of DPHM in patients with cirrhotic liver disease suggests the role of hepatic metabolism in human DPHM clearance as well (Meredith et al., 1984). Liver microsomes from rats, mice, guinea-pigs and rabbits appear to rapidly N-demethylate DPHM (Roozemond et al., 1965; Kataoka and Takabatake, 1971). Incubation of DPHM with liver microsomes from rat, guinea-pig and rabbit results in the formation of methylamine but not dimethylamine (Yamada et al., 1993). This indicates that biotransformation of DPHM proceeds via an N-demethylation and subsequent deamination, rather than direct oxidative deamination of the dimethylamino group (Yamada et al., 1993). It is, however, not clear whether the direct oxidative deamination of N-demethyl DPHM or its further N-
demethylation to N,N-didemethyl DPHM and subsequent deamination is quantitatively the more important pathway.

In rhesus monkeys, the major urinary DPHM metabolites include unchanged DPHM (2-10%), N-demethyl (5-11%) and N,N-didemethyl (3-13%) analogs of DPHM, DPHM N-oxide (7-15%), an acid metabolite (diphenylmethoxyacetic acid, DPMA) (4-20%), a trace of benzhydrol (1-2%), a glutamine conjugate of DPMA (35-59%), and an uncharacterized glucuronide (0-14%) (Drach and Howell, 1968; Drach et al., 1970). Thus, in this species, N-demethylation followed by oxidative deamination to DPMA and subsequent conjugation of DPMA with glutamine appears to be the major metabolic pathway. Dogs appear to have a similar sequence of metabolic reactions, except that DPMA is conjugated to glycine and this pathway accounts for ~40% of the dose (Drach et al., 1970). Drach et al., (1970) also demonstrated the presence of unchanged DPHM, N-demethyl DPHM, N,N-didemethyl DPHM, and DPHM-N-oxide in rat urine. However, in contrast to the rhesus monkey and dog, neither the free nor the conjugated form of DPMA was detectable in rat urine (Drach et al., 1970); thus, the exact nature of the majority of DPHM metabolites in this species still remains to be determined. The metabolic pathways of DPHM in man have also not been fully established; however, the urinary metabolites identified thus far include small amounts of unchanged DPHM and N,N-didemethyl DPHM, and relatively large amounts of N-demethyl DPHM and DPMA, the latter being the major metabolite (Chang et al., 1974). DPMA is excreted both in its free and conjugated form in humans but the exact nature of the conjugate(s) is not known (Chang et al., 1974). Recently, a quaternary ammonium glucuronide conjugate of DPHM has been identified in human urine and it may account for ~2-15% of the total administered dose (Luo et al., 1991; Luo et al., 1992; Fischer and Breyer-Pfaff, 1997).
Human liver microsomes have also been shown to form this quaternary ammonium glucuronide in significant amounts (Breyer-Pfaff et al., 1997).

DPHM has been found to interact by forming a complex with the enzyme systems, cytochrome P-450 (Bast et al., 1990) and monoamine oxidase (Yoshida et al., 1989; 1990). Both these enzyme systems are known to metabolize a number of xenobiotics; however, it is not yet clear which enzyme system or isoform thereof, is responsible for the metabolism of DPHM. In the rat, it has been demonstrated that DPHM and its structural analogues (e.g., orphenadrine) form a metabolic-intermediate complex with cytochrome P-450 2B1/2B2 and 2C6, and thus may act as inhibitors of these isozymes (Rekka et al., 1989; Reidy et al., 1989; Bast et al., 1990). In this regard, DPHM has been shown to significantly inhibit the clearance of diltiazam in the isolated perfused rat liver (Hussain et al., 1994).

1.4.4 DPHM in Pregnancy

A number of surveys of drug use during pregnancy indicate that on average 10-20% of women take antihistamine containing preparations at some point during pregnancy (Peckham and King, 1963; Forfar and Nelson, 1973; Doering and Stewart, 1978; Piper et al., 1987). DPHM is one of the commonly used antihistamines and is taken by 3-7% of pregnant women (Piper et al., 1987; Briggs et al., 1990; Smith et al., 1994; Bologa et al., 1994). The high incidence of DPHM use during pregnancy is evident from the fact that it appears on the lists of 'Drugs and Chemicals Most Commonly Used by Pregnant Women' and 'Drugs of Choice for Pregnant Women' compiled during 1994 at the Hospital for Sick Children in Toronto, Canada (Smith et al., 1994; Bologa et al., 1994).
The predominant therapeutic applications of DPHM during pregnancy are similar to its uses in the normal population. These include the symptomatic treatment of allergic conditions such as rhinitis and contact dermatitis, motion sickness, pruritis, as a hypnotic, and as a cough and cold remedy (Forfar and Nelson, 1973; Ahmed and Kaplan, 1981; Smith et al., 1994). In addition, it is also used in the treatment of certain pregnancy-specific conditions such as nausea and vomiting during the first trimester and a pregnancy-related urticarial condition in late gestation (Nagoette et al., 1996; Ahmad and Kaplan, 1981; The Drugs and Pregnancy Study Group, 1994). Common coughs, colds, nausea and vomiting are among the most common complaints during pregnancy (de Jong-van den Berg et al., 1993; Irl et al., 1997). In one survey, DPHM was administered to pregnant women for an average duration of ~19 days over the entire gestation (Forfar and Nelson, 1973). DPHM, despite initial reports of possible association with an increased incidence of oral clefts, is now considered to have no significant teratogenic potential (Saxen, 1974; Briggs et al., 1990; Bologna et al., 1994). There appear to be no systematic studies on the placental transfer and fetal effects of DPHM in the human during the later part of gestation.

1.4.5 DPHM Disposition in Chronically-Instrumented Pregnant Sheep: Earlier Studies

During the course of the last few years, a series of studies have been conducted in this lab to examine the placental transfer, comparative maternal-fetal pharmacokinetics and metabolism, and fetal effects of DPHM in chronically-instrumented pregnant sheep (Yoo, 1989; Tonn, 1995). Rapid placental transfer of DPHM across the sheep placenta was demonstrated after i.v. bolus administration to the mother, with peak fetal plasma concentrations occurring within 5 min after injection. The fetal-to-maternal AUC ratio of
the drug averaged 0.85 indicating significant fetal DPHM exposure after maternal drug administration (Yoo et al., 1986a). Also, the drug did not appear to accumulate or persist to any significant extent in the fetal circulation and the observed maternal and fetal elimination half-lives were similar (40-50 min). Maternal and fetal placental and non-placental clearances of DPHM were determined after separate maternal and fetal steady-state drug administration using a 2-compartment pharmacokinetic model of the maternal-fetal unit (Figure 1.1) (Yoo et al., 1993; Szeto et al., 1982a). The estimates of maternal and fetal placental and non-placental clearances of DPHM along with those of other drugs studied in pregnant sheep are given in Table 1.1. The most significant features of these data are the generally higher magnitudes of fetal weight-normalized placental and non-placental clearances compared to the mother. Also, the fetal non-placental drug elimination capacities for most drugs are remarkable in comparison to the mother. The magnitude of CL$_{fm}$ compared to CL$_{mf}$ is also higher for all other drugs where estimates are available, except acetaminophen. One property of the 2-compartment model is that if maternal and fetal plasma protein binding of the drug is similar and the mode of its placental transfer is via passive diffusion, the magnitude of its placental clearance in the two directions should be equal. This point is discussed at further length in chapter 3. Some of the drugs listed in Table 1.1 do not bind significantly to maternal and fetal plasma proteins (e.g., morphine) and yet exhibit a higher CL$_{fm}$ compared to CL$_{mf}$. For others, this CL$_{fm}$-CL$_{mf}$ difference still remains after the differences in maternal and fetal plasma protein binding of the drug are taken into account (e.g., DPHM, methadone) (Yoo et al., 1993; Szeto et al., 1982b). These findings are surprising considering the fact that all these drugs appear to undergo placental transfer via passive diffusion.
Table 1.1 – Average values of fetal and maternal placental (CL_{fm} and CL_{mf}, respectively) and non-placental clearances (CL_{f0} and CL_{mo}, respectively) of various drugs studied in pregnant sheep during the last part of gestation.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Clearance (ml/min/kg)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CL_{fm}^a</td>
<td>CL_{mf}^a</td>
<td>CL_{f0}^a</td>
<td>CL_{mo}^b</td>
<td></td>
</tr>
<tr>
<td>DPHM^c</td>
<td>116.8</td>
<td>37.2</td>
<td>88.8</td>
<td>43.5</td>
<td></td>
</tr>
<tr>
<td>Morphine^d</td>
<td>19.4</td>
<td>8.3</td>
<td>42.0</td>
<td>39.7</td>
<td></td>
</tr>
<tr>
<td>Methadone^e</td>
<td>101.1</td>
<td>32.2</td>
<td>70.9</td>
<td>26.3</td>
<td></td>
</tr>
<tr>
<td>Labetalol^f</td>
<td>23.4</td>
<td>7.3</td>
<td>27.1</td>
<td>30.5</td>
<td></td>
</tr>
<tr>
<td>Acetaminophen^g</td>
<td>30.5</td>
<td>31.1</td>
<td>10.8</td>
<td>14.6</td>
<td></td>
</tr>
<tr>
<td>Metoclopramide^h</td>
<td>103.9</td>
<td>72.0</td>
<td>27.8</td>
<td>46.1</td>
<td></td>
</tr>
<tr>
<td>Ritodrine^i</td>
<td>9.2</td>
<td>-</td>
<td>52.8</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Indomethacin^j</td>
<td>2.0</td>
<td>-</td>
<td>4.0</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

^a clearance are ml/min/kg estimated fetal body weight; ^b clearance is ml/min/kg maternal body weight; ^c from Yoo et al., (1993); ^d and ^e clearance data from Szeto et al. (1982b) and are weight-normalized to maternal and fetal body weights of 70 kg and 3 kg, respectively; ^f from Yeleswaram et al. (1993) and clearances were determined using separate maternal and fetal bolus drug administration; ^g from Wang et al. (1986a); ^h from Riggs et al. (1990); ^i from Wright et al. (1991), determined using the Fick principle and only fetal steady-state drug infusion; ^j Krishna et al. (1995), determined using the Fick principle and only fetal steady-state drug infusion.

Similar to other amine drugs, DPHM accumulates in fetal tracheal and amniotic fluids, with drug concentrations in fetal tracheal fluid being 4-5 times higher compared to fetal plasma (Riggs et al., 1987; Yoo, 1989; Rurak et al., 1991). The concentration of DPHM in amniotic fluid increases progressively during infusion and subsequently declines more slowly than fetal and maternal plasma (Yoo, 1989). Drug appearance in the amniotic fluid may result from fetal renal and tracheal fluid excretion of the drug into this compartment. In addition, there could be direct drug transfer from the fetal blood into the amniotic fluid via the fetal chorioallantoic membranes (Rurak et al., 1991). After injection of DPHM into the amniotic fluid, the drug is preferentially taken up by the fetus.
via fetal swallowing and absorption, and exchange between amniotic fluid and fetal circulation via the fetal membranes (Yoo, 1989). Thus, the drug present in amniotic fluid may be recirculated in the fetal lamb via these mechanisms (Yoo, 1989).

The above results indicate that the fetus is capable of eliminating a substantial amount of DPHM via non-placental routes. Subsequent studies have attempted to examine the components of this high CL_f0. Since DPHM was found to accumulate in the tracheal fluid to a high extent, Yoo (1989) examined the fetal pulmonary clearance of the drug and found this to be a minor component of CL_f0 (~8% of CL_f0). An extension of the assessment of the contribution of various fetal organs to DPHM elimination are the recent studies employing simultaneous administration of stable-isotope labeled ([²H_{10}]DPHM, with deuterium labels on the two aromatic rings) and unlabeled DPHM (Tonn, 1995). Tonn et al., (1996) examined the in vivo first-pass uptake/metabolism of DPHM by the fetal and adult sheep liver. After randomized simultaneous but separate administration of [²H_{10}]DPHM and DPHM via the portal and i.v. routes, it was demonstrated that hepatic first-pass extraction of DPHM in adult sheep was ~95%. This indicates that hepatic uptake and/or metabolism may be a major component of DPHM clearance in adult sheep. The study of fetal hepatic uptake of DPHM was much more complicated due to the unique geometry of the fetal hepatic circulation and multiple number of blood flow inputs into the fetal liver. The fetal liver receives ~75% of its blood supply from the umbilical vein, ~20% from the portal vein and ~5% from the hepatic artery (Holzman, 1984). Also, ~50% of the umbilical venous blood flow returning from the placenta passes through the fetal liver before reaching the fetal circulation and the rest bypasses the fetal liver via the ductus venosus (Figure 1.3) (Edelstone et al., 1978). Since drugs transfer across the placenta and reach the fetal circulation via the umbilical
vein, the fetal liver may exert a 'partial first-pass effect' on the drug present in the umbilical vein (Figure 1.3). This fetal hepatic first-pass uptake of the drug from the umbilical vein, if present, may be the most significant factor in minimizing fetal drug exposure. Thus, fetal hepatic first-pass extraction of the drug from the umbilical vein was examined by simultaneous bolus or infusion administration of stable-isotope labeled and unlabeled forms of the drug at umbilical and inferior vena caval sites (Figure 1.3). In contrast to the adult sheep, no fetal hepatic first-pass extraction of DPHM was observed after umbilical venous administration (Tonn et al., 1996).

The previous studies of Yoo et al., (1993) involved maternal and fetal drug infusions on different days separated by a washout period in order to calculate maternal and fetal placental and non-placental clearances. It has been suggested that any time-related developmental changes occurring in the rapidly growing fetus at this stage of gestation and inter-occasion variability in drug kinetics can bias the results from such experiments (Rurak et al., 1991). In order to eliminate this potential bias in calculated pharmacokinetic parameters, Tonn (1995) employed a simultaneous steady-state infusion of DPHM and $[^2\text{H}_10]$-DPHM to the mother and the fetus, respectively, to re-examine the placental and non-placental clearances. In general, results similar to those of Yoo et al., (1993) were obtained; the higher magnitudes of $\text{CL}_{fm}$ and $\text{CL}_{fo}$ as compared to corresponding maternal clearance parameters were still observed (Tonn, 1995). In addition, it was demonstrated in these studies that fetal renal clearance of DPHM accounts for ~2% of $\text{CL}_{fo}$. 
Figure 1.3 - A diagrammatic sketch of the fetal circulation showing the position of the fetal liver and sites of drug administration for the assessment of fetal first-pass hepatic drug uptake from the umbilical vein.
During the above simultaneous maternal-fetal steady-state infusion studies, maternal and fetal plasma concentrations of diphenylmethoxyacetic acid (DPMA or [\(^{2}\)H\(_{10}\)]-DPMA), a major DPHM metabolite in many species, were also measured. After the administration of [\(^{2}\)H\(_{10}\)]-DPHM to the fetus, much higher concentrations of [\(^{2}\)H\(_{10}\)]-DPMA were detected in the fetal arterial plasma compared to the mother, indicating that fetus can form this metabolite in utero. In contrast to the parent drug, the [\(^{2}\)H\(_{10}\)]-DPMA metabolite was not detectable in fetal tracheal or amniotic fluids. In addition, weight-normalized fetal renal clearance of this metabolite was much lower compared to the mother (Tonn, 1995). A similar phenomenon has been observed in this lab with another carboxylic acid drug, indomethacin (Krishna et al., 1995), and may reflect a limited ability of the fetal lamb kidney to excrete organic acids (Elbourne et al., 1990). The likelihood of maternal and fetal secondary metabolism of DPMA to its amino acid or other conjugates, as in other species, was not investigated in these studies. Other possible metabolites of DPHM such as N-demethyl DPHM and N,N-didemethyl DPHM were not detected in maternal or fetal plasma and urine in any significant amounts.

Thus, over the years a considerable amount of information has been gathered on the disposition and metabolism of DPHM in the ovine maternal-fetal unit. However, the study of the components of high fetal CL\(_{f0}\) has not yielded quantitatively significant information and major routes of CL\(_{f0}\) are still not clear. The results obtained also raise the question as to whether the estimated CL\(_{f0}\)'s for DPHM and other drugs are in fact related to intrinsic fetal drug clearance capacity, or are some feature of the intrauterine environment, or are perhaps just the product of a pharmacokinetic modeling exercise. In addition, the metabolic routes of DPHM in the mother and the fetus have not been fully elucidated. Thus, a meaningful comparison of maternal and fetal drug
elimination/metabolism capacity and its role in determining fetal drug exposure is not possible.

1.5 Valproic Acid

Valproic acid (2-propylpentanoic acid, VPA) is a low molecular weight (144.2 Da) antiepileptic drug with a unique branched-chain fatty acid structure (Figure 1.4) (Davis et al., 1994; Baillie and Sheffels, 1995). It is considerably less lipophilic compared to DPHM (octanol/water partition coefficient 398) and has a pKa of 4.8. It is available for clinical 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 1.4 - Chemical structure of VPA.](image)

1.5.1 Therapeutic Use, Pharmacology and Adverse Effects

VPA possesses a broad spectrum of clinical efficacy against several types of epilepsy including generalized seizures (such as tonic-clonic, myoclonic and absence), some partial seizures (such as simple, complex and secondarily generalized), and
The precise mechanism(s) of action of VPA remains poorly understood. A mechanism involving potentiation of \(\gamma\)-aminobutyric acid (GABA) neurotransmission has been the focus of major scientific investigation. Based on the results from numerous animal studies, it has been suggested that VPA increases brain concentrations of the inhibitory neurotransmitter, GABA, via its effects on the enzymes involved in GABA production or degradation. In this context, VPA has been shown to result in increased synthesis of GABA via activation of glutamic acid decarboxylase (GAD) (Lösch, 1981; Lösch, 1989). Blockade of GABA degradation by inhibition of GABA-transaminase (GABA-T) and succinic semialdehyde dehydrogenase (SSADH), two enzymes involved in successive degradation steps of GABA, has also been demonstrated (Lösch and Vetter, 1985; Lösch, 1993; Zeise \textit{et al.}, 1991). Lastly, an enhancement of potassium-induced GABA release into the synapse has also been suggested as one of the possible mechanisms (Gram \textit{et al.}, 1988). The other proposed mechanisms of action include, inhibition of \(\gamma\)-hydroxybutyric acid (GHB) (a minor but epileptogenic metabolic product of GABA itself) release (Vayer \textit{et al.}, 1988), inhibition of NMDA receptor-mediated excitation by the actions of aspartate (Zeise \textit{et al.}, 1991), and a nonspecific membrane action which reduces high frequency repetitive firing of neurons through effects on sodium and/or potassium channels (Slater and Johnson, 1978; McLean and MacDonald, 1986). There is ample evidence in the literature both supporting and contradicting all of these postulated mechanisms. It is almost certain that more than one molecular mechanism is necessary to explain the broad-spectrum activity of VPA against a variety of epileptic disorders.
The most common adverse effects of VPA therapy can be broadly classified into two categories: dose-related side effects and idiosyncratic reactions (Davis et al., 1994; Dreifuss, 1995). The dose-related adverse effects include gastrointestinal side effects (nausea, vomiting and gastrointestinal distress), excessive weight gain, hair loss, CNS effects (tremor, drowsiness, acute confusional states and irritability), and metabolic effects (hypocarnitinemia, hyperammonememia). Many of these side effects can be minimized/eliminated by lowering the dose or by a more gradual increase in dose or by administering the drug in its enteric-coated form. The most serious side effect of VPA therapy is a rare but fatal idiosyncratic hepatotoxicity characterized by microvesicular steatosis and necrosis of the liver (Dreifuss, 1995). In one survey, the overall incidence of valproate induced hepatic failure was 1 in 49,000, and patients on polytherapy with VPA and other antiepileptic drugs appeared to be more susceptible (Dreifuss et al., 1989). A higher incidence of fatalities due to hepatic failure appears to occur in patients <2 years of age, including those on monotherapy (1 in 7000) and especially on polytherapy (1 in 800) (Dreifuss et al., 1989; Bryant and Dreifuss, 1996). However, the overall incidence of VPA-induced hepatotoxicity and especially that in <2 years old population appears to have decreased over the years due to the recognition of susceptibility and subsequent changes in prescribing patterns (Dreifuss et al., 1987; Dreifuss et al., 1989; Dreifuss, 1995).

1.5.2 Basic Pharmacokinetics

VPA is rapidly and completely absorbed after oral administration in humans with an absolute bioavailability of 90-100% (Klotz and Antonin, 1977; Perucca et al., 1978a).
Peak plasma concentrations occur within 1-3 hours for rapid-release dosage forms and within 3-8 h for enteric-coated formulations (Klotz and Antonin, 1977; Gugler et al., 1977; Davis et al., 1994). The drug has a relatively small volume of distribution (0.1-0.2 L/kg in adults and 0.15-0.4 L/kg in neonates, infants and children), indicating that it is likely confined to the vascular space and extracellular fluids (Gugler and von Unruh, 1980; Gugler et al., 1977; Perucca et al., 1978a & b; Gal et al., 1988; Irvine-Meek et al., 1982; Hall et al., 1985; Herngren et al., 1991; Cloyd et al., 1993). This appears to be primarily related to its high degree of ionization at physiological pH (pKa 4.8) and also to its extensive serum/plasma protein binding (~90% at therapeutic concentrations) (Levy and Shen, 1995; Davis et al., 1994). The major binding protein for VPA in plasma/serum appears to be albumin (Kober et al., 1980). The serum/plasma protein binding of VPA is nonlinear and exhibits a concentration dependency, leading to increased unbound fractions at higher total plasma concentrations (Gugler and Mueller, 1978; Yu, 1984; Cramer et al., 1986; Scheyer et al., 1990; Cloyd et al., 1993; Levy and Shen, 1995). VPA has been shown to easily enter the CNS in spite of its high degree of ionization in the blood. Brain and CSF concentrations in humans, as well as animals, range from 60-100% of the serum unbound concentrations and are to some extent related to the serum protein binding of the drug (Levy and Shen, 1995). Carrier-mediated, probenecid-sensitive transport processes have been suggested to play a significant role in CNS transport of VPA (Levy and Shen, 1995).

The elimination half-life of VPA ranges from 9-18 h in humans (Nau et al., 1982; Zaccara et al., 1988; Davis et al., 1994; Perucca et al., 1978a & b; Gugler et al., 1977; Bowdle et al., 1980; Bialer et al., 1985; Schapel et al., 1980; Hoffman et al., 1981). VPA is a low clearance drug with plasma clearances in the range of 1.0-3.0 ml/min/kg and
0.1-0.3 ml/min/kg based on unbound and total drug concentrations, respectively. VPA clearance is generally higher in children and epileptic patients on polytherapy as compared to adults on monotherapy (Perucca et al., 1978b; Gugler et al., 1977; Bowdle et al., 1980; Bialer et al., 1985; Schapel et al., 1980; Hoffman et al., 1981). The clearance of VPA is dose-dependent, with possible nonlinearities due to alterations in the unbound fraction of the drug and/or saturable metabolism with increasing dose (Bowdle et al., 1980; Gómez Bellver et al., 1993). The major route of VPA elimination in humans is via hepatic metabolism and on average only 1-3% of the total dose is excreted unchanged in urine (Gugler et al., 1977; Gugler and von Unruh, 1980; Dickinson et al., 1989).

1.5.3 Metabolism

In spite of the deceptively simple branched short-chain fatty acid structure of VPA, its metabolic fate is extremely complex. Because of its chemical structure, VPA enters into metabolic pathways normally reserved for fatty-acid oxidation (e.g., mitochondrial β-oxidation). In addition, it is also metabolized via the common pathways of xenobiotic metabolism (e.g., glucuronidation, cytochrome P-450 mediated oxidation). The level of complexity in VPA metabolism is evident from the fact that there are approximately 50 known VPA metabolites, with at least 16 being observed consistently in humans (Kassahun et al., 1990; Baillie and Sheffels, 1995). The complex and ever-changing subject of VPA metabolism has been extensively reviewed (Gugler and von Unruh, 1980; Granneman et al., 1984a; Zaccara et al., 1988; Baillie and Rettenmeier, 1989; Baillie and Levy, 1991; Ponchaut and Veitch, 1993; Baillie and Scheffels, 1995) and only a very brief overview will be presented here. The major routes of VPA metabolism
can be broadly divided into 3 categories: glucuronidation, mitochondrial β-oxidation and microsomal oxidative metabolism including desaturation and ω- and ω-1 oxidations (Figure 1.5) (Baillie and Scheffels, 1995).

Figure 1.5 – Metabolic pathways of VPA. Dotted arrows indicate pathways for which direct experimental evidence is lacking.
1.5.3.1 Glucuronidation

Glucuronidation is the major route of VPA metabolism and results in the formation of 1-O-acyl-β-D-ester linked glucuronide (Dickinson et al., 1979a; Dickinson et al., 1984). In different studies, on average 20-40% (individual subject range 10-70%) of the total dose has been recovered in urine as VPA-glucuronide in humans (Gugler et al., 1977; Dickinson et al., 1989; Levy et al., 1990). Also, it has been suggested that the contribution of the glucuronide pathway to total VPA metabolism may increase as a function of increasing dose due to saturation of the β-oxidation pathway (Granneman et al., 1984a; Pollack et al., 1986; Dickinson et al., 1989).

1.5.3.2 Mitochondrial β-Oxidation

A significant fraction of the VPA dose is also metabolized via the β-oxidation pathway in humans. The VPA metabolites formed via the mitochondrial β-oxidation pathway include 2-n-propyl-2-pentenoic acid (2-ene VPA) (formed predominantly as the E-isomer), 2-n-propyl-3-pentenoic acid (3-ene VPA) (predominantly 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 3-keto VPA metabolite is a prominent urinary metabolic product of VPA in humans and may account for 10-60% of the total administered dose (Dickinson et al., 1989; Levy et al., 1990; Sugimoto et al., 1996). Small amounts (~1-3% of dose) of other β-oxidation metabolites, (E)-2-ene and 3-OH VPA are also detectable in human urine. Structurally, all these metabolites resemble the intermediates of fatty acid β-oxidation. The fact that these metabolites are
excreted in urine in appreciable quantities, while similar intermediates from the β-
oxidation of straight-chain endogenous fatty-acids are not, indicates that the branched-
chain nature of VPA renders it a poor substrate for its complete oxidation via this
pathway (Li et al., 1991). Further experiments conducted via selective deuterium
labeling of VPA have demonstrated that 3-OH VPA is not an exclusive product of the β-
oxidation pathway (Rettenmeier et al., 1987). Instead, it has a dual origin and may also
be formed via cytochrome P-450 mediated microsomal hydroxylation (Prickett and
Baillie, 1984; Rettenmeier et al., 1987). Also, 3-keto VPA originates via direct oxidation
of 2-ene VPA rather than from the oxidation of 3-OH VPA (Rettenmeier et al., 1987).
The (E)-3-ene VPA metabolite arises from the isomerization of (E)-2-ene VPA, and may
be further metabolized to (E,E)-2,3'-diene VPA via β-oxidation (Bjorge and Baillie, 1991).
Also, (E)-2-ene VPA, (E)-3-ene VPA and (E,E)-2,3'-diene VPA are interconvertible via isomerization and reduction processes, and thus all may serve as
precursors of 3-keto VPA (Bjorge and Baillie, 1991). Presumably because of its entry
into the β-oxidation pathways, VPA has been found to be an inhibitor of fatty acid β-
oxidation via competition with β-oxidation enzymes (Bjorge and Baillie, 1985).

1.5.3.3 Microsomal Metabolism

The products of microsomal ω- and ω-1 hydroxylation of VPA are 2-n-propyl-5-
hydroxypentanoic acid (5-OH VPA) and 2-n-propyl-4-hydroxypentanoic acid (4-OH
VPA), respectively (Prickett and Baillie, 1984). Further oxidation of 5-OH VPA leads to
2-propylglutaric acid (2-PGA) while that of 4-OH VPA results in the formation of 2-n-
propyl-4-oxopentanoic acid (4-keto VPA) and 2-propylsuccinic acid (2-PSA) (Granneman et al., 1984b).

In addition to 2-ene and 3-ene VPA above, another monounsaturated metabolite of VPA is 2-n-propyl-4-pentenoic acid (4-ene VPA). It has been shown that in contrast to 2-ene and 3-ene VPA metabolites, 4-ene VPA is formed via a distinct microsomal cytochrome P-450 mediated desaturation reaction (Rettie et al., 1987; Rettie et al., 1988). More recently, studies with recombinant human cytochrome P-450 enzymes have demonstrated that CYP2C9 and CYP2A6 enzymes catalyze the formation of 4-ene VPA (Sadeque et al., 1997). The study of 4-ene-VPA metabolite formation and its subsequent fate has received considerable attention because of its possible involvement in VPA-induced idiosyncratic hepatotoxicity. This metabolite structurally resembles a known hepatotoxin, 4-pentenoic acid, and has been shown to cause cytochrome P-450 destruction (Prickett and Baillie, 1986), inhibition of β-oxidation (Bjorge and Baillie, 1985), and microvesicular steatosis in rats (Kesterson et al., 1984; Granneman et al., 1984b). In addition, the formation of this metabolite is enhanced in epileptic patients on polytherapy with other antiepileptic drugs, the population more susceptible to VPA-induced hepatotoxicity (Levy et al., 1990). The 4-ene VPA metabolite can be subsequently metabolized via mitochondrial β-oxidation to form the diunsaturated metabolite (E)-2,4-diene VPA; although the latter species can also arise via microsomal desaturation of (E)-2-ene VPA (Kassahun and Baillie, 1993; Kassahun et al., 1994). One hypothesis of VPA-induced hepatotoxicity is that subsequent oxidative metabolism of 4-ene VPA, possibly via (E)-2,4-diene VPA formation, leads to the generation of chemically reactive and potentially toxic intermediates that are capable of reacting with and depleting mitochondrial glutathione stores (Rettenmeier et

The plasma and urine concentrations of VPA metabolites exhibit extremely high inter-individual variability. However, the major VPA metabolites in plasma are generally (E)-2-ene VPA, (E,E)-2,3′-diene VPA and 3-keto VPA (Rettenmeier et al., 1989; Kassahun et al., 1990). At therapeutic VPA plasma concentrations (40-100 μg/ml), the plasma concentrations of these metabolites are usually within the range of 1-10 μg/ml. Other metabolites such as 3-ene VPA, 4-keto VPA, 3-OH VPA, 4-OH VPA and 5-OH VPA are also present in significant concentrations (0.5-2 μg/ml) (Rettenmeier et al., 1989; Granneman et al., 1984a). The 4-ene VPA, (E)-2,4-diene VPA, 2-PSA and 2-PGA metabolites are usually present only in trace amounts in plasma (Rettenmeier et al., 1989; Kassahun et al., 1990); however, plasma concentrations of 4-ene VPA may be elevated in patients on polytherapy (Levy et al., 1990). The major urinary metabolites of VPA are VPA-glucuronide (10-70% of the dose) and 3-keto VPA (10-60% of the dose) (Dickinson et al., 1989; Levy et al., 1990; Sugimoto et al., 1996). Other metabolites such as (E,E)-2,3′-diene VPA, 3-OH VPA, 4-OH VPA, 5-OH VPA, 4-keto VPA and 2-PGA may account for 1-5% of the administered VPA dose (Dickinson et al., 1989; Levy et al., 1990). The 4-ene VPA, (E)-2,4-diene VPA, (E)-2-ene VPA, 3-ene VPA, and 2-PSA metabolites are minor urinary products and each accounts for 0-0.5% of the total VPA dose (Dickinson et al., 1989; Levy et al., 1990).
In addition to the above metabolites, a number of other minor VPA metabolites have also been identified. These include carnitine and glycine conjugates of VPA, a VPA coenzyme A thioester, 2-OH VPA and a 4,5-epoxide metabolite of 4-ene VPA (Baillie and Sheffels, 1995). The conjugation of VPA with carnitine may be significant in light of the fact that VPA therapy is associated with a secondary carnitine deficiency (Coulter, 1991).

1.5.4 Epilepsy, Pregnancy and Valproic Acid

Epilepsy occurs in 0.6-1% of the normal population and 0.5% of all pregnancies occur in epileptic women (Martin and Millac, 1993). Due to its prevalence and high risk, epilepsy is considered the most common major neurologic complication of pregnancy (Rochester and Kirchner, 1997). There are approximately 12,000 births per year to epileptic women in United States alone, and approximately 95% of these women are on antiepileptic therapy during pregnancy (Vorhees et al., 1988). VPA is one of the 4 major drugs (phenytoin, phenobarbital, carbamazepine and VPA) used to treat epilepsy in the pregnant population (Lindhout and Omtzigt, 1994; Malone and D'Alton, 1997). Unfortunately, the incidence of major birth defects in children born to epileptic women is 2-3 times greater compared to the normal population (Kelly, 1984; Yerby, 1994). The occurrence of fetal malformations has been associated with the use of all 4 major antiepileptic drugs. However, causal relationships have not been established because the possible confounding effects of the disease itself on organogenesis are not clear (Lindhout and Omtzigt, 1994; Malone and D'Alton, 1997). All of the above anticonvulsants are associated with major cardiovascular defects, orofacial clefts, genitourinary defects and dysmorphic syndromes (Malone and D'Alton, 1997).
addition, the use of VPA appears to be associated with a 20-fold higher risk of neural tube defects (spina bifida aperta) compared to the general population (Lindhout and Omtzigt, 1994; Yerby, 1991). A fetal valproate syndrome consisting of dysmorphic features such as flat nasal bridge, up-turned nasal tip, down-turned mouth, low-set ears, microencephaly, thin overlapping fingers or toes, and flat orbits has also been reported (DiLiberti et al., 1984). In addition to the teratogenic effects associated with the use of VPA and other antiepileptic drugs, there are also reports that prenatal exposure to these compounds may result in alterations in cognitive function and behaviour during postnatal life (Trimble, 1990). At least in one study, children exposed prenatally to VPA showed poorer motor performance and impaired neurological function at 6 years of age as compared to controls (Koch et al., 1996).

VPA has been shown to undergo extensive placental transfer in animals as well as humans (Nau, 1986; Kondo et al., 1987; Nau et al., 1984; Nau et al., 1981; Ishizaki et al., 1981; Dickinson et al., 1979b; Nau and Krauer, 1986; Dickinson et al., 1980). In one study in a single pregnant rhesus monkey (Dickinson et al., 1980), VPA appeared rapidly in fetal blood after maternal i.v. bolus administration and reached concentrations comparable to those in maternal blood within 15 min. Also, during the terminal phase, the average fetal-to-maternal plasma concentration ratio was 1.3, indicating a high degree of fetal exposure (Dickinson et al., 1980). Similar to the monkey, cord-to-maternal blood VPA concentration ratios in humans at birth range from 0.5 to 4.6 (Kondo et al., 1987; Nau et al., 1984; Nau et al., 1981; Ishizaki et al., 1981; Dickinson et al., 1979b; Nau and Krauer, 1986; Nau et al., 1982). It has been demonstrated that the plasma protein binding of VPA gradually decreases in the mother over the course of gestation, whereas that in the fetus gradually increases, such that at birth fetal VPA plasma protein binding
exceeds that in the mother (Nau and Krauer, 1986). Also, there is a further reduction in maternal plasma protein binding of the drug at birth due to elevated plasma free fatty acids (Nau et al., 1984; Nau and Krauer, 1986). These phenomena result in fetal accumulation of the drug and a greater than unity cord-to-maternal blood VPA ratio at birth (Nau et al., 1984). The decrease in maternal plasma protein binding of VPA and other anticonvulsants with advancing gestation also results in a significant increase in their clearance and a fall in steady-state plasma concentrations (Yerby et al., 1992).

Similar to VPA, the 2-ene and 3-keto VPA metabolites have also been measured in higher concentrations in cord and newborn blood compared to maternal blood (Nau et al., 1981; Nau et al., 1984; Kondo et al., 1987). A mechanism related to maternal-fetal plasma protein binding differences may be operative in this case also; however, this remains to be demonstrated.

Rapid placental transfer of VPA has also been demonstrated in the perfused human placental model where placental clearance of VPA was ~95% of that of antipyrine (a flow limited marker for placental transfer) (Fowler et al., 1989). In contrast, the placental clearance rate of VPA-glucuronide was only ~13% of that of antipyrine, possibly due to its extremely hydrophilic nature (Fowler et al., 1989).

In some of the above studies in pregnant women at birth, the elimination of VPA from neonates was also followed (Nau et al., 1981; Nau et al., 1984; Kondo et al., 1987; Ishizaki et al., 1981; Dickinson et al., 1979b). In addition, there are 2 more studies of VPA pharmacokinetics in the immediate newborn period (Irvine-Meek et al., 1982; Gal et al., 1988). In these studies, elimination half-lives of VPA in newborns were in the range of
15.1-80 h, and are approximately 2-8 fold longer compared to adults. Similar findings have been reported for newborns of other species such as rats and guinea-pigs (Haberer and Pollack, 1994; Yu et al., 1985; Yu et al., 1987). This indicates a much reduced elimination capacity for the drug during the immediate newborn period in all species studied till date.

1.5.5 VPA Disposition in Pregnant Sheep: Earlier Studies

A study was conducted in this lab to examine the placental transfer of VPA in chronically-catheterized pregnant sheep during late gestation (Gordon et al., 1995). After maternal i.v. bolus administration, VPA appeared rapidly in plasma (within 2 min) and the average fetal drug exposure index based on fetal-to-maternal AUC ratio was 0.41 (range 0.29 – 0.61). Similarly, after separate fetal i.v. bolus administration, VPA was rapidly detectable in maternal plasma (within 2 min). During both maternal as well as fetal administration, maternal and fetal plasma concentrations of the drug declined in parallel with each other with an apparent elimination half-life of ~2-4 h, indicating that the drug does not persist in the fetal circulation. VPA did not appear to accumulate in amniotic and fetal tracheal fluids in contrast to a number of amine drugs studied earlier in this model. A number of human VPA metabolites, such as (E)-2-ene VPA, (Z)-2-ene VPA, (E)-3-ene VPA, (Z)-3-ene VPA, 4-ene VPA, 3-keto VPA, 4-keto VPA, 3-OH VPA, 4-OH VPA, 5-OH VPA and 2-PGA were also detectable in maternal sheep serum. Some metabolites (e.g., (E)-2-ene VPA, 4-ene VPA, 3-keto VPA, 4-OH VPA and 5-OH VPA) were also detected in fetal serum, albeit at lower concentrations compared to the mother. In contrast to the human, however, the diunsaturated VPA metabolites (e.g., (E,E)-2,3'-diene VPA and (E)-2,4-
diene VPA) were not detected in sheep, indicating a possible species difference (Gordon et al., 1995).

1.6 Rationale

The continued use of drugs during pregnancy to treat maternal and fetal disease states and the problem of maternal illicit substance abuse necessitate a better understanding of the factors determining maternal-fetal drug disposition and fetal drug exposure. In the above discussion, a point has been made that such studies cannot be conducted in humans due to practical and ethical constraints, and small animal models do not provide detailed data on maternal-fetal concentration relationships due to technical difficulties related to their small size and blood volume. The chronically-instrumented pregnant sheep is the most commonly used model to study maternal-fetal drug disposition and fetal physiologic functions during late gestation due to the ability to study the fetal lamb for days or weeks in its normal intrauterine environment. Also, the size of the sheep fetus permits serial sampling of blood and other fluids, thereby allowing detailed study of drug disposition in the fetus, surrounding fluid compartments and the mother (Rurak et al., 1991). In comparison to non-human primate models, sheep are much more economical, and are safer and easier to work with. In certain aspects, there are clear differences between the sheep and the human, such as the difference in placental permeability to polar drugs due to differences in placental structure. However, we feel that these differences are not likely to be very great for the lipophilic compound, DPHM, and the relatively polar but low molecular weight, VPA. Also, as with all other animal models, there are likely to be differences in ovine drug metabolizing enzymes and different drug clearance rates compared to the human. However, the overall advantages of sheep for
the study of maternal-fetal disposition of lipophilic and low molecular weight compounds far outweigh any disadvantages.

The extent of fetal drug exposure after maternal administration, although a clinically useful index, is the result of a complex interaction of a host of pharmacokinetic factors related to the placenta, the mother and the fetus. The determination of maternal and fetal placental and non-placental clearances partitions these factors into their individual contributions in determining fetal drug exposure, and also provides valuable information on in utero fetal drug elimination capacity compared to the mother. For all drugs studied in pregnant sheep, the fetal ability to eliminate drugs via non-placental pathways is remarkable (Table 1.1). However, for none of these drugs have the exact routes of fetal non-placental clearance been fully elucidated, and for many of them, the routes responsible for the majority of maternal non-placental clearance do not appear to fully account for fetal non-placental clearance. This includes studies with DPHM in this lab where there were large apparent differences in hepatic uptake of the drug in adult and fetal sheep (Tonn et al., 1996). Also, for acetaminophen, glucuronidation and sulfation of the drug appear to be responsible for ~97% of maternal non-placental clearance but account for only ~33% of fetal non-placental clearance (Wang et al., 1986a). Similar findings have been observed with ritodrine where ~35% of the fetal dose could be accounted for by placental transfer to the mother and fetal glucuronidation of the drug (Wright et al., 1991). Thus, it appears important to fully elucidate the components of fetal non-placental clearance of at least one compound so as to validate that this parameter is attributable to actual fetal drug elimination capacity and is not a feature of the intra-uterine environment or a by-product of the pharmacokinetic modeling. This
includes the identification of fetal organs responsible for drug elimination as well as of the exact metabolic pathways contributing to this drug elimination.

At the outset of this project, we felt that there could be some confounding factors associated with the geometry of the fetal circulation and fetal blood flow patterns that may have led to an apparent lack of any fetal hepatic DPHM uptake from the umbilical vein in previous studies in this lab (Tonn et al., 1996). Thus, we decided to re-examine this fetal hepatic DPHM uptake from the umbilical vein and its contribution to fetal non-placental DPHM clearance. Also, it was felt essential to examine and compare the metabolic pathways responsible for DPHM non-placental clearance and their relative capacity in the mother and the fetus.

A detailed analysis of the importance of various placental, maternal and fetal pharmacokinetic factors in determining fetal drug exposure has not been performed for any drug. Also, the last 3rd of gestation is a very dynamic period in terms of fetal development and numerous changes in fetal physiological variables occur during this time. These include possible changes in maternal and fetal plasma protein binding of drugs, development of fetal drug metabolism capacity and renal excretion, and alterations in fetal circulatory and hemodynamic processes with advancing gestation. All these variables could differentially affect the individual placental, maternal and fetal components of drug disposition in the maternal-placental-fetal unit and hence affect fetal drug exposure. Hence, we examined the inter-relationships between maternal and fetal placental and non-placental clearances of DPHM, plasma protein binding, umbilical blood flow and placental transfer and fetal drug exposure as a function of gestational age.
VPA is a drug with several contrasting features compared to DPHM. These include a relatively greater polarity, acidic nature, much lower clearance and possible nonlinearities in its pharmacokinetics due to the phenomena of saturable plasma protein binding and metabolism. Thus, the comparative study of DPHM and VPA may provide information on the relative importance of different factors in determining fetal exposure to these two extreme classes of drugs (e.g., low vs. high polarity; low vs. high clearance). In addition, in many species, both these drugs are metabolized via many distinct drug metabolism pathways. Thus, the study of the maternal-fetal metabolism of DPHM and VPA may provide information on the ontogenetic development of fetal xenobiotic metabolism in general.

Lastly, both DPHM and VPA appear to be relatively widely used during pregnancy. VPA undergoes extensive placental transfer in humans and is also occasionally administered to epileptic newborns to treat seizures that are refractory to other drugs (Gal et al., 1988). Although data on placental transfer of DPHM in humans are not available, it is likely as rapid and extensive as in sheep. Thus, a detailed study of the maternal-fetal pharmacokinetics and metabolism, placental transfer and fetal exposure of these two drugs is relevant in its own right.
1.7 Objectives

The major objectives of the research presented in this thesis were:

1. To identify the components of maternal and fetal DPHM non-placental clearance in sheep, including the organs and metabolic pathways responsible for drug elimination.
2. To examine the inter-relationships between maternal and fetal placental and non-placental clearances, plasma protein binding, placental transfer and fetal exposure of DPHM as a function of advancing gestation.
3. To study steady-state placental transfer, fetal exposure, and pharmacokinetics and metabolism of VPA in the sheep maternal-fetal unit.
4. To study VPA disposition during the immediate newborn period in comparison to that in the mother and the fetus.
5. To draw parallels and comparisons between DPHM and VPA in terms of the factors affecting their placental transfer and fetal exposure.
6. To compare the development of fetal (and also newborn in case of VPA) drug metabolism and renal excretion capacity in comparison to the adult for these two drugs.
During the studies described in subsequent chapters, a number of parent drug and metabolite assay methods were employed. The analysis of DPHM and $[^2\text{H}_{10}]$-DPHM, and DPMA and $[^2\text{H}_{10}]$-DPMA was accomplished using two gas-chromatographic-mass spectrometric (GC-MS) methods developed previously in this lab (Tonn et al., 1993; Tonn et al., 1995). In addition, VPA and its metabolites were also quantified using a previously developed GC-MS method (Yu et al., 1995). During the course of these studies, a new high-performance liquid chromatographic-tandem mass spectrometric (LC-MS/MS) analysis method was developed for the simultaneous quantitation of DPHM, $[^2\text{H}_{10}]$-DPHM, and their corresponding N-oxide metabolites, DPHM-N-oxide (DPHMNOX) and $[^2\text{H}_{10}]$-DPHM-N-oxide ($[^2\text{H}_{10}]$-DPHMNOX), respectively. A brief summary of the procedures and specifications of the previously developed methods, and a detailed account of the LC-MS/MS method is presented in this chapter.

2.1 Materials

Reference standards, chemicals, reagents and other materials used during the studies described in this thesis, along with the information on their purity (where applicable) and source, are listed below. Unless otherwise specified, the materials were used without further purification or modification. Diphenhydramine hydrochloride [2-(diphenylmethoxy)-N,N-dimethylethylamine] (>99% purity), orphenadrine hydrochloride [N,N-dimethyl-2-[(2-methylphenyl)phenyl]ethyamine] (>99% purity), diphenylacetic acid (>98% purity), ethylenediaminetetraacetic acid (EDTA), sucrose,
tris(hydroxymethyl)aminomethane (Trizma® Base), \(\beta\)-nicotinamide-adenine dinucleotide phosphate (reduced) tetr sodium salt ca. 98\% (NADPH), uridine-5'\-diphosphogluconic acid (UDPGA), \(\beta\)-glucuronidase (Glucurase®), sodium 2-propylpentanoate (sodium valproate) and cellophane dialysis membrane “sacks” (molecular weight cutoff = 12,000 daltons) were purchased from Sigma Chemical Co. (St. Louis, MO., U.S.A.).

The DPMA metabolite was synthesized and purified using a previously described method (Tonn et al., 1995). Deuterated analogues of DPHM hydrochloride (i.e., \([^{2}H_{10}]\)-DPHM hydrochloride), and DPMA (i.e., \([^{2}H_{10}]\)-DPMA) were also synthesized and purified as described previously (Tonn et al., 1993; Tonn et al., 1995). DPHM-N-oxide hydrochloride (>98\% purity) was generously provided by Parke-Davis Pharmaceutical Research Division (Ann Arbor, MI, U.S.A.). Deuterium-labeled DPHM-N-oxide hydrochloride \([^{2}H_{10}]\)-DPHM-N-oxide.HCl) was synthesized and purified as described later in this chapter.

The metabolites of valproic acid 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). These metabolites include: (E)-2-propyl-2-pentenoic acid [(E)-2-ene VPA], (E)-2-propyl-3-pentenoic acid [(3)-ene VPA], 2-propyl-4-pentenoic acid (4-ene VPA), 2-propyl-3-hydroxypentanoic acid (3-OH-VPA), 2-propyl-4-hydroxypentanoic acid (4-OH-VPA), 2-[(E)-1'-propenyl]-(E)-2-pentenoic acid [(E,E)-2,3'-diene VPA], (E)-2-propyl-2,4-pentadienoic acid [(E)-2,4-diene VPA], 2-propylglutaric acid (2-PGA) and 2-propylsuccinic acid (2-PSA). The metabolites 2-propyl-3-oxopentanoic acid (3-keto-VPA) (Kassahun et al., 1990), 2-propyl-4-
oxopentanoic acid (4-keto-VPA) (Kassahun et al., 1990) and 2-propyl-5-hydroxypentanoic acid (5-OH-VPA) (Rettenmeier et al., 1985) were also synthesized according to previously reported procedures.

The following heptadeuterated compounds (utilized as internal standards for VPA and VPA-metabolite analysis) were synthesized as described elsewhere (Zheng, 1993): 2-[\textsuperscript{2}H\textsubscript{7}]propylpentanoic acid ([\textsuperscript{2}H\textsubscript{7}]VPA), 2-[\textsuperscript{2}H\textsubscript{7}]propyl-2-pentenoic acid (2-ene[\textsuperscript{2}H\textsubscript{7}]VPA), 2-[\textsuperscript{2}H\textsubscript{7}]propyl-4-pentenoic acid (4-ene[\textsuperscript{2}H\textsubscript{7}]VPA), 2-[\textsuperscript{2}H\textsubscript{7}]propyl-3-oxopentanoic acid (3-keto[\textsuperscript{2}H\textsubscript{7}]VPA), 2-[\textsuperscript{2}H\textsubscript{7}]propyl-4-oxopentanoic acid (4-keto[\textsuperscript{2}H\textsubscript{7}]VPA), 2-[\textsuperscript{2}H\textsubscript{7}]propyl-3-hydroxypentanoic acid (3-OH[\textsuperscript{2}H\textsubscript{7}]VPA), and 2-[\textsuperscript{2}H\textsubscript{7}]propyl-5-hydroxypentanoic acid (5-OH[\textsuperscript{2}H\textsubscript{7}]VPA).

Deuterated benzene ([\textsuperscript{2}H\textsubscript{6}]benzene, 99.96% isotopic purity) for the synthesis of deuterated DPHM and its metabolites was obtained from Cambridge Isotope Laboratories (Andover, MA, U.S.A.). Ammonium acetate, sodium carbonate, glacial acetic acid, disodium hydrogen orthophosphate (dibasic), potassium dihydrogen orthophosphate (monobasic), potassium chloride, sodium hydroxide pellets, magnesium chloride, and hydrochloric acid were obtained from BDH Chemicals (Toronto, Ontario, Canada) and were of analytical reagent grade. Anhydrous aluminum chloride, anhydrous sodium sulfate, bromoacetic acid, carbon tetrachloride, magnesium chloride, petroleum ether, sodium metal, and para-toluene sulfonic acid utilized during synthesis procedures were also purchased from BDH Chemical Co. (Toronto, Ontario, Canada).

Chloroperoxybenzoic acid, 2-methylglutaric acid (2-MGA), triton-X 100, and deuterium oxide were obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). Sequanal grade
triethylamine (TEA) and N-methyl-N-(tert-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) were purchased from Pierce Chemical Co. (Rockville, IL, U.S.A.).

Acetonitrile, ethylacetate, methanol, methylene chloride, toluene, ethanol, isopropanol, acetone, diethyl ether, ethyl acetate and n-hexane were purchased from Caledon Laboratories (Georgetown, Ontario, Canada) and were of distilled in glass HPLC or GC grade.

The Bradford protein assay kit was purchased from Bio-Rad Laboratories (Mississauga, Ontario, Canada). Centrifree® micropartition devices were from Amicon (Amicon, Inc., Danver, MA, U.S.A.). Ultra-high purity grade helium, hydrogen, and nitrogen were utilized (Praxair, Vancouver, BC, Canada).

Deionized high purity water (referred to as ‘distilled or deionized water’ in text) was produced on-site by reverse osmosis and subsequent filtration using a Milli-Q® water system (Millipore, Bedford, MA, U.S.A.).

Materials used during sheep experiments were as follows. Veramix Sheep Sponges (Tuco Products Co., Orangeville, Ontario, Canada); Pregnant Mares’ Serum Gonadotropin (Ayerst Laboratories, Montreal, Quebec, Canada); thiopental sodium injectable 1 g/vial; sodium chloride for injection USP (Abbott Laboratories, Montreal, Quebec, Canada); injectable ampicillin (250 mg/vial) (Novopharm, Toronto, Ontario, Canada); injectable atropine sulfate (0.6 mg/mL) (Glaxo Laboratories, Montreal, Quebec, Canada); heparin 1000 units/mL (Organon Canada Ltd., West Hill, Ontario, Canada); halothane (Ayerst Laboratories, Montreal, Quebec, Canada); and lidocaine.
2% (Astra Pharma Inc., Mississauga, Ontario, Canada). All injectable drug formulations were purchased from BC Women’s Hospital Pharmacy, Vancouver, BC, Canada.

Also used were: syringe needles and plastic disposable Luer-Lok® Syringes for drug administration and sample collection (Becton-Dickinson Canada, Mississauga, Ontario, Canada); nylon syringe filters (0.22 μm) (MSI, Westboro, MA, U.S.A.); heparinized blood gas syringes (Marquest Medical Products Inc., Englewood, CO, U.S.A.); heparinized Vacutainer® tubes (Vacutainer Systems, Rutherford, NJ, U.S.A.); 15 ml Pyrex® disposable culture tubes (Corning Glass Works, Corning, NY, U.S.A.); polytetrafluoroethylene (PTFE) lined screw caps (Canlab, Vancouver, BC, Canada.); silicone or polyvinyl rubber tubing for catheter preparation (Dow Corning, Midland, MI, U.S.A.); and cryovials and closures (Nalgene Company, Rochester, NY, U.S.A.).

2.2 Instrumentation

2.2.1 Gas Chromatography-Mass Spectrometry

A Hewlett-Packard (HP) model 5890 (Series II) gas chromatograph equipped with a split-splitless capillary inlet system, a HP Model 7673 autoinjector, a HP Model 5971A quadrupole mass selective detector, and a Vectra 486 25T Computer with MS DOS® HP Model G1030A workstation software was utilized for all GC-MS assays (Hewlett-Packard, Avondale, PA, U.S.A.). The gas-chromatograph was equipped with either a DB1701 (30 m x 0.25 mm i.d.; film thickness 0.25 μm; 5% phenylmethylsilicone and 7% cyanopropylsilicone; J&W Scientific, Folsom, CA, U.S.A.; DPHM[²H₁₀]-DPHM and VPA+VPA metabolites assay methods) or an HP Ultra-2 (25 m x 0.25 mm i.d.; film
thickness 0.25 μm; 5% phenylmethylsilicone; DPMA/[²H₁₀]DPMA assay method) cross-linked fused silica capillary column. Samples were injected into the GC-MS via a 4 x 78 mm deactivated Pyrex® glass inlet liner and a Thermogreen LB-2® silicone rubber septum.

2.2.2 High-Performance Liquid Chromatography-Tandem Mass-Spectrometry

The LC-MS/MS instrumentation consisted of a HP 1090 II liquid chromatograph (Hewlett-Packard, Avondale, PA, U.S.A.) interfaced to a Fisons VG Quattro I Triple Quad Tandem-Mass Spectrometer (Micromass, Cheshire, UK). The operation of both instruments and mass-spectrometric data acquisition were controlled with a Windows-NT® based Pentium Pro 200 MHz personal computer using the mass-spectrometry data handling software, MassLynx® (MicroMass, Cheshire, UK). Chromatographic separations were carried out on a YMC propyl amino (NH₂, 100 mm x 2.0 mm ID, 5 μm) column (YMC, Inc., Wilmington, NC, U.S.A.) at ambient temperature. The HPLC autoinjector syringe and sample loop volumes were 25 and 250 μl, respectively.

2.2.3 Spectrophotometer

A HP 8452A diode array spectrophotometer equipped with a Vectra® computer interface was used for spectrophotometric measurements for the microsomal protein assay.

2.2.4 Physiological Monitoring
Physiological monitoring of the animals was performed using a Beckman R-711 Dynograph Recorder (Beckman Instruments, Inc., Palo Alto, CA, U.S.A.), disposable DTX pressure transducers (Spectramed, Oxnard, CA, U.S.A.), cardiotachometer (Model 9857, Sensormedics, Anaheim, CA, U.S.A.), and transit-time blood flow transducers (Transonic Systems Inc., Ithaca, NY, U.S.A.). An Apple Ile computer and data acquisition system consisting of an interactive systems analog to digital converter (Daisy Electronics, Newton Square, PA, U.S.A.), and a clock card (Mountain Software, Scott's Valley, CA, U.S.A.) were used for online data acquisition from the polygraph recorder. Blood pH, \( \text{Po}_2 \), and \( \text{Pco}_2 \) were measured using an IL 1306 pH/Blood gas analyzer (Allied Instrumentation Laboratory, Milan, Italy). Blood \( \text{O}_2 \)-saturation and hemoglobin concentration were determined using a Hemoximeter (Radiometer, Copenhagen, Denmark). Blood glucose and lactate concentrations were measured with a 2300 STAT plus glucose/lactate analyzer (Y.S.I. Inc., Yellow Springs, OH, U.S.A.).

### 2.2.5 Other Equipment

Also utilized were: a vortex-type mixer and incubation oven (Isotemp model 350) (Fisher Scientific Industries, Springfield, MA, U.S.A.); IEC model 2K centrifuge (Damon/IEC division, Needham Hts., MA, U.S.A.); rotating-type mixer (Labquake model 415-110, Lab Industries, Berkeley, CA, U.S.A.); infusion pumps (Harvard model 944, Harvard Apparatus, Millis, MA, U.S.A.); DIAS Roller pump (DIAS, Ex154, DIAS Inc. Kalamazoo, MI, U.S.A.); high speed centrifuge model J2-21, ultra-centrifuge model L8-60M or L5-50, JA-17 fixed angle rotor, Ti 50.2 fixed angle rotor (Beckman Instruments, Inc., Palo Alto, CA, U.S.A.).
2.3 Analysis of DPHM and \[^2\text{H}_{10}\]-DPHM in Biological Fluids

This was accomplished using the GC-MS assay method developed previously in this lab, using orphenadrine as the internal standard (Tonn et al., 1993). Briefly, appropriate volumes of biological fluids (e.g., plasma, urine etc.) were alkalinized with 0.5 ml of 1M NaOH, and extracted for 20 min with 7 ml of a hexane-2% isopropanol mixture containing 0.05M TEA on a rotary mixer. The organic extract was separated and dried under a gentle stream of nitrogen at room temperature. The residue was reconstituted in 150 μl of dry toluene containing 0.05M TEA and 2 μl was injected into the GC-MS using the splitless mode of sample introduction (purge time 1.5 min). Chromatographic separations were performed on a DB1701 (30 m x 0.25 mm i.d., 0.25 μm film thickness) fused silica capillary column with helium as the carrier gas at a 12.5 psi column head pressure. The GC operating conditions were as follows. The injection port temperature was 180°C. The oven temperature program consisted of an initial temperature of 140°C for 1 min, a 30°C/min ramp to 200°C, and another 17.5°C/min ramp to 265°C where it was held for 5.0 min. This resulted in a total run time of 12.7 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. Ion fragment m/z 165 was used to monitor both DPHM and orphenadrine, whereas \[^2\text{H}_{10}\]-DPHM was monitored using m/z 173. The calibration curve concentration range for this assay is 2.0–200.0 ng/ml for both DPHM and \[^2\text{H}_{10}\]-DPHM. During earlier validation, the inter- and intra-day variability and bias of this assay were <20% at the limit of quantitation (LOQ) and <10% at all other concentrations (Tonn et al., 1993). In addition, the extraction recoveries of
DPHM and $[\text{H}_{10}]$-DPHM are nearly complete with the above procedure and analyte stability under the conditions of the assay has been established (Tonn et al., 1993).

2.4 Analysis of DPMA and $[\text{H}_{10}]$-DPMA in Biological Fluids

The biological fluid concentrations of the DPHM metabolite, DPMA, and its deuterated analog, $[\text{H}_{10}]$-DPMA, were also measured using the previously developed GC-MS analytical method (Tonn et al., 1995). Briefly, appropriate volumes of biological fluids (e.g., plasma, urine etc.) were acidified with 0.4 ml of 1M HCl, and extracted for 20 min with 5 ml of toluene on a rotary mixer. The organic extract was separated and dried under a gentle stream of nitrogen at 40°C, and the residue was reconstituted in 200 μl of dry toluene. The reconstituted residue was derivatized with 25 μl MTBSTFA (N-methyl-N-(tert-butyldimethylsilyl)trifluoroacetamide) at 60°C for 1 h in order to form tert-butyldimethylsilyl (t-BDMS) derivatives of DPMA and $[\text{H}_{10}]$-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 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, 183, 177 were used to monitor diphenylacetic acid (DPAA; internal standard),
DPMA and [\(^{2}\text{H}_{10}\)]-DPMA, respectively. The calibration curve concentration range for this assay is 2.5–250.0 ng/ml for both DPMA and [\(^{2}\text{H}_{10}\)]-DPMA. During earlier validation, the 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 extraction recoveries of DPMA and [\(^{2}\text{H}_{10}\)]-DPMA using the above procedure are in the range of ~75-80%, and the analytes are stable under the conditions of the assay (Tonn et al., 1995).

2.5 Analysis of VPA and its Metabolites in Biological Fluids

The biological fluid concentrations of VPA and 12 of its metabolites [(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] were measured during our studies of VPA disposition in maternal, fetal and newborn sheep. A previously developed GC-MS analytical method was utilized for these measurements (Yu et al., 1995). Briefly, the procedure involves acidification of biological fluid samples to pH 3.0-3.5 with 1M HCl. The samples are then extracted twice with 3 ml ethyl acetate on a rotary mixer for 30 min each. The absorbed water from the combined ethyl acetate extract is removed by vortexing with anhydrous sodium sulfate, and the dry extract is concentrated to ~100 µl under a gentle stream of nitrogen. A 50 µl aliquot of MTBSTFA is then added, samples are derivatized by heating at 60°C for 1 h, and 1 µl is injected into the GC-MS 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 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 250°C. The oven
temperature program 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). This resulted in a total run time of 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. The calibration curve concentration range for VPA and various metabolites, the internal standards utilized for each compound, and the ions monitored are presented in Table 2.1.

<table>
<thead>
<tr>
<th>Analytes to be quantitated</th>
<th>Internal Standards</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analyte</td>
<td>Calibration curve range (µg/ml)</td>
</tr>
<tr>
<td>VPA</td>
<td>0.025-20.0</td>
</tr>
<tr>
<td>(E)-2-ene VPA</td>
<td>0.01-8.0</td>
</tr>
<tr>
<td>(E)-3-ene VPA</td>
<td>0.0025-2.0</td>
</tr>
<tr>
<td>4-ene VPA</td>
<td>0.0025-2.0</td>
</tr>
<tr>
<td>(E,E)-2,3',diene VPA</td>
<td>0.0025-2.0</td>
</tr>
<tr>
<td>(E)-2,4-diene VPA</td>
<td>0.0075-6.0</td>
</tr>
<tr>
<td>3-keto VPA</td>
<td>0.02-4.0</td>
</tr>
<tr>
<td>4-keto VPA</td>
<td>0.0025-2.0</td>
</tr>
<tr>
<td>3-OH VPA</td>
<td>0.02-2.0</td>
</tr>
<tr>
<td>4-OH VPA</td>
<td>0.04-4.0</td>
</tr>
<tr>
<td>5-OH VPA</td>
<td>0.02-2.0</td>
</tr>
<tr>
<td>2-PSA</td>
<td>0.01-2.0</td>
</tr>
<tr>
<td>2-PGA</td>
<td>0.01-2.0</td>
</tr>
</tbody>
</table>
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).

2.6 Simultaneous LC-MS/MS Analysis of DPHM, [\(^{2}\text{H}_{10}\)]-DPHM, DPHM-N-Oxide and [\(^{2}\text{H}_{10}\)]-DPHM-N-Oxide in Biological Fluids

As mentioned above, a LC-MS/MS analytical method was developed for the simultaneous quantitation of DPHM, [\(^{2}\text{H}_{10}\)]-DPHM, DPHM-N-oxide (DPHMNOX) and [\(^{2}\text{H}_{10}\)]-DPHM-N-oxide ([\(^{2}\text{H}_{10}\)]-DPHMNOX) in biological fluids obtained from pregnant sheep. This was essential for the study of the DPHM-N-oxide pathway of DPHM metabolism in the mother and the fetus. Development and validation of this assay is described below in detail.

2.6.1 Methods

2.6.1.1 Synthesis and Purification of Deuterium-Labeled DPHM-N-oxide Hydrochloride

The [\(^{2}\text{H}_{10}\)]-DPHMNOX metabolite was synthesized from [\(^{2}\text{H}_{10}\)]-DPHM by its oxidation with 3-chloroperoxybenzoic acid using a slight modification of the method described for the synthesis of S- and N-oxides of phenothiazine antipsychotics (Jaworski et al., 1993). For this purpose, [\(^{2}\text{H}_{10}\)]-DPHM hydrochloride was converted to its free base by alkalization of an aqueous solution of the hydrochloride salt with sodium hydroxide. The free [\(^{2}\text{H}_{10}\)]-DPHM base was extracted with diethyl ether and the solvent was evaporated under vacuum. The [\(^{2}\text{H}_{10}\)]-DPHM base (10 mmol) was then dissolved in 30 ml of dry dichloromethane. To this solution, 3-chloroperoxybenzoic acid (12 mmol) was
added and the mixture was stirred in an ice bath for 1 h. At the end of the reaction, unreacted 3-chloroperoxybenzoic acid was consumed by addition of an excess of TEA (12 mmol). The crude product was purified by flash column chromatography over silica gel using a benzene and methanol (85:15) solvent mixture as the eluant. The fraction containing $[^2\text{H}_{10}]-\text{DPHMNOX}$ was collected, and the solvent was removed under vacuum. The residue was washed repeatedly with hexane and cold methanol (-20°C). The washed residue was dissolved in dry acetone, cooled to -20°C, and $[^2\text{H}_{10}]-\text{DPHMNOX}$ hydrochloride was precipitated by the addition of isopropanol saturated with hydrogen chloride gas. The precipitate was recrystallized from acetone to give a white crystalline powder. The final product gave a single spot on TLC and only one peak on a number of HPLC columns under a variety of elution conditions, indicating acceptable purity for the synthesized metabolite. Also, the HPLC retention time and daughter ion mass spectrum of the purified metabolite were similar to that of the Parke-Davis DPHMNOX standard, except with an expected 10 a.m.u difference in certain fragment masses (Figure 2.1; also see below).

2.6.1.2 Standard Stock Solutions

An aqueous stock solution of analytes containing 2.5 µg/ml of DPHM, 2.6 µg/ml of $[^2\text{H}_{10}]-\text{DPHM}$ (to account for the mass of the deuterium labels), 1.0 µg/ml of DPHMNOX and 1.04 µg/ml of $[^2\text{H}_{10}]-\text{DPHMNOX}$ (to account for the mass difference of the deuterium labels) was prepared by dissolving appropriate amounts of the analytes (based on free base) in deionized water. Two additional solutions were prepared that were 10 and 25 fold dilutions of the above standard stock. The internal standard (I.S.)
solution containing 250.0 ng/ml orphenadrine was prepared by dissolving an accurately weighed amount of orphenadrine hydrochloride in deionized water.

### 2.6.1.3 Sample Extraction

The analytes of interest were extracted from the biological fluid samples using a single step liquid-liquid extraction procedure. Sheep plasma or urine samples (up to 1.0 ml) or the spiked standards were pipetted into clean borosilicate glass tubes with polytetrafluoroethylene (PTFE) lined caps. The sample volume was adjusted to 1.0 ml with deionized water. A 100 µl aliquot of the I.S. solution (containing 25.0 ng orphenadrine) was added to each sample and the samples were alkalinized (pH 11.0) by adding 0.5 ml of a saturated sodium carbonate solution. Ethyl acetate (6 ml) containing 0.05 M TEA was then added to each sample and the tubes were capped. Samples were vortex mixed for 10 sec, extracted with a slow rotary motion on a rotary shaker for 20 min, cooled to -20°C for 10 min (to break any emulsion formed during mixing), and then centrifuged at 3000 g for 10 min. The top organic ethyl acetate layer was separated, transferred to a clean set of tubes, and evaporated to dryness under a gentle stream of nitrogen at 25°C using a Zymark Turbo Vap® LV Evaporator (Zymark Corporation, Hopkinton, MA, USA). The residue was reconstituted in 200 µl of a 9:1 mixture of acetonitrile:water and the tubes were vortex mixed for 30 sec. Samples were transferred to HPLC autosampler vials with 0.35 ml disposable glass inserts and a 10 µl volume was injected into the HPLC.

### 2.6.1.4 High Performance Liquid Chromatography
The samples were chromatographed on a HP 1090 II LC using a 100 mm x 2.0 mm ID, 5 μm YMC amino (-NH₂) column (YMC, Inc., Wilmington, NC, U.S.A.) employing normal-phase chromatography. Precolumn filters, with replaceable 2 μm frits, were installed in the LC between the sample loop and the column. Gradient elution was used to achieve a quick run time as well as optimal retention of the compounds on the HPLC column. The chromatographic run began with a 95:5 mixture of acetonitrile: 2 mM ammonium acetate buffer containing 1% glacial acetic acid (pH 3.0). The proportion of the aqueous buffer was increased to 25% in a 6 min linear gradient, held for 0.5 min, brought back to the initial 95:5 proportion at 7.0 min, and again held for 3 min before the next injection. The mobile phase flow rate was 0.4 ml/min with a 50:50 split to the mass-spectrometer and waste. This HPLC procedure resulted in a total run time of 10 min for all 5 compounds of interest.

2.6.1.5 Electrospray Tandem Mass-Spectrometry (MS/MS)

The effluent from the HPLC column was split, and 50% (0.2 ml/min) was introduced into the Fisons VG Quattro I Triple Quad Tandem-Mass Spectrometer for detection of the analytes. Nitrogen was used as the nebulizing and bath gas. The compounds were ionized in the positive ion electrospray mode and detected using multiple reaction monitoring (MRM). The ion transitions monitored were m/z 256 → m/z 167 (DPHM), m/z 266 → m/z 177 ([²H₁₀]-DPHM), m/z 272 → m/z 167 (DPHMNOX), m/z 282 → m/z 177 ([²H₁₀]-DPHMNOX), and m/z 270 → m/z 181 (orphenadrine). These transitions were selected based on the predominant fragmentation pattern of various compounds in their daughter ion spectra (Figure 2.1). The dwell time for each transition was set at 0.2
sec with an inter-channel delay of 20 milliseconds to provide optimal sampling of each peak of interest (12-15 scans/peak). Collision induced dissociation (CID) was achieved with argon at a pressure of $3 \times 10^{-4}$ mbar in the collision cell. For maximal sensitivity, the collision energy, ion source temperature and cone voltage of the mass-spectrometer were optimized at 70 eV, 110 °C, and 30 V, respectively.

2.6.1.6 Calibration Curves and the Regression Model

Calibration standards (at concentrations of 0.2, 0.5, 1.0, 2.5, 5.0, 10.0, 25.0, 50.0, 125.0 and 250.0 ng/ml for both amines, and at 0.4, 1.0, 2.0, 4.0, 10.0, 20.0, 50.0 and 100.0 ng/ml for both N-oxides) were prepared by adding appropriate amounts of the prepared standard stock solutions to 1 ml of blank ovine plasma or urine. The I.S. (25.0 ng orphenadrine) was then added to each sample and the samples were extracted and analyzed using LC-MS/MS as described above. Weighted linear regression (weighting factor $= 1/y^2$) was performed between the ratio of peak area of each analyte to that of the I.S. vs. the corresponding spiked concentration in order to reduce bias at the lower concentrations. Linearity of calibration curves was demonstrated by calculating the regression bias. This was accomplished by analyzing 6 sets of calibration curve samples and back calculating the concentration of each standard from the obtained slope, intercept and the peak area ratios. The bias (%) was calculated as:

$$\% \text{ Bias} = \frac{\text{Back Calculated Concentration} - \text{Nominal Concentration}}{\text{Nominal Concentration}} \times 100$$

A bias of $< \pm 15\%$ at each concentration was considered evidence of linearity of the calibration curves.
2.6.1.7 Extraction Recovery

Absolute recoveries of all analytes in plasma and urine were determined at four different concentrations (2.0, 5.0, 50.0, and 250.0 ng/ml for both amines, and 2.0, 5.0, 50.0 and 100.0 ng/ml for both N-oxides) representing the entire range of the calibration curves. Two sets of samples, the control group and the recovery group, were prepared. Both sets of samples were prepared by spiking 1.0 ml of blank ovine plasma or urine with 25.0 ng of I.S. In addition, the samples in the recovery group were also spiked with known amounts of analytes (as above) at this point. All the samples were then subjected to the extraction procedure described earlier. After drying of the ethyl acetate organic extract, the samples in the control group were also spiked with the above amounts of analytes from an aqueous analyte stock solution. These control group samples were again dried under a gentle stream of nitrogen at 25°C using the Zymark Turbo Vap® LV Evaporator. All samples were then reconstituted as described above (Section 2.6.1.3) and injected into the LC-MS/MS system. The concentrations of the analytes in the control and recovery group samples were measured against the extracted duplicate standard curves prepared in the corresponding biological matrix. The absolute recovery was calculated as the ratio of measured concentration of recovery samples to that of the corresponding control samples at each different analyte concentration.

2.6.1.8 Analyte Stability in Biological Fluid Samples

A number of tests were carried out in order to establish the stability of the analytes under the routine sample handling conditions in the lab. This included the following:
**Bench-Top Stability:** Blank plasma was spiked with 250.0 ng/ml of each amine and 100.0 ng/ml of each N-oxide. The samples were left on the bench-top overnight (12 h at ~25 °C) and processed the next day.

**Freeze-Thaw Stability:** Blank plasma was spiked with the analytes as above. The samples were repeatedly frozen (at -20°C) and thawed (on bench-top at ~28 °C) for a total of three cycles, and then analyzed for drug and metabolite concentration.

**Stability in Saturated Sodium Carbonate Solution:** Stability of the analytes in the saturated sodium carbonate solution was evaluated because some tertiary amine N-oxides are known to decompose in plasma after alkalinization (Hubbard *et al.*, 1985; Midha *et al.*, 1993; Lin *et al.*, 1994). Plasma samples spiked at the above concentrations were mixed with 0.5 ml of saturated sodium carbonate solution and left on the bench-top for 1 h. Samples were then processed using the described procedure.

**Autosampler Stability:** Processed samples on the autosampler tray were injected repeatedly 3 times during a 48 h period after extraction. The area counts of peaks and their ratios to those of the I.S. were evaluated.

**Freezer Stability:** Plasma samples were spiked with known concentrations of analytes as above, and processed after a 3 month storage at -20°C.

In all stability tests, the concentration of analytes in the samples was measured after appropriate treatment and compared to the nominal values. The analytes were considered “stable” if the measured concentration after the treatment was within ±10% of the nominal value.
Method Validation

Method validation was performed by evaluating the intra- and inter-assay variance and bias (inaccuracy) in the quantitation of quality control samples (QC's). The QC samples were prepared by spiking blank plasma or urine with analytes at concentrations representing the limit of quantitation, and low, medium and high range of the standard curve. Thus, for amines, QC's were prepared at concentrations of 0.2, 1.0, 5.0, 50.0 and 250.0 ng/ml (QC's 1-5, Tables 2.2 and 2.3), whereas those for N-oxides had concentrations of 0.4, 2.0, 20.0 and 100.0 ng/ml (QC's 2-5, Tables 2.2 and 2.3).

Intra-assay variance and bias were estimated by quantitating six QC's at each concentration using a duplicate standard curve in one batch. For inter-assay variance and bias, six batches of samples, each consisting of six QC's at each concentration and a duplicate standard curve were analyzed on six separate days.

The assay method was also independently cross-validated for the quantitation of DPHM and $[{}^{2}H_{10}]$-DPHM with our earlier GC-MS assay (Tonn et al., 1993). This was accomplished by comparing the results obtained from the two methods for the analysis of plasma samples spiked with 3 different concentrations (5.0, 50.0 and 250.0 ng/ml) of DPHM and $[{}^{2}H_{10}]$-DPHM. The cross-validation of the quantitation of N-oxide metabolites could not be performed because to our knowledge no methods have been reported for the measurement of these compounds in biological fluids.

Application of the Assay to a Sample Study of DPHM, $[{}^{2}H_{10}]$-DPHM, DPHMNOX and $[{}^{2}H_{10}]$-DPHMNOX Disposition in the Ovine Maternal-Fetal Unit
A pregnant sheep (125 d gestation, term 145 d) was surgically prepared under halothane anesthesia by placing fluid sampling polyvinyl catheters and other monitoring devices (e.g., ultrasonic blood flow probe) in maternal and fetal blood vessels as described earlier (Rurak et al., 1988). After a recovery period (4 days), an equimolar dose of DPHM (2.5 mg) and \([^{2}\text{H}_{10}]\)-DPHM (2.6 mg) was simultaneously administered as a bolus via the fetal lateral tarsal vein. Serial fetal (~2 ml each) and maternal (~ 4 ml each) femoral arterial blood samples were collected at 5, 10, 15, 20, 30, 40, 50, 60, 75, 90, 105, 120, 140, 160, 180, 210, 240, 300 and 360 min after drug administration. Plasma was separated by centrifugation and the plasma samples were stored in borosilicate glass tubes at -20°C until analysis. Blank plasma samples were also collected just before drug administration (-5 min) for use in calibration curve sample preparation. Maternal and fetal plasma samples were then analyzed for DPHM, \([^{2}\text{H}_{10}]\)-DPHM, DPHMNOX and \([^{2}\text{H}_{10}]\)-DPHMNOX concentrations using the LC-MS/MS method described above.

2.6.2 Results and Discussion

The ability to simultaneously administer unlabeled and stable-isotope labeled drugs and metabolites has significantly improved our ability to study maternal-fetal drug disposition in a scientifically unbiased, efficient and cost-effective way (Tonn et al., 1996; Kumar et al., 1997; also see Chapter 3). The focus of studies described in this thesis was to study the factors affecting fetal drug exposure and to elucidate the in-utero fetal development and functional capacity of various drug-metabolizing enzyme systems as compared to the adult. We planned to achieve this by studying in vivo maternal-fetal drug pharmacokinetics and metabolite formation using a combination of stable-isotope
labeled compounds and mass-spectrometry. As mentioned previously, the GC-MS assays to quantitate DPHM, DPMA (major DPHM metabolite in many species), and their corresponding deuterated analogs (\([^{2}H_{10}]\)-DPHM and \([^{2}H_{10}]\)-DPMA) were developed earlier in this lab. DPHM-N-oxide is also a major metabolite of DPHM in many species, accounting for \(~10-20\%\) of the administered dose (rat, dog, rhesus monkey) (Drach and Howell, 1968; Drach et al, 1970). Hence, we wished to examine the relative capacity of this pathway in maternal and fetal sheep. The rationale for studying DPHM-N-oxide also lies with the fact that N-oxides are commonly formed via microsomal flavin-containing-monoxygenases (Poulsen and Ziegler, 1995; Cashman et al., 1995). In contrast to the cytochrome P450 and phase II conjugation enzymes, there is almost no information in the literature on the extent of development of these enzymes and the pharmacokinetics of tertiary amine N-oxides in the fetus of any species.

The study of the DPHM N-oxide metabolite in pregnant sheep required a rapid, sensitive and selective assay method capable of analyzing low concentrations (in the range of ng/ml) of DPHMNOX and its stable-isotope labeled analog, \([^{2}H_{10}]\)-DPHMNOX, in biological fluids (e.g., plasma, urine). Since the volume of fluids (e.g., blood) that can be sampled from the fetus is limited, we felt it would be advantageous to include the parent drug as well as its deuterated analog in this assay so as to be able to analyze all these compounds simultaneously in a single run. The tertiary-amine-N-oxide metabolites are generally unstable at the high temperatures encountered in gas-chromatography (Midha et al., 1991). The LC-MS/MS technique offers an obvious choice for the analysis of such compounds in biological matrices due to its sensitivity, selectivity, and chromatography and ionization at a relatively lower temperature. Hence, we developed and validated an LC-MS/MS method for simultaneous
quantitation of DPHM, DPHMNOX and their deuterium-labeled analogues in plasma and urine samples obtained from chronically-instrumented pregnant sheep.

2.6.2.1 High Performance Liquid Chromatography of Diphenhydramine and the N-Oxide Metabolite

Simultaneous analysis of the tertiary amine, diphenhydramine (DPHM and [2H10]-DPHM), and its N-oxide metabolite (DPHMNOX and [2H10]-DPHMNOX), in a single run using LC-MS/MS, presented an interesting analytical challenge. The parent amines and their N-oxide metabolites have widely differing polarities. In general, tertiary amine N-oxides are some of the most polar drug metabolites, whereas the parent amines have a predominantly lipophilic character (Midha et al., 1991). This presented us with some difficulties with respect to the choice of a single stationary phase for the optimal chromatographic retention of both the parent drug and the metabolites. The N-oxide metabolites exhibited little retention on many conventional reversed phase columns such as C18, C8, C2, Phenyl, and Cyano. The extreme reversed-phase conditions (>99% aqueous content in the mobile phase) necessary for only a minimal retention of the N-oxides led to severe "sticking" of the parent amines to the column and subsequent slow elution. This resulted in extremely long run times, severe peak tailing for the parent amines, and also "carry-over" problems. Since, reversed phase chromatography utilizes interactions between the lipophilic moieties of the analytes with the nonpolar stationary phase for analyte retention on the HPLC column, we concluded that the N-oxide metabolites do not exhibit sufficient lipophilic character for optimal interaction with these nonpolar stationary phases.
Some investigators have used "ion-pairing" as an approach to improve the retention of tertiary amine N-oxides on reversed phase columns in HPLC analytical methods with ultraviolet detection (Koyoma et al., 1993). This, however, was not possible with our method because electrospray mass-spectrometry prohibits the use of non-volatile additives such as ion-pairing agents. This led us to consider the use of normal phase chromatography in order to utilize the polar component of the analyte molecules for their interaction with and retention on a relatively polar column. For normal phase chromatography, the propyl amino (-NH₂) phase provides a useful alternative to silica. In contrast to silica, the -NH₂ phase is compatible with the aqueous components of the mobile phase. This stationary phase essentially allows the use of the same solvents as in traditional reversed phase chromatography (in different proportions compared to reversed phase mode, with water being the stronger solvent) without any restriction to purely organic mobile phases (e.g., hexane, dichloromethane) as is the case with silica. This is important because a complete lack of an aqueous or buffer component in the mobile phase could lead to inadequate ionization of the analytes during the electrospray ionization process in the mass spectrometer.

In our experiments, both the parent amines and their N-oxide metabolites exhibited excellent retention on a relatively short (column length 10 cm) -NH₂ column (Figure 2.2B). However, due to greatly different polarities of the parent drug and metabolites, and in order to achieve a balance between adequate retention of all analytes and a rapid analysis time (~ 10-15 min), gradient elution was found to be necessary. In general, low mobile phase flow rates (~50-100 μl/min) provide maximal sensitivity in LC-MS/MS assays. However, it is difficult to use gradient elution at these low flow rates because of a relatively large void volume between the HPLC pump and the column (~1
ml in the HP 1090 series II chromatograph). Hence, we decided to use a higher mobile phase flow rate (0.4 ml/min) with a 50:50 split to the mass-spectrometer and waste. This provided sufficient sensitivity for all analytes for our purposes and a rapid run time (10 min) for all five compounds of interest (Figure 2.2B). Using normal phase chromatography and the described gradient time-table, the relatively lipophilic amines, orphenadrine, DPHM and $[^2\text{H}_{10}]$-DPHM, eluted earlier (at 3.06, 3.12, and 3.12 min, respectively) compared to the more polar N-oxides (at 5.27 min). The variations in retention times of different analytes within a single run were <5%. Although the two groups of compounds (tertiary amines and N-oxides) were well resolved from each other, it was neither possible nor necessary to chromatographically resolve different compounds within the amine or the N-oxide group (Figure 2.2B; also see below).

Tertiary amine drugs may exhibit severe peak-tailing on many reversed phase columns. However, the use of an -NH$_2$ column, in addition to providing optimal retention for all the compounds, also resulted in excellent symmetrical peak shape (Figure 2.2B). Use of the acidic ammonium acetate buffer, pH 3.0 (2 mM ammonium acetate and 1% acetic acid) instead of pure water led to an improvement in peak shape for all the compounds and significantly reduced peak tailing for the parent amines.

**2.6.2.2 Tandem Mass Spectrometric (MS/MS) Detection of Analytes**

Figure 2.1 shows daughter ion mass spectra of all the analytes and the internal standard, orphenadrine, in the positive ion electrospray mode. All analytes appear to predominantly fragment at the ether linkage on the aliphatic side chain of the molecules, thus forming analogous daughter ions. Some additional fragmentation also takes place
and the corresponding m/z assignments are depicted in Figure 2.1. Our initial attempts to quantitate these compounds involved the use of single ion recording (SIR) by monitoring the [M+H]^+ ion for each analyte in the first quadrupole of the mass spectrometer. However, for some ions, this resulted in significant cross-over of signal from one channel to the other (e.g., m/z 266 and m/z 270 for [\(^2\)H\(_{10}\)]-DPHM and orphenadrine, respectively), especially at higher analyte concentrations. Mass-spectrometric resolution of these interferences was important because it was not possible to chromatographically resolve many of these compounds from one another due to very subtle differences in their chemical structure (Figures 2.1 and 2.2). The use of multiple reaction monitoring mode (MRM) helped to eliminate these interferences by utilizing the enhanced selectivity of MS/MS detection as compared to the single mass spectrometer configuration in SIR. Also, the MRM mode provided much cleaner baselines compared to SIR, leading to an increase in signal to noise ratio. The molecular ion [M+H]^+ and the daughter ion formed by fragmentation at the ether linkage are the major ions in the mass spectra of all the analytes (Figure 2.1) and would be expected to provide maximal sensitivity for the analysis of these compounds. Based on this, the mass transitions of m/z 256 → m/z 167 (DPHM), m/z 266 → m/z 177 ([\(^2\)H\(_{10}\)]-DPHM), m/z 272 → m/z 167 (DPHMNOX), m/z 282 → m/z 177 ([\(^2\)H\(_{10}\)]-DPHMNOX), and m/z 270 → m/z 181 (orphenadrine) were selected for the detection of the compounds of interest (Figures 2.1 and 2.2).

A number of mass-spectrometric parameters such as ion source temperature, argon pressure in the collision cell, collision energy of the collision gas, and cone voltage were optimized to achieve maximal sensitivity. In general, lower ion source temperatures would be safer in terms of the stability of the N-oxide metabolites. In our experiments,
Figure 2.1 - Positive ion electrospray daughter ion mass-spectra of DPHM, $[^2\text{H}_{10}]$DPHM, DPHM-N-Oxide, $[^2\text{H}_{10}]$DPHM-N-Oxide, and the internal standard, orphenadrine.
Figure 2.2 - LC-MS/MS MRM ion chromatograms of: (A) an extracted blank sheep plasma sample, and (B) an extracted plasma calibration standard containing 0.4 ng/ml each of $^2$H$_{10}$-DPHM-N-oxide and DPHM-N-oxide, and 1 ng/ml each of $^2$H$_{10}$-DPHM and DPHM. The y-axis scales in panel (A) have been magnified to clearly show the baselines at different MRM ion transitions. The HPLC and MS/MS conditions and specifications are described in the text.
lower temperatures (80 - 90°C), however, resulted in a loss of sensitivity and deposition of material in the mass spectrometer, thus requiring frequent cleaning of the ion source. An increase in ion source temperature beyond 110°C did not offer any significant increase in sensitivity and the N-oxide metabolites appeared to decompose at temperatures higher than 150°C. Thus, an ion source temperature of 110°C was considered optimal for final analysis. Similarly, the argon pressure in the collision cell, the collision energy of the argon molecules and the cone voltage were optimized for maximal sensitivity at 3 X 10^-4 mbar, 70 eV and 30 V, respectively.

2.6.2.3 Extraction Method and Recovery

A number of organic solvents and solvent mixtures such as dichloromethane, dichloromethane containing 2% isopropanol, hexane containing 2% isopropanol, toluene, and ethyl acetate were evaluated for maximizing the extraction recovery of the analytes. Triethylamine (TEA; 0.05 M) was included in all the solvent systems in order to prevent nonspecific binding of the analytes to glassware (Tonn et al., 1993) (also see below). All solvent systems were very efficient in terms of extracting the parent amines. However, due to the extreme polarity of the N-oxide metabolites, their recoveries were low (ranging from <20% for the hexane-2% isopropanol mixture to ~50% for the dichloromethane-2% isopropanol mixture). Extraction with an ethyl acetate-0.05 M TEA mixture provided maximal recoveries for the N-oxide metabolites and also resulted in clean extracts devoid of any chromatographic or mass-spectrometric interference from the biological matrix (Figure 2.2A). Thus, the ethyl acetate-0.05 M TEA mixture was chosen as the final extraction solvent. The recoveries for all the analytes were consistent (coefficient of variation <10% for the parent amines and <15% for the N-
oxides) and independent of the analyte concentration; hence an overall mean recovery was calculated. The mean recoveries for DPHM, [\(^{2}\text{H}_{10}\)]DPHM, DPHMNOX and [\(^{2}\text{H}_{10}\)]DPHMNOX from ovine plasma were 79.7 ± 6.6 %, 76.6 ± 6.6 %, 71.6 ± 9.4 % and 69.0 ± 8.3 %, respectively. Similar recoveries were obtained for the extraction of urine samples.

Earlier we observed a dramatic improvement in DPHM and [\(^{2}\text{H}_{10}\)]DPHM extraction recovery by the addition of 0.05M TEA to a hexane and 2% isopropanol solvent mixture, possibly due to a reduction in nonspecific binding of the tertiary amines to active sites on glassware (Tonn et al., 1993). A similar phenomenon was observed with ethyl acetate extraction; the recovery of all analytes was reduced by ~20-30% when 0.05M TEA was not included in the extraction solvent.

Some investigators have demonstrated decomposition of the N-oxide metabolites in plasma on alkalinization with sodium hydroxide (Hubbard et al., 1985; Midha et al., 1991; Lin et al., 1994). These metabolites, however, appear to be stable with the use of saturated sodium carbonate solution (Hubbard et al., 1985; Lin et al., 1994). Hence, we decided to use a saturated sodium carbonate solution for alkalinization of samples in this method. The DPHM N-oxide metabolites were found to be stable in sodium carbonate (see below). Also, the use of a saturated sodium carbonate solution resulted in cleaner extracts as compared to sodium hydroxide as evidenced by a reduction in baseline noise.
Analyte stability in biological samples under conditions encountered in their routine handling is of paramount importance in order to accurately study drug disposition. Thus, a number of studies were conducted to evaluate stability of the analytes in biological fluid samples under conditions simulated to match routine sample handling. The mean analyte concentrations measured in samples subjected to a 12 h bench-top stability test, 3 freeze-thaw cycles, 1 h exposure to saturated sodium carbonate solution, and a 3 month freezer storage at -20 °C were found to be within ±10% of the nominal concentrations with an acceptable coefficient of variation (<10%; according to acceptability standards established for bioanalytical assays by Shah et al., 1992). There was no change in analyte to I.S. peak area ratios of various compounds during repeated injections of the processed samples on the autosampler tray for up to 48 h after extraction. Based on these results, it was concluded that the analytes would be sufficiently stable in biological samples during actual freezer storage and analysis conditions.

Calibration Curves and the Regression Model

The calibration curves for all the analytes showed good linearity in the concentration range tested (0.2 - 250.0 ng/ml for parent amines and 0.4 - 100.0 ng/ml for N-oxides). Representative calibration curves of all 4 analytes are shown in Figure 2.3. Weighted linear regression (weighting factor = 1/y^2) was performed on all the calibration curve data in order to reduce bias at the lower concentrations. This weighting function resulted in an acceptable regression bias (Shah et al., 1992) for all analytes at the lower
as well as the upper limits of the calibration curves (-11.6 % and +1.5 % for DPHM; -11.3% and -1.5% for [²H₁₀]-DPHM; -7.1% and +3.0% for DPHMNOX; +9.4% and -8.6% for [²H₁₀]-DPHMNOX at the lower and upper end of the calibration curve, respectively), again indicating good linearity. The peak response for the N-oxides was nonlinear at concentrations greater than 100.0 ng/ml. This could be due to fact that at higher concentrations, partial dimerization of the N-oxides was observed. The slopes of the [²H₁₀]-DPHM and [²H₁₀]-DPHMNOX standard curves were consistently higher compared to those of DPHM and DPHMNOX, respectively. This indicates that the fragmentation patterns of the compounds may have been quantitatively altered by the presence of deuterium labels.

The LOQ's of 0.2 ng/ml and 0.4 ng/ml were established for the parent amines and N-oxides, respectively, based on a signal to noise ratio of at least 20 and an acceptable variability and bias (Shah et al., 1992) at this concentration (see below). The LOQ of 0.2 ng/ml for DPHM and [²H₁₀]-DPHM achieved in this assay method is an order of magnitude lower than that of our earlier GC-MS method (2.0 ng/ml) (Tonn et al., 1993) indicating a significant improvement in sensitivity.

2.6.2.6 Method Validation

The validation of the assay involved estimation of intra- and inter-assay variability and bias in ovine plasma and urine. The results from plasma validation are presented in Tables 2.2 and 2.3. Variability and bias data in urine were similar and are not presented here. The intra- and inter-assay variabilities (CV's) for all analytes were ≤15% below a concentration of 2.0 ng/ml and < 10% at all other concentrations. The mean intra- and
Figure 2.3 – Representative calibration curves of: (A) DPHM and $[^2\text{H}_{10}]$-DPHM, and (B) DPHM-N-oxide and $[^2\text{H}_{10}]$-DPHM-N-oxide, in sheep plasma.
inter-assay bias (inaccuracies) ranged from -6% to +12% of the nominal concentration for the amines and from -10% to +14% for the N-oxides over the calibration curve concentration range (Tables 2.2 and 2.3).

Table 2.2 - Intra-assay variability and bias of the LC-MS/MS analytical method for DPHM, \([^{2}\text{H}_{10}]\)-DPHM, DPHM-N-oxide and \([^{2}\text{H}_{10}]\)-DPHM-N-oxide in ovine plasma.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>QC-1</th>
<th>QC-2</th>
<th>QC-3</th>
<th>QC-4</th>
<th>QC-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nominal Concentrationa</td>
<td>0.20</td>
<td>1.0</td>
<td>5.0</td>
<td>50.0</td>
<td>250.0</td>
</tr>
<tr>
<td>Measured Concentrationa</td>
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<td>1.04</td>
<td>5.0</td>
<td>55.3</td>
<td>239.9</td>
</tr>
<tr>
<td>S.D.</td>
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<td>0.05</td>
<td>0.32</td>
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<td>6.8</td>
</tr>
<tr>
<td>C.V. (%)</td>
<td>9.7</td>
<td>5.1</td>
<td>6.4</td>
<td>3.3</td>
<td>2.8</td>
</tr>
<tr>
<td>Bias (%)</td>
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<td>+3.8</td>
<td>+0.2</td>
<td>+10.6</td>
<td>-4.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Analyte</th>
<th>QC-1</th>
<th>QC-2</th>
<th>QC-3</th>
<th>QC-4</th>
<th>QC-5</th>
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<tr>
<td>Nominal Concentrationa</td>
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<td>1.0</td>
<td>5.0</td>
<td>50.0</td>
<td>250.0</td>
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<td>S.D.</td>
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<td>0.05</td>
<td>0.25</td>
<td>1.3</td>
<td>8.7</td>
</tr>
<tr>
<td>C.V. (%)</td>
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<td>4.4</td>
<td>5.0</td>
<td>2.3</td>
<td>3.6</td>
</tr>
<tr>
<td>Bias (%)</td>
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<td>+0.8</td>
<td>+12.0</td>
<td>-3.4</td>
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<table>
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<th>QC-3</th>
<th>QC-4</th>
<th>QC-5</th>
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<td>C.V. (%)</td>
<td>13.3</td>
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<td>9.6</td>
<td>7.0</td>
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</tr>
<tr>
<td>Bias (%)</td>
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<td>+14.0</td>
<td>+9.2</td>
<td>-5.3</td>
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</table>

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<tr>
<th>Analyte</th>
<th>QC-1</th>
<th>QC-2</th>
<th>QC-3</th>
<th>QC-4</th>
<th>QC-5</th>
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<td>94.9</td>
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<tr>
<td>Measured Concentrationa</td>
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<td>2.09</td>
<td>21.7</td>
<td>94.9</td>
<td></td>
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<td>8.3</td>
<td>5.0</td>
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</tr>
<tr>
<td>Bias (%)</td>
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<td>+4.4</td>
<td>+8.6</td>
<td>-5.1</td>
<td></td>
</tr>
</tbody>
</table>

^a – All concentrations are in ng/ml.

The cross-validation of the LC-MS/MS assay with our earlier GC/MS method yielded excellent agreement between the two methods. The concentrations of DPHM and
[²H₁₀]-DPHM measured in 1.0 ml aliquots of the plasma samples spiked at 5.0, 50.0 and 250.0 ng/ml by the two analysis methods were highly correlated (Pearson correlation coefficient, r = 1.0 at all the concentrations) and were not significantly different from each other (unpaired t-test, p>0.05). This indicates that the plasma concentrations of both the parent amines can be measured with a high degree of confidence by either of these two methods. However, as discussed above, the current method offers the

Table 2.3 - Inter-assay variability and bias of the LC-MS/MS analytical method for DPHM, [²H₁₀]-DPHM, DPHM-N-oxide and [²H₁₀]-DPHM-N-oxide in ovine plasma.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>QC-1</th>
<th>QC-2</th>
<th>QC-3</th>
<th>QC-4</th>
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<td></td>
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<tr>
<td>Nominal Concentration</td>
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<td>1.0</td>
<td>5.0</td>
<td>50.0</td>
<td>250.0</td>
</tr>
<tr>
<td>Measured Concentration*</td>
<td>0.21</td>
<td>1.06</td>
<td>5.5</td>
<td>54.5</td>
<td>237.7</td>
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<td>0.04</td>
<td>0.3</td>
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</tr>
<tr>
<td>C.V. (%)</td>
<td>5.6</td>
<td>3.5</td>
<td>5.9</td>
<td>1.7</td>
<td>1.8</td>
</tr>
<tr>
<td>Bias (%)</td>
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<td>-4.9</td>
</tr>
<tr>
<td>[²H₁₀]-DPHM</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nominal Concentration</td>
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<td>1.0</td>
<td>5.0</td>
<td>50.0</td>
<td>250.0</td>
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<tr>
<td>Measured Concentration*</td>
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<td>5.5</td>
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<td>1.9</td>
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<tr>
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<td>+8.8</td>
<td>+10.1</td>
<td>+9.6</td>
<td>-4.7</td>
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<tr>
<td>DPHM-N-oxide</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nominal Concentration</td>
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<td>2.0</td>
<td>20.0</td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td>Measured Concentration*</td>
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<td>3.3</td>
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<tr>
<td>C.V. (%)</td>
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<td>6.1</td>
<td>2.8</td>
<td>3.6</td>
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<tr>
<td>Bias (%)</td>
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<td>+12.0</td>
<td>+10.4</td>
<td>-9.1</td>
<td></td>
</tr>
<tr>
<td>[²H₁₀]-DPHM-N-oxide</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nominal Concentration</td>
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<td>20.0</td>
<td>100.0</td>
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<tr>
<td>Measured Concentration*</td>
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<tr>
<td>C.V. (%)</td>
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<td>12.2</td>
<td>2.8</td>
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<td></td>
</tr>
<tr>
<td>Bias (%)</td>
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<td>+0.9</td>
<td>+10.4</td>
<td>-8.0</td>
<td></td>
</tr>
</tbody>
</table>

a – All concentrations are in ng/ml.
advantage of a lower LOQ (0.2 ng/ml vs. 2.0 ng/ml) compared to the earlier method. In addition, this method can also simultaneously quantitate the N-oxide metabolite of the drug with good sensitivity and selectivity.

2.6.2.7 Application of the Assay to a Sample Study of DPHM, $[^2\text{H}_{10}]$-DPHM, DPHMNOX and $[^2\text{H}_{10}]$-DPHMNOX Disposition in the Ovine Maternal-Fetal Unit

The developed assay was applied to a pharmacokinetic study in one chronically-catheterized pregnant sheep, designed to test the bioequivalency of DPHM and $[^2\text{H}_{10}]$-DPHM in the fetal lamb in terms of the parent amine disposition, formation of DPHMNOX and $[^2\text{H}_{10}]$-DPHMNOX from the two compounds, and the clearance of DPHMNOX and $[^2\text{H}_{10}]$-DPHMNOX. This bioequivalency is extremely important in order to obtain meaningful pharmacokinetic data by utilizing the stable-isotope labeled drug. Figure 2.4 shows the fetal femoral arterial plasma profiles of DPHM, $[^2\text{H}_{10}]$-DPHM, DPHMNOX, and $[^2\text{H}_{10}]$-DPHMNOX after simultaneous equimolar fetal intravenous bolus administration of DPHM (2.5 mg) and $[^2\text{H}_{10}]$-DPHM (2.6 mg). These data show that plasma concentrations of DPHM and $[^2\text{H}_{10}]$-DPHM decline rapidly after drug administration and are virtually superimposable. This indicates approximately equal rates of clearance of these two compounds from the fetal circulation. Also, the corresponding N-oxide metabolites (DPHMNOX and $[^2\text{H}_{10}]$-DPHMNOX) were detectable in fetal plasma (Figure 2.4). The concentration of metabolites increased gradually and then declined rapidly in parallel with the parent drug. Similar to the parent drugs, the concentrations of the two metabolites were virtually identical indicating a lack of any effect of the deuterium labels on this metabolic pathway. Thus, these data demonstrate the absence of any isotope-effect in the disposition of $[^2\text{H}_{10}]$-DPHM compared to DPHM,
and also for $[^2\text{H}_{10}]$-DPHMNOX compared to DPHMNOX. In addition to fetal plasma, the maternal femoral arterial plasma samples collected at the corresponding time points were also analyzed for the labeled and unlabeled parent drug and the metabolite. However, all compounds were present at much lower concentrations (near LOQ) compared to the fetal plasma. The higher plasma concentrations of the N-oxide metabolites in fetal plasma compared to maternal plasma indicate the ability of the fetus to form this metabolite after DPHM and $[^2\text{H}_{10}]$-DPHM administration (also see Chapter 3).
In conclusion, we have developed and validated an LC-MS/MS assay for the simultaneous quantitation of DPHM, [\textsuperscript{2}H\textsubscript{10}]DPHM, DPHMNOX and [\textsuperscript{2}H\textsubscript{10}]DPHMNOX in plasma and urine samples obtained from chronically-instrumented pregnant sheep. The assay is rapid (fast sample processing, a 10 min run time), sensitive and selective (no interference from biological matrices, LOQ's of 0.2 and 0.4 ng/ml for amines and N-oxides, respectively) and robust (acceptable variability and bias, sample stability). This assay and three previously developed GC-MS analytical methods (for DPHM and [\textsuperscript{2}H\textsubscript{10}]DPHM, DPMA and [\textsuperscript{2}H\textsubscript{10}]DPMA, and VPA and its metabolites) were utilized in the studies of DPHM and VPA disposition in pregnant sheep presented in Chapters 3-5 of this thesis.
Chapter 3

Organs and Metabolic Pathways of Diphenhydramine Clearance in Maternal and Fetal Sheep

A number of studies were conducted to elucidate the role of different organs and metabolic pathways in maternal and fetal DPHM clearance. Firstly, as discussed in the Introduction section (Chapter 1), we felt that there could be some confounding factors associated with the geometry of the fetal circulation and its blood flow patterns that might have led to the earlier conclusion of no apparent DPHM uptake by the fetal liver. In order to reexamine this issue, additional data were obtained from the fetal plasma samples collected during the previous study of fetal hepatic first-pass uptake of DPHM from the umbilical venous blood (Tonn et al., 1996). Subsequent to the demonstration of significant fetal hepatic uptake of DPHM, the impact of this phenomenon on the estimation of maternal and fetal placental and non-placental clearances of DPHM, and also of other drugs in general, was assessed. Hence, maternal and fetal DPHM clearance and metabolite (DPMA) formation data obtained during an earlier study, where DPHM and $[^{2}H_{10}]$-DPHM were infused simultaneously but separately to the mother and the fetus, respectively (Tonn, 1995), are also presented in the context of fetal hepatic uptake of the drug from the umbilical vein.

In the previous study (Tonn et al., 1996), a high hepatic first-pass extraction of DPHM was demonstrated in adult non-pregnant sheep after simultaneous but separate administration of DPHM and $[^{2}H_{10}]$-DPHM via the portal venous (p.v.) and i.v. routes. During the current studies, the adult sheep plasma samples collected during the previous study were used to obtain additional metabolite (DPMA and $[^{2}H_{10}]$-DPMA)
plasma concentration data. A detailed theoretical analysis of these metabolite data in combination with the previous parent drug concentration data are presented later in this chapter to provide evidence for a significant role of the gut in DPHM systemic clearance in adult sheep. Additional studies were subsequently conducted to directly confirm this gut uptake of DPHM in adult sheep.

In terms of the pathways of DPHM clearance, studies were conducted to examine the role of DPMA and DPHM-N-oxide metabolites (two major pathways of DPHM clearance in many species) in maternal and fetal DPHM elimination.

### 3.1 Methods

#### 3.1.1 Animals and Surgical Preparation

All studies described in this thesis 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. In order to obtain time-dated pregnant sheep, ewes’ estrus cycle was synchronized by inhibition of their spontaneous ovulation. This was accomplished by the administration of medroxyprogesterone acetate for two weeks via an intravaginal pessary (Veramix Sheep Sponge®). At the end of this two-week period, ovulation was induced by an intramuscular injection of 500 I.U. pregnant mares' serum gonadotropin. The ewes were then placed with a ram for 1-2 days to result in time-dated pregnancies. Animals were typically brought into the research facility at least 3-4 days prior to surgery for their acclimatization and were kept indoors in large pens in the company of other sheep. A standard diet and free access to water was provided to all
sheep. The surgical preparation of animals employed for different studies presented in this chapter is described below:

3.1.1.1 Study A: Paired Maternal (DPHM) and Fetal \([^{2}\text{H}_{10}]-\text{DPHM}\) Infusions for the Determination of Placental and Non-Placental Clearances

Five pregnant Dorset Suffolk cross-bred ewes, with a maternal body weight of 70.5 ± 7.7 kg (mean ± S.D.), were surgically prepared between 119-127 d gestation (122 ± 2 d, term ~145 d). 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 \textit{i.v.} dose of atropine was administered \textit{via} the jugular vein to control salivation. Anesthesia was induced by \textit{i.v.} administration of 1 g pentothal sodium \textit{via} the jugular vein. The ewe was immediately intubated and maintained on halothane (1-2%) and nitrous oxide (70%) in oxygen anesthesia. An \textit{i.v.} infusion of 500 ml 5% dextrose solution containing 500 mg ampicillin was administered over ~45-60 min \textit{via} the jugular vein. A midline abdominal incision was made after cleansing and disinfecting the ewe's abdomen with 10% povidone-iodine solution, and the uterus was exposed. Access to the head and hind quarters of the fetus was gained \textit{via} two separate uterine incisions made carefully in the areas devoid of major blood vessels and placental cotyledons. Polyvinyl or silicone rubber catheters (Dow Corning, Midland, MI) were implanted in both fetal femoral arteries and lateral tarsal veins, a fetal carotid artery, fetal trachea, fetal urinary bladder (\textit{via} a suprapubic incision), and the amniotic cavity (catheter i.d. 1.02 mm and o.d. 2.16 mm). Electrodes (Cooper Corporation, Chatsworth, CA) were implanted biparietally on the dura to record the fetal electrocorticogram (ECoG). In four animals, a transit-time 4SB blood flow transducer (Transonic Systems, Inc., Ithaca, NY) was placed around the common umbilical artery to measure umbilical blood flow. The
amniotic fluid lost during surgery was replaced with warm sterile irrigation saline and the uterine and abdominal incisions were closed in layers. Catheters were also implanted in a maternal femoral artery and vein (catheter i.d. 1.02 mm and o.d. 2.16 mm).

3.1.1.2 Study B: Study of Fetal Hepatic First-Pass Uptake of DPHM from the Umbilical Vein

Additional data were obtained from the fetal plasma samples from eight animals out of a total of 11 employed in the previous study (Tonn et al., 1996). The surgical preparation for these animals was similar to that described above, with the exception that fetal and maternal bladder catheters and electrodes for monitoring fetal behavior were not implanted.

3.1.1.3 Study C: Hepatic Uptake of DPHM in Adult Non-Pregnant Sheep

A total of five adult non-pregnant sheep (body weight 70.1 ± 10.5 kg) were employed in these studies. Aseptic surgical procedures, similar to those described above for pregnant sheep, were also employed for non-pregnant sheep. Femoral arterial and venous catheters were implanted in all five sheep. In addition, a catheter was implanted in a branch of one of the mesenteric veins with the catheter tip advanced to the main mesenteric vein in the direction of the hepatic portal vein.

3.1.1.4 Study D: Gut Uptake of DPHM from the Systemic Circulation in Adult Non-Pregnant Sheep

A total of four adult non-pregnant sheep (body weight 64.0 ± 7.9 kg) were used for these studies. Femoral arterial and venous catheters were implanted as above. In addition, a
catheter was implanted in the main hepatic portal venous trunk just prior to its entry into
the liver, as described below. A longitudinal abdominal incision was made in order to gain
access to various compartments of the ruminant sheep stomach. A prominent branch of
the main gastric vein was identified either on the surface of the rumen or the omasum, and
a segment of the intact vessel was then carefully isolated from the surface of the stomach
compartment. An ~12-18 inch length of a sterile polyvinyl catheter was then advanced into
this vessel towards the direction of liver, via the main gastric vein and into the hepatic
portal vein. The catheter was secured in place using sterile silk sutures and was anchored
to the surface of the rumen or the omasum. The position of the portal venous catheter
was verified in all animals by autopsy at the end of each experiment.

3.1.1.5 Study E: Contribution of DPMA Formation to DPHM Non-Placental
Clearance in Maternal and Fetal Sheep

A total of five pregnant Dorset-Suffolk cross-bred ewes, with a maternal body weight of
82.4 ± 14.1 kg (mean ± S.D.), were surgically prepared between 118-129 days gestation
for the purpose of these studies. Surgical procedure was similar to that describe above for
Study A. The sites where sterile polyvinyl catheters were implanted include both fetal
femoral arteries and lateral tarsal veins, and a maternal femoral artery and vein. Catheters
were also implanted in the fetal trachea, amniotic cavity and in three animals in the fetal
urinary bladder (via a suprapubic incision). Other physiological monitoring devices were
not implanted in these animals.

3.1.1.6 Study F: Disposition of the DPHM-N-oxide Metabolite in the Maternal-
Fetal Unit
Samples from four out of the five animals above in Study E were utilized for this part of the studies. Maternal and fetal plasma and urine concentrations of the DPHM-N-oxide metabolite were measured in these four animals.

In all animals, the catheters and/or blood flow probe cables and electrode cables for monitoring fetal behavior were tunneled subcutaneously and exteriorized via a small incision on the flank of the ewe, and were stored in a denim pouch when not in use. All catheters were flushed daily with approximately 3 ml of sterile 0.9% sodium chloride containing 12 units of heparin per ml to maintain their patency. Intramuscular injections of ampicillin 500 mg were given to the ewe on the day of surgery and for 3 days post-operatively. In pregnant animals, ampicillin (500 mg) was also given into the amniotic cavity immediately following surgery and daily thereafter for the duration of the preparation. Following surgery, animals were kept in holding pens with other sheep and were given free access to food and water. The sheep were allowed to recover for 3-8 days prior to experimentation. Following the recovery period, the sheep were moved to a monitoring pen adjacent to and in full view of the holding pen for experimentation purposes. In experiments requiring collection of maternal urine, a Foley® bladder catheter was inserted via the urethra of the ewe on the morning of the experiment and attached to a sterile polyvinyl bag for cumulative urine collection.

3.1.2 Experimental Protocols

3.1.2.1 Study A: Paired Maternal (DPHM) and Fetal ([$^2$H$_{10}$]-DPHM) Infusions for the Determination of Placental and Non-Placental Clearances
Experiments were conducted at 125-133 d gestation (128.8 ± 3.2 d) (term ~145 d). Simultaneous infusions of DPHM and [\(^{2}\)H\(_{10}\)]-DPHM to the ewe and fetus, respectively, were administered to all five sheep. DPHM was given as a 20 mg i.v. bolus loading dose over 1.0 min, followed immediately by an infusion of 670 \(\mu g/min\) via the maternal femoral vein. Simultaneously, a 5.0 mg i.v. bolus loading dose of [\(^{2}\)H\(_{10}\)]-DPHM was given via the fetal lateral tarsal vein over 1.0 min, followed by an infusion of the compound at 170 \(\mu g/min\). Simultaneous blood samples were collected from the fetal (1.5 ml) and maternal (3.0 ml) femoral arterial catheters at 30, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330, and 360 min during the infusion and at 30, 60, 120, 180, 240, 360 min and 8, 12, 18, 24, 30 and 40 h post-infusion. Fetal femoral arterial samples (0.6 ml) were also collected at the same time intervals for blood gas analysis and measurement of glucose and lactate concentrations. Fetal carotid and umbilical venous blood samples (1.5 ml) were collected at 30, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330, and 360 min during the infusion period. All fetal blood removed for sampling was replaced at intervals during the experiment by an equal volume of maternal blood obtained prior to the start of the experiment. Serial amniotic and tracheal fluid (3.0 ml) and cumulative maternal urine samples were obtained at 60, 120, 180, 240, 300, and 360 min during the infusion and at 60, 120, 180, 240, 360 min and 8, 12, 18, 24, 30 and 40 h post-infusion.

### Study B: Study of Fetal Hepatic First-Pass Uptake of DPHM from the Umbilical Vein

These experiments were conducted between 124-138 d gestation (128.1 ± 4.9 d). In order to assess the fetal first-pass hepatic uptake for DPHM from the umbilical vein, two different types of administration protocols were employed:
1. In four animals, simultaneous but separate randomized bolus injections of $[^{2}\text{H}_{10}]$-DPHM and DPHM (5.0 mg each) were administered via the common umbilical vein and fetal lateral tarsal vein (which drains directly into the inferior vena cava) (see Figure 1.3 for position of the administration routes relative to the fetal liver).

2. In the other four animals, 90 min i.v. infusions of the compounds (60 µg/min each, preceded by a 2.0 mg each i.v. bolus) via the same routes were employed (see Figure 1.3 for position of the administration routes relative to the fetal liver).

During bolus experiments, samples of fetal arterial blood (~2 ml) were collected at 5, 10, 15, 20, 30, 40, 50, 60, 75, 90, 105 min and 2, 2.5, 3, 3.5, 4, 5, 6, 8, 10 and 12 h after the drug administration. During infusion studies, fetal femoral arterial blood samples were collected at 5, 15, 30, 45, 60, 75 and 90 min after the beginning of infusion. In the present study, fetal plasma samples from the above fetal bolus (n=4) and infusion (n=4) experiments were used for the measurement of $[^{2}\text{H}_{10}]$-DPMA and DPMA concentrations. The parent drug plasma concentration data were taken from the previous study (Tonn et al., 1996).

3.1.2.3 Study C: Hepatic Uptake of DPHM in Adult Non-Pregnant Sheep

Equimolar amounts of DPHM and $[^{2}\text{H}_{10}]$-DPHM (equivalent to 50 mg DPHM) were administered simultaneously but separately via the femoral (intravenous or i.v. route) and mesenteric vein (portal venous or p.v. route) catheters in a randomized manner. Serial samples of femoral arterial plasma (~ 3 ml) were collected at 5, 10, 20, 30, and 40 min, and 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 10 and 12 h after drug injection.
3.1.2.4 Study D: Gut Uptake of DPHM from the Systemic Circulation in Adult Non-Pregnant Sheep

DPHM gut uptake was measured under steady-state conditions in four adult sheep with implanted portal venous catheters. A 20 mg \textit{i.v.} bolus loading dose of DPHM was administered at the beginning of the experiment \textit{via} the femoral venous catheter. This was followed immediately by an infusion of unlabeled DPHM at a rate of 670 $\mu$g/min for 6 h \textit{via} the same route. Simultaneous femoral arterial (before the gut) and portal venous (after the gut) blood samples (~3 ml each) were collected every hour for the entire duration of DPHM infusion (6 h) in order to estimate the steady-state gut extraction ratio of the drug.

3.1.2.5 Study E: Contribution of DPMA Formation to DPHM Non-Placental Clearance in Maternal and Fetal Sheep

All experiments were conducted between 124-140 d gestation. Two sets of experiments were carried out on all five pregnant sheep.

1. \textbf{Maternal Administration Experiments}: In three animals (E2174, E4227 and E4230), a 6 h steady-state DPHM infusion at 670 $\mu$g/min, combined with initial 20.0 mg DPHM and 2.5 mg $[^2\text{H}_{10}]$-DPMA boluses, was administered \textit{via} the maternal femoral vein catheter. In the other two animals (E1225A and E303Y), the isotope labels on the compounds were reversed, \textit{i.e.}, $[^2\text{H}_{10}]$-DPHM infusion (at 670 $\mu$g/min) was administered in combination with $[^2\text{H}_{10}]$-DPHM (20.0 mg) and DPMA (2.5 mg) boluses.

2. \textbf{Fetal Administration Experiments}: Similar to the maternal experiments above, in three animals (E2174, E4227 and E4230), a 6 h steady-state DPHM infusion at 170
μg/min, combined with initial 5.0 mg DPHM and 0.85 mg [²H₁₀]-DPMA boluses, was administered via the fetal lateral tarsal vein catheter. In the other two animals (E1225A and E303Y), [²H₁₀]-DPHM infusion (at 170 μg/min) was administered in combination with [²H₁₀]-DPHM (5.0 mg) and DPMA (0.85 mg) boluses.

In both maternal and fetal experiments, simultaneous serial blood samples were collected from the fetal (1.5 ml) and maternal (3.0 ml) femoral arterial catheters at 5, 10, 20, 30, 45, 60, 90, 120, 180, 240, 300 and 360 min during the infusion, and at 5, 15, 30, 60, 120, 180, 240, 360 min and 9, 12, 18, 30, 42, 54, 66, 78, 90 h post-infusion. Fetal femoral arterial samples (0.5 ml) were also collected at the same time intervals for blood gas analysis and measurement of glucose and lactate concentrations. All fetal blood removed for sampling during the experiment was replaced, at intervals, by an equal volume of blood obtained from the mother prior to the start of the experiment (during the first day) or from another ewe (after the first day). Cumulative samples of maternal urine were also collected every hour for the first 8 h, and along with each blood sample beyond 10 h. Fetal urine was allowed to drain by gravity into a sterile bag, an aliquot (3.0 ml) was sampled for drug analysis, and the rest was returned to the amniotic cavity via the amniotic catheter after recording the total volume. Fetal urine samples were collected at the same intervals as the maternal urine samples above but only during the first 24 h of the experimental protocol.

3.1.2.6 Study F: Disposition of the DPHM-N-oxide Metabolite in the Maternal-Fetal Unit

As indicated above, maternal and fetal plasma and urine concentrations of DPHMNOX (or [²H₁₀]-DPHMNOX) were measured in four out of five animals employed for study E.
All maternal, fetal and adult non-pregnant sheep blood samples collected for drug and metabolite analysis were placed into heparinized Vacutainer® tubes (Becton-Dickinson, Rutherford, NJ) and gently mixed. The blood samples were then centrifuged at 2000 g for 10 min. The plasma supernatant was removed and placed into clean borosilicate test tubes with polytetrafluoroethylene (PTFE)-lined caps. Urine samples were also placed into clean borosilicate test tubes. All samples were stored frozen at -20°C until the time of analysis.

All doses were prepared by dissolving accurately weighed amounts of the drug (and the DPMA or [²H₉]-DPMA metabolite in study E) in sterile water for injection and were sterilized by filtering through a 0.22 μm nylon syringe filter into a capped empty sterile injection vial. The doses of [²H₉]-DPHM and [²H₁₀]-DPMA were corrected for the mass-difference due to the presence of deuterium labels as compared to the unlabeled drug and metabolite.

3.1.3 Physiological Recording and Monitoring Procedures

During study A, from at least 24 h before to at least 24 h after the infusion period, fetal amniotic, tracheal and femoral arterial pressures, heart rate, electrocortical activity and urine production rate were continuously monitored. In the animals with an implanted umbilical flow transducer, umbilical blood flow was measured with a Transonic model T201 transit-time flow meter (Transonic Systems, Inc., Ithaca, NY). Fetal urine flow rate was estimated using a computer controlled roller pump assembly developed in our laboratory. The fetal bladder catheter was allowed to drain by gravity into a sterile reservoir (10 ml
syringe barrel) to which a disposable DTX transducer was connected. When, due to urine
collection, the pressure in the reservoir increased above a preset level (usually 3 mm Hg),
the computer activated a roller pump (DIAS, Ex154, DIAS Inc., Kalamazoo, MI) which
pumped a calibrated volume of urine from the reservoir back to the amniotic cavity (via the
amniotic catheter) during control periods. During the experimental period, the urine was
collected into a sterile sample collection syringe, at appropriate sampling intervals a 5 ml
aliquot was taken and the rest returned to the amniotic cavity after recording the total
volume. The cumulative volume pumped per min, which equals fetal urine production rate
per min, was stored on diskette.

All of these physiological data as well as the pharmacokinetic data on DPHM (or $[^2]H_{10}$-
DPHM) disposition in amniotic and tracheal fluids and maternal and fetal urine from Study
A have been reported previously (Tonn, 1995) and will not be presented here.

In all pregnant sheep experiments, the fetal blood pH, $P_{O_2}$, and $P_{CO_2}$, $O_2$-saturation, and
hemoglobin, glucose and lactate concentrations were measured as described in Chapter 2
(Section 2.2.4).

3.1.4 Plasma Protein Binding of DPHM and $[^2]H_{10}$-DPHM in the Mother and the
Fetus

The plasma protein binding/unbound fraction of DPHM (or $[^2]H_{10}$-DPHM) was measured ex
vivo in pooled fetal and maternal steady-state plasma samples using an equilibrium
dialysis procedure, as described by Yoo et al. (1993).
3.1.5 Drug and Metabolite Analysis

The concentrations of DPHM, $[^2H_{10}]-DPHM$, DPMA and $[^2H_{10}]-DPMA$ in all biological fluids were measured using previously developed GC-MS analytical methods (Tonn et al., 1993; Tonn et al., 1995) (see Chapter 2, Sections 2.3 and 2.4). The plasma and urine concentrations of DPHMNOX and $[^2H_{10}]-DPHMNOX$ were measured using the LC-MS/MS analytical method developed during the course of these studies (see Chapter 2, Section 2.6).

3.1.6 Pharmacokinetic Analysis

3.1.6.1 Study A: Paired Maternal (DPHM) and Fetal ($[^2H_{10}]-DPHM$) Infusions for the Determination of Placental and Non-Placental Clearances

The maternal and fetal steady-state arterial plasma DPHM and $[^2H_{10}]-DPHM$ concentration data obtained from Study A were treated according to a 2-compartment open model in order to estimate the placental and non-placental clearance parameters of DPHM and $[^2H_{10}]-DPHM$ in the ewe and fetus, respectively. This model assumes steady-state plasma concentrations and drug elimination from both the maternal and fetal compartments (Szeto et al., 1982a). Clearances were calculated from the following equations (1-6) as described previously (Szeto et al., 1982a).

\[
CL_{mm} = \frac{k_0}{[C_m - C_m^* (C_m'/C')]}
\]  \hspace{1cm} (1)

\[
CL_f = \frac{k_0'}{[C_f - C_m^* (C_f/C_m)]}
\]  \hspace{1cm} (2)
\( CLmf = CLff \times \left( \frac{Cf}{Cm} \right) \)  

(3)

\( CLfm = CLmm \times \left( \frac{Cm'}{Cf'} \right) \)  

(4)

\( CLmo = CLmm - CLmf \)  

(5)

\( CLfo = CLff - CLfm \)  

(6)

\( CLmm \) and \( CLff \) are maternal and fetal total body clearances, respectively. \( CLmf \) is the maternal-to-fetal placental clearance, whereas \( CLfm \) is the fetal-to-maternal placental clearance. \( CLmo \) and \( CLfo \) are maternal and fetal non-placental clearances, respectively.

The symbols \( k_0 \) and \( k_0' \) denote the drug infusion rates to the mother (DPHM) and the fetus ([\(^{2}H_{10}\)-DPHM]), respectively. \( Cm \) and \( Cf \) are the steady-state plasma DPHM concentrations in the mother and the fetus after maternal DPHM administration, and \( Cm' \) and \( Cf' \) are the steady-state maternal and fetal plasma \([^{2}H_{10}]\)-DPHM concentrations after fetal \([^{2}H_{10}]\)-DPHM administration.

The net maternal and fetal steady-state clearances of the drug were calculated by dividing the appropriate infusion rate by the respective steady-state plasma concentration (i.e., \( CLm(\text{net}) = k_0/Cm; \) and \( CLf(\text{net}) = k_0'/Cf' \)). The \( CLm(\text{net}) \) and \( CLf(\text{net}) \) are related to 2-compartment clearances as: \( CLm(\text{net}) \times Cm = CLmm \times Cm - CLmf \times Cf; \) and \( CLf(\text{net}) \times Cf' = CLff \times Cf' - CLmf \times Cm' \).
The fetal first-pass hepatic extraction ratio ($E_H$) for the maternally derived DPHM in the umbilical vein was indirectly estimated using equation (7). This equation assumes sole hepatic formation of the DPMA and $[^2H_{10}]$-DPMA metabolites in the fetus and also no placental transfer of the DPMA metabolite formed in the mother (see Appendix I for theoretical basis and derivation):

$$E_H = \frac{\left( \frac{AUC_{0-\infty}^{DPMA}}{AUC_{0-\infty}^{DPHM}} \right) - \left( \frac{AUC_{0-\infty}^{[^2H_{10}]DPMA}}{AUC_{0-\infty}^{[^2H_{10}]DPHM}} \right)}{\left( \frac{AUC_{0-\infty}^{DPMA}}{AUC_{0-\infty}^{DPHM}} \right)}$$

(7)

$AUC_{0-\infty}^{DPHM}$, $AUC_{0-\infty}^{[^2H_{10}]DPHM}$, $AUC_{0-\infty}^{DPMA}$, $AUC_{0-\infty}^{[^2H_{10}]DPMA}$ are the area under the fetal femoral arterial plasma concentration vs. time profiles (from time 0 to $\infty$) of DPHM, $[^2H_{10}]$-DPHM, DPMA and $[^2H_{10}]$-DPMA, respectively. DPHM was infused to the mother and hence it reaches the fetus via the umbilical vein, whereas $[^2H_{10}]$-DPHM was directly infused to the fetus via the lateral tarsal vein.

3.1.6.2 Study B: Study of Fetal Hepatic First-Pass Uptake of DPHM from the Umbilical Vein

For these fetal hepatic first-pass experiments, fetal systemic availability of DPHM (or $[^2H_{10}]$-DPHM) after umbilical venous administration was calculated from the parent drug data as before (Tonn et al., 1996):

$$F = \frac{AUC_{\text{parent(UV)}}}{AUC_{\text{parent(TV)}}}$$

(8)
where, respective AUC values refer to fetal arterial plasma AUC of the parent drug after umbilical (UV) or tarsal venous (TV) administration.

Fetal hepatic extraction ratio ($E_H$) of DPHM (or $^2H_{10}$-DPHM) after umbilical venous administration was calculated as:

$$E_H = 1 - \frac{F}{9}$$

(9)

In these experiments, fetal hepatic extraction ratio of DPHM (or $^2H_{10}$-DPHM) was also calculated from the parent drug and metabolite (DPMA and $^2H_{10}$-DPMA) data in an analogous fashion to equation (7) as:

$$E_H = 1 - \frac{\left(\frac{AUC_{\text{metabolite(UV)}}}{AUC_{\text{parent(UV)}}}\right) - \left(\frac{AUC_{\text{metabolite(TV)}}}{AUC_{\text{parent(TV)}}}\right)}{\left(\frac{AUC_{\text{metabolite(UV)}}}{AUC_{\text{parent(UV)}}}\right)}$$

(10)

where, $AUC_{\text{metabolite(UV)}}$ and $AUC_{\text{metabolite(TV)}}$ refer to fetal arterial plasma AUC's of the metabolite after umbilical and tarsal venous administration of the parent drug, respectively. In this study (Study B), all AUC's were only available from time 0 to the time of the last sampling point.

Fetal systemic availability using this extraction ratio data was also calculated using equation (9).
3.1.6.3 Study C: Hepatic Uptake of DPHM in Adult Non-Pregnant Sheep

In the following equations, subscripts 'parent' and 'metabolite' refer to the 'parent drug' and 'metabolite', respectively.

The systemic total body clearance of drug was calculated as:

$$\text{CL}_{ib} = \frac{(\text{Dose})_{\text{i.v.}}}{(\text{AUC}_{\text{parent}})_{\text{i.v.}}} \quad (11)$$

where, \text{i.v.} refers to intravenous administration of the parent drug, and the AUC term is the femoral arterial plasma AUC.

The hepatic first-pass extraction ratio ($E_H$) of drug is given by:

$$E_H = 1 - \frac{(\text{AUC}_{\text{parent}})_{\text{p.v.}}}{(\text{AUC}_{\text{parent}})_{\text{i.v.}}} \quad (12)$$

where, \text{p.v.} refers to the portal venous administration of the parent drug.

The fraction of the intravenously administered drug metabolized in the liver was calculated from the arterial AUC's of the DPMA metabolite after \text{p.v.} and \text{i.v.} administration of the parent drug, as:
This equation assumes linear pharmacokinetics, sole hepatic formation of the metabolite, and complete metabolism of the p.v. dose in the liver.

Total hepatic blood flow (portal venous + hepatic arterial) was estimated from the relationships of the well-stirred model of hepatic elimination (Wilkinson and Shand, 1975; Pang and Gillette, 1978):

\[ Q_H = \frac{1}{\frac{[AUC_{\text{parent}}^{0-\infty}]}{\text{Dose}_{i.v.}} - \frac{[AUC_{\text{parent}}^{0-\infty}]}{\text{Dose}_{p.v.}}} } \]  \hspace{1cm} (14)

where, i.v. and p.v. refer to the doses and respective arterial AUC's during intravenous or portal venous administration of the parent drug.

The amount of intravenously administered drug eventually delivered to the 'hepato-portal' system (gut + liver) was calculated from the estimated hepatic blood flow and its systemic arterial AUC, as:

\[ \text{Amount delivered to the "hepato-portal" system} = Q_H \times (AUC_{\text{parent}}^{0-\infty})_{i.v.} \]  \hspace{1cm} (15)

This was subsequently converted to the percentage of administered dose delivered to the 'hepato-portal' system.
3.1.6.4 Study D: Gut Uptake of DPHM from the Systemic Circulation in Adult Non-Pregnant Sheep

Steady-state systemic total body clearance of the drug is calculated as:

\[(\text{CL}_{\text{to}})_{ss} = \frac{k_0}{(C_{ss})_{MA}}\]  \hspace{1cm} (16)

where, \(k_0\) is the DPHM infusion rate, and \((C_{ss})_{MA}\) is the steady-state femoral arterial plasma DPHM concentration.

The steady-state gut extraction ratio of the drug \((E_g)\) is calculated as:

\[E_g = 1 - \frac{(C_{ss})_{PV}}{(C_{ss})_{MA}}\]  \hspace{1cm} (17)

where, \((C_{ss})_{MA}\) is the steady-state DPHM concentration in the femoral arterial plasma (before the gut), and \((C_{ss})_{PV}\) is the steady-state DPHM concentration in the portal venous plasma (after the gut).

3.1.6.5 Study E: Contribution of DPMA Formation to DPHM Non-Placental Clearance in Maternal and Fetal Sheep

The maternal and fetal net, total, placental and non-placental clearances of DPHM (or \([^{2}H_{10}]\)-DPHM) were calculated as above in study A.

Other pharmacokinetic parameters were calculated by the equations described below (Kaplan et al., 1973; Pang et al., 1979; Wagner, 1993). All AUC and AUMC (area under...
the first-moment curve) terms in these equations refer to those in femoral arterial plasma.

Fraction of the total parent drug dose converted to the metabolite (DPMA or \([^{2}\text{H}_{10}]-\text{DPMA}\)) \textit{in vivo} (in the mother or the fetus) or the formation clearance of the metabolite as a fraction of the total body clearance of the parent drug:

\[
F_m = \frac{\text{AUC}_{\infty}^{\infty} \text{formed metabolite}}{\text{Dose}_{\text{parent drug}}} \div \frac{\text{AUC}_{\infty}^{\infty} \text{preformed metabolite}}{\text{Dose}_{\text{preformed metabolite}}}
\]

where, 'formed metabolite' refers to the metabolite generated \textit{in vivo} from the parent drug and 'preformed metabolite' refers to the synthesized metabolite administered \textit{per se}.

The formation clearance of the metabolite as a fraction of maternal or fetal non-placental clearance (\(F_m\)) was calculated by dividing the \(F_m\) value obtained above by the fractional contribution of the maternal or fetal non-placental clearance to the corresponding total body clearance.

Mean residence time of the preformed metabolite:

\[
\text{MRT}_{\text{preformed metabolite}} = \frac{\text{AUMC}_{\infty}^{\infty} \text{preformed metabolite}}{\text{AUC}_{\infty}^{\infty} \text{preformed metabolite}}
\]

Mean residence time of the metabolite formed \textit{in vivo}:
\[ MRT_{\text{formed metabolite}} = \left( \frac{\text{AUMC}^{0-\infty}}{\text{AUC}^{0-\infty}} \right)_{\text{formed metabolite}} - \left( \frac{\text{AUMC}^{0-\infty}}{\text{AUC}^{0-\infty}} \right)_{\text{parent drug}} \]  

(20)

Mean residence time of the parent drug:

\[ MRT_{\text{parent drug}} = \left( \frac{\text{AUMC}^{0-\infty}}{\text{AUC}^{0-\infty}} \right)_{\text{parent drug}} - \frac{k_0 \cdot \tau^2}{2(k_0 \cdot \tau + D_{\text{bolus}})} \]  

(21)

where, \( k_0 \), \( \tau \), and \( D_{\text{bolus}} \) are the infusion rate, infusion duration, and initial bolus loading dose of the parent drug, respectively.

Total body clearance (\( CL_{\text{tb}} \)) of the preformed metabolite or parent drug:

\[ CL_{\text{tb}} = \frac{\text{Total i.v. Dose}}{\text{AUC}^{0-\infty}} \]  

(22)

Steady-state volume of distribution (\( V_{dss} \)) of the preformed metabolite:

\[ (V_{dss})_{\text{preformed metabolite}} = (CL_{\text{tb}})_{\text{preformed metabolite}} \cdot MRT_{\text{preformed metabolite}} \]  

(23)

And, \( V_{dss} \) of the parent drug:

\[ (V_{dss})_{\text{parent drug}} = (CL_{\text{tb}})_{\text{parent drug}} \cdot MRT_{\text{parent drug}} \]  

(24)
The terminal elimination half-life ($t_{1/2p}$) of the parent drug as well as the preformed metabolite was obtained from a 2-compartment model fitting of the data using nonlinear least-squares regression software WinNonlin (Scientific Consulting, Inc., Apex, NC). Maternal renal clearance values for the parent drug, the preformed metabolite and the formed metabolite were calculated by dividing the total amount of each compound excreted in maternal urine by the respective maternal plasma AUC$^{0-\infty}$. Fetal renal clearances for all these compounds were calculated by dividing the total amount excreted in fetal urine during the 24 h sampling period by the respective fetal plasma AUC$^{0-24h}$.

3.1.6.6 Study F: Disposition of the DPHM-N-oxide Metabolite in the Maternal-Fetal Unit

The maternal and fetal renal clearances of DPHMNOX (or $[^2H_{10}]$-DPHMNOX) were calculated in a fashion similar to DPMA (or $[^2H_{10}]$-DPMA) above.

Maternal and fetal plasma AUC's and AUMC's for the parent drug and metabolites in all studies (A - F) from time 0 to the last sampling point were calculated using the linear trapezoidal rule (Gibaldi and Perrier, 1982). The AUC was then extrapolated to time infinity by adding the factor, $C_{last}/K; C_{last}$ is the plasma concentration of the drug or metabolite at the last sampling time ($t_{last}$), and $K$ is the terminal elimination rate constant. The factor for the extrapolation of AUMC to time infinity was $(C_{last}{t_{last}}/K+C_{last}/K^2)$ (Gibaldi and Perrier, 1982).

3.1.7 Statistical Analysis
All values are reported as mean ± S.D. In all pregnant sheep studies, the fetal weight in utero at the time of experimentation was estimated from the weight at birth and the time interval between the experiment and birth (Koong et al., 1975). The achievement of steady-state in maternal, fetal or adult non-pregnant sheep plasma was established according to two criteria: i) the slope of the plasma concentration vs. time curve should not be significantly different from zero, and, ii) the coefficient of variation of the measured concentrations should be <15%. Maternal and fetal pharmacokinetic parameters in studies A and E were compared against each other using an unpaired t-test. In study C, the adult sheep femoral arterial plasma AUC’s of the parent drug or metabolite after i.v. and p.v. drug administration, were compared using a paired t-test. The steady-state DPHM concentrations in femoral arterial and portal venous plasma during gut uptake studies (study D) were also compared against each other using a paired t-test. The significance level was p < 0.05 in all cases.

3.2 Results

3.2.1 Study A: Paired Maternal (DPHM) and Fetal ([2H10]-DPHM) Infusions for the Determination of Placental and Non-Placental Clearances

The five experiments involving simultaneous 6 h infusions of DPHM and [2H10]-DPHM to ewe and fetus, respectively, were carried out at 125-133 days gestation (128.8 ± 3.2 d). Estimated fetal weight on the day of experiment was 2.46 ± 0.21 kg. During the control period, the fetal femoral arterial values for pH, PO2, PCO2, O2-saturation, hemoglobin, glucose and lactate concentrations were 7.36 ± 0.02, 22.6 ± 1.65 mm Hg, 47.3 ± 0.5 mm Hg, 55.3 ± 23.8%, 10.0 ± 0.3 g/dl, 0.98 ± 0.09 mM and 0.70 ± 0.11 mM, respectively. There were no consistent changes in any of these variables during or after the infusion
period. Likewise umbilical blood flow (281 ± 39 ml/min/kg, n=3) was not consistently altered during the experiment.

3.2.1.1 Maternal and Fetal Plasma DPHM and $[^2H_{10}]$-DPHM Concentrations, and Placental and Non-Placental Clearance Values

The average plasma concentrations of DPHM and $[^2H_{10}]$-DPHM in maternal and fetal femoral arterial plasma are illustrated in Figure 3.1. Steady-state was achieved in maternal as well as fetal plasma after 120 min according to the established criteria. Thus, maternal and fetal steady-state plasma concentrations were calculated as the average concentration during the 150-360 min period. The mean steady-state concentrations of DPHM were 260.8 ± 42.3 and 45.7 ± 38.9 ng/ml in maternal and fetal femoral arterial plasma, respectively, while the mean concentrations of $[^2H_{10}]$-DPHM in the same vessels were 44.6 ± 12.9 and 244.0 ± 94.7 ng/ml, respectively. The total maternal and fetal femoral arterial steady-state concentrations of DPHM (i.e., labeled and unlabeled DPHM) were 305.4 ± 54.7 and 289.6 ± 128.9 ng/ml.

As in previous studies (Yoo, 1989), there was accumulation of DPHM (both labeled and unlabeled) in fetal tracheal and amniotic fluids. The average lung fluid to FA plasma drug concentration ratio was 4.0 ± 1.7 for DPHM and 4.5 ± 1.6 for $[^2H_{10}]$-DPHM. The corresponding ratios in amniotic fluid (i.e., amniotic fluid/FA) were 0.6 ± 0.2 and 0.8 ± 0.2 for labeled and unlabeled drug, respectively. Following the infusion, the concentrations of DPHM and $[^2H_{10}]$-DPHM in all fluids declined rapidly with terminal plasma elimination half-lives of 70.5 ± 6.9 and 51.8 ± 7.2 min in the ewe and fetus, respectively. Details of these data have been presented previously (Tonn, 1995).
Figure 3.1 – Average plasma concentrations of DPHM and $[^2\text{H}_{10}]$-DPHM in maternal and fetal femoral arterial plasma during and following simultaneous i.v. infusions of DPHM (670 μg/min) to the ewe and $[^2\text{H}_{10}]$-DPHM (170 μg/min) to the fetus (n=5).

The calculated net, total, non-placental and placental plasma clearances in the mother and the fetus are presented in Table 3.1. The weight-normalized estimates of $\text{CL}_{\text{f(\text{net})}}$ (314.8 ± 101.5 ml/min/kg), $\text{CL}_{\text{ff}}$ (324.2 ± 104.3 ml/min/kg), $\text{CL}_{\text{fm}}$ (214.4 ± 68.8 ml/min/kg) and $\text{CL}_{\text{fo}}$ (109.8 ± 49.8 ml/min/kg) were all significantly higher than the corresponding maternal values for $\text{CL}_{\text{m(\text{\text{net})}}}$ (37.2 ± 4.6 ml/min/kg), $\text{CL}_{\text{mm}}$ (38.3 ± 5.1 ml/min/kg), $\text{CL}_{\text{mf}}$ (50.3 ± 29.6 ml/min/kg) and $\text{CL}_{\text{mo}}$ (36.6 ± 4.2 ml/min/kg) (unpaired t-test, $p < 0.05$ in all cases). The non-placental contribution to net DPHM clearance averaged 98.5 ± 0.9% and 34.4 ± 9.4% in ewe and fetus, respectively, and again these were significantly different. In ewes 122z and 2181, the presence of a functional umbilical venous catheter also allowed calculation
of the fetal DPHM extraction across the placenta. The umbilical extraction ratios for \(^{2}\text{H}_{10}\)-DPHM were 0.64 and 0.51 in E122z and E2181, respectively, indicating net drug transfer from the fetus to the mother.

3.2.1.2 Fetal and Maternal DPMA and \(^{2}\text{H}_{10}\)-DPMA Plasma Concentrations

A mean concentration vs. time plot of DPMA and \(^{2}\text{H}_{10}\)-DPMA in maternal and fetal femoral arterial plasma is shown in Figure 3.2. Although concentrations of DPHM and \(^{2}\text{H}_{10}\)-DPHM reached steady-state at ~120 minutes from the start of the infusion (Figure 3.1), the plasma levels of DPMA and \(^{2}\text{H}_{10}\)-DPMA did not reach steady-state during the whole duration of infusion and continued to increase for 30-120 minutes post-infusion. At all time points during the infusion period, the concentration of \(^{2}\text{H}_{10}\)-DPMA was higher in the fetus than in the mother, whereas for the unlabeled metabolite the situation was reversed. The peak concentrations of DPMA in maternal and fetal plasma averaged 137.4 ± 42.5 and 92.8 ± 42.6 ng/ml, respectively, while the peak maternal and fetal plasma concentrations of \(^{2}\text{H}_{10}\)-DPMA were 28.7 ± 9.7 and 135.0 ± 49.7 ng/ml, respectively. The time at which the peak levels occurred post-infusion was 18.0 ± 16.4 min for both labeled and unlabeled DPMA in the ewe, whereas in the fetus it was 87.0 ± 40.2 min. Following the peak, the fetal metabolite levels declined much more slowly than in the ewe. The elimination half-life of the metabolite in the fetus (15.2 ± 5.6 h) was significantly longer compared to the mother (3.0 ± 0.5 h) (unpaired t-test, p < 0.01). In the 2 animals with functional umbilical venous catheters, the extraction ratio of \(^{2}\text{H}_{10}\)-DPMA across the fetal side of the placenta averaged -0.06 ± 0.04. This value is not significantly different from zero, but is different from the umbilical extraction ratio for \(^{2}\text{H}_{10}\)-DPHM given above. Finally, DPMA or \(^{2}\text{H}_{10}\)-DPMA metabolites were not detected in amniotic or fetal lung fluid.
Figure 3.2 – Average maternal and fetal femoral arterial plasma concentrations of DPMA and \(^{2}H_{10}\)-DPMA during and following simultaneous i.v. infusion of DPHM (670 µg/min) to the ewe and \(^{2}H_{10}\)-DPHM (170 µg/min) to the fetus (n=5).

Table 3.2 gives the AUC values for DPHM, \(^{2}H_{10}\)-DPHM, DPMA and \(^{2}H_{10}\)-DPMA in maternal and fetal femoral arterial plasma. The fetal AUC\textsubscript{DPMA}/AUC\textsubscript{DPHM} ratio (8.20 ± 3.62) was significantly higher than the corresponding AUC ratio for \(^{2}H_{10}\)-DPMA/\(^{2}H_{10}\)-DPHM (2.24 ± 1.19) (paired t-test, p < 0.02). In the ewe, however, there was the opposite situation. The AUC\textsubscript{DPMA}/AUC\textsubscript{DPHM} ratio (0.62 ± 0.15) was significantly less than the corresponding ratio for \(^{2}H_{10}\)-DPMA/\(^{2}H_{10}\)-DPHM (0.96 ± 0.26) (paired t-test, p < 0.02), although the magnitude of the difference is much smaller than that in the fetus. Table 3.2 also gives the estimates of the fetal hepatic first-pass extraction ratio for maternally derived DPHM calculated using equation 7 (section 3.1.6.1), with the mean value for this parameter being 0.71 ± 0.16.
Table 3.1 - Weight-normalized estimates of net (CL_{m(net)} and CL_{f(net)}), total (CL_{mm} and CL_{tt}), placental (CL_{mf} and CL_{fm}), and non-placental (CL_{mo} and CL_{fo}) clearances in the mother and the fetus, respectively, in 5 experiments involving simultaneous i.v. infusion of DPHM to the mother and [^{2}H_{10}]-DPHM to the fetus.

<table>
<thead>
<tr>
<th>Ewe No.</th>
<th>GA (days)</th>
<th>CL_{m(net)}^{b}</th>
<th>CL_{mm}^{b}</th>
<th>CL_{mo}^{b}</th>
<th>CL_{mf}^{c}</th>
<th>CL_{f(net)}^{c}</th>
<th>CL_{tt}^{c}</th>
<th>CL_{fo}^{c}</th>
<th>CL_{fm}^{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td>2101</td>
<td>133</td>
<td>36.4</td>
<td>36.8</td>
<td>36.1</td>
<td>25.2</td>
<td>309.0</td>
<td>312.6</td>
<td>138.0</td>
<td>174.6</td>
</tr>
<tr>
<td>122z</td>
<td>127</td>
<td>40.2</td>
<td>41.5</td>
<td>39.4</td>
<td>48.0</td>
<td>445.4</td>
<td>459.7</td>
<td>177.2</td>
<td>282.5</td>
</tr>
<tr>
<td>2177</td>
<td>128</td>
<td>37.8</td>
<td>39.0</td>
<td>37.3</td>
<td>54.6</td>
<td>383.6</td>
<td>396.4</td>
<td>106.7</td>
<td>289.7</td>
</tr>
<tr>
<td>2181</td>
<td>131</td>
<td>41.7</td>
<td>43.8</td>
<td>40.5</td>
<td>97.8</td>
<td>198.9</td>
<td>209.1</td>
<td>75.4</td>
<td>133.6</td>
</tr>
<tr>
<td>2241</td>
<td>125</td>
<td>29.9</td>
<td>30.6</td>
<td>29.7</td>
<td>26.0</td>
<td>237.3</td>
<td>243.4</td>
<td>51.6</td>
<td>191.8</td>
</tr>
<tr>
<td>Mean</td>
<td>128.8</td>
<td>37.2</td>
<td>38.3</td>
<td>36.6</td>
<td>50.3</td>
<td>314.8*</td>
<td>324.2*</td>
<td>109.8*</td>
<td>214.4*</td>
</tr>
<tr>
<td>±S.D.</td>
<td>±3.2</td>
<td>±4.6</td>
<td>±5.1</td>
<td>±4.2</td>
<td>±29.6</td>
<td>±101.6</td>
<td>±104.3</td>
<td>±49.8</td>
<td>±68.8</td>
</tr>
</tbody>
</table>

\(^{a}\) - GA-gestational age; \(^{b}\) - clearance values are in ml/min/kg maternal body weight; \(^{c}\) - clearance values are in ml/min/kg estimated fetal body weight; * significantly greater than the corresponding maternal value (p<0.05).

CL_{m(net)}: Maternal net clearance; CL_{mm}: Maternal total clearance; CL_{mf}: Maternal placental clearance; CL_{mo}: Maternal non-placental clearance; CL_{f(net)}: Fetal net clearance; CL_{tt}: Fetal total clearance; CL_{fo}: Fetal placental clearance; CL_{fm}: Fetal non-placental clearance.
Table 3.2 - Maternal and fetal femoral arterial AUC values for unlabeled and labeled forms of DPHM and DPMA, metabolite/parent drug AUC ratios and estimates of fetal hepatic extraction of the maternally derived DPHM in 5 maternal-fetal paired infusion experiments.

<table>
<thead>
<tr>
<th>Ewe No.</th>
<th>Site&lt;sup&gt;a&lt;/sup&gt;</th>
<th>AUC&lt;sub&gt;0-∞&lt;/sub&gt; Values (ng.min/ml)</th>
<th>AUC Ratio</th>
<th>Fetal Hepatic First-Pass Extraction Ratio for DPHM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>DPHM</td>
<td>[&lt;sup&gt;2&lt;/sup&gt;H&lt;sub&gt;10&lt;/sub&gt;]-DPHM</td>
<td>DPMA</td>
</tr>
<tr>
<td>E2101</td>
<td>MA</td>
<td>87278.0</td>
<td>12645.7</td>
<td>62067.8</td>
</tr>
<tr>
<td></td>
<td>FA</td>
<td>7270.7</td>
<td>87988.8</td>
<td>95138.8</td>
</tr>
<tr>
<td>E1222</td>
<td>MA</td>
<td>95396.9</td>
<td>14089.4</td>
<td>40862.4</td>
</tr>
<tr>
<td></td>
<td>FA</td>
<td>10888.4</td>
<td>50871.6</td>
<td>116584.0</td>
</tr>
<tr>
<td>E2177</td>
<td>MA</td>
<td>88534.1</td>
<td>17516.2</td>
<td>44028.7</td>
</tr>
<tr>
<td></td>
<td>FA</td>
<td>16805.3</td>
<td>83072.2</td>
<td>110297.6</td>
</tr>
<tr>
<td>E2181</td>
<td>MA</td>
<td>94710.2</td>
<td>15986.8</td>
<td>76879.8</td>
</tr>
<tr>
<td></td>
<td>FA</td>
<td>45697.4</td>
<td>149357.8</td>
<td>299601.8</td>
</tr>
<tr>
<td>E2241</td>
<td>MA</td>
<td>128464.1</td>
<td>26465.9</td>
<td>84048.4</td>
</tr>
<tr>
<td></td>
<td>FA</td>
<td>14847.2</td>
<td>109036.0</td>
<td>60600.8</td>
</tr>
<tr>
<td>Mean ±S.D.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MA</td>
<td></td>
<td>98876.7</td>
<td>17340.8</td>
<td>61577.4</td>
</tr>
<tr>
<td>±16929.1</td>
<td>±5425.6</td>
<td>±19212.1</td>
<td>±3230.2</td>
<td>±0.15</td>
</tr>
<tr>
<td>FA</td>
<td></td>
<td>19101.8</td>
<td>96065.3</td>
<td>136444.6</td>
</tr>
<tr>
<td>±15314.5</td>
<td>±36348.5</td>
<td>±93750.8</td>
<td>±106765.4</td>
<td>±3.62**</td>
</tr>
</tbody>
</table>

<sup>a</sup> - MA- maternal femoral arterial plasma; FA – fetal femoral arterial plasma.

- significantly higher than the corresponding DPHM/DPMA ratio in MA (p < 0.05); ** - significantly higher than the corresponding [<sup>2</sup>H<sub>10</sub>]-DPMA/[<sup>2</sup>H<sub>10</sub>]-DPHM ratio in FA. (p < 0.05).
3.2.2 Study B: Study of Fetal Hepatic First-Pass Uptake of DPHM from the Umbilical Vein

Figure 3.3 illustrates the representative fetal plasma concentration vs. time plots for unlabeled and labeled forms of DPHM and DPMA measured from samples obtained during the previous study of fetal hepatic first-pass DPHM uptake after umbilical venous administration (Tonn et al., 1996). Figure 3.3A shows the data from a bolus experiment where $[^2\text{H}_{10}]$-DPHM was administered via the umbilical vein, whereas Figure 3.3B shows the data from an infusion study in which unlabeled DPHM was infused via the umbilical route. In both experiments, there were no consistent differences in the fetal arterial plasma concentrations of DPHM and $[^2\text{H}_{10}]$-DPHM. In contrast, the plasma concentration of the form of DPMA derived from the drug administered via the umbilical vein was consistently higher than that derived from the drug given via the lateral tarsal vein. Table 3.3 gives the fetal femoral arterial plasma AUC values for labeled and unlabeled DPHM and DPMA. The estimates of fetal first-pass hepatic extraction ratio based upon intact drug concentrations were obtained using equations 8 and 9 (section 3.1.6.2), and the calculated mean value of $-0.06 \pm 0.17$ is not significantly different from zero (unpaired t-test, $p > 0.1$), as reported previously (Tonn et al., 1996). In contrast, the estimates of fetal hepatic extraction ratio obtained using the AUC values for DPMA and $[^2\text{H}_{10}]$-DPMA in combination with those of DPHM and $[^2\text{H}_{10}]$-DPHM and equation 10 (section 3.1.6.2) indicate significant drug uptake by the fetal liver from the umbilical vein. The mean fetal hepatic extraction ratio was $0.44 \pm 0.14$, and this was significantly lower than the value of $0.71 \pm 0.16$ obtained above in the paired maternal-fetal infusion experiments (unpaired t-test, $p < 0.005$) (Study A; see Table 3.2).
Figure 3.3 - A). Representative fetal femoral arterial plasma concentrations of DPHM, $[^{2}H_{10}]$-DPHM, DPMA and $[^{2}H_{10}]$-DPMA after simultaneous i.v. bolus administration of DPHM and $[^{2}H_{10}]$-DPHM (5 mg each) to the fetus (E#989). DPHM was given via the fetal tarsal vein and $[^{2}H_{10}]$-DPHM via the umbilical vein. B). Representative fetal femoral arterial plasma concentrations of DPHM, $[^{2}H_{10}]$-DPHM, DPMA and $[^{2}H_{10}]$-DPMA after simultaneous steady-state infusion of DPHM and $[^{2}H_{10}]$-DPHM (60μg/min each) to the fetus (E#2164). DPHM was infused via the fetal umbilical vein and $[^{2}H_{10}]$-DPHM via the fetal lateral tarsal vein.
Table 3.3 - Fetal femoral arterial AUC values of labeled and unlabeled DPHM and DPMA after simultaneous but separate umbilical and tarsal venous administration of DPHM and $[^2]H_{10}$-DPHM, and the estimates of fetal hepatic extraction ratio of DPHM in umbilical venous first-pass experiments.

<table>
<thead>
<tr>
<th>Ewe No.</th>
<th>Protocol</th>
<th>AUC$_{0-\text{last}}$ Values (ng.min/ml)</th>
<th>Extraction Ratio (Parent Drug Data)</th>
<th>Availability (Parent Drug Data)</th>
<th>Extraction Ratio (Metabolite Data)</th>
<th>Availability (Metabolite Data)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E989$^a$</td>
<td>Bolus</td>
<td>DPHM 4658.0 [${}^2$H$<em>{10}$]-DPHM 4305.2 DPMA 5787.2 [${}^2$H$</em>{10}$]-DPMA 9372.0</td>
<td>0.08</td>
<td>0.92</td>
<td>0.43</td>
<td>0.57</td>
</tr>
<tr>
<td>E208$^a$</td>
<td>Bolus</td>
<td>DPHM 3861.4 [${}^2$H$<em>{10}$]-DPHM 4022.5 DPMA 4251.0 [${}^2$H$</em>{10}$]-DPMA 7239.8</td>
<td>-0.04</td>
<td>1.04</td>
<td>0.39</td>
<td>0.61</td>
</tr>
<tr>
<td>E499$^b$</td>
<td>Bolus</td>
<td>DPHM 3334.5 [${}^2$H$<em>{10}$]-DPHM 2718.7 DPMA 6952.5 [${}^2$H$</em>{10}$]-DPMA 2820.8</td>
<td>-0.23</td>
<td>1.23</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td>E1143$^b$</td>
<td>Bolus</td>
<td>DPHM 3298.1 [${}^2$H$<em>{10}$]-DPHM 2481.0 DPMA 11096.1 [${}^2$H$</em>{10}$]-DPMA 6526.9</td>
<td>-0.33</td>
<td>1.33</td>
<td>0.22</td>
<td>0.78</td>
</tr>
<tr>
<td>E1142$^a$</td>
<td>Infusion</td>
<td>DPHM 8462.1 [${}^2$H$<em>{10}$]-DPHM 6625.7 DPMA 794.7 [${}^2$H$</em>{10}$]-DPMA 2103.1</td>
<td>0.22</td>
<td>0.78</td>
<td>0.70</td>
<td>0.30</td>
</tr>
<tr>
<td>E1242$^a$</td>
<td>Infusion</td>
<td>DPHM 3240.8 [${}^2$H$<em>{10}$]-DPHM 3268.5 DPMA 521.1 [${}^2$H$</em>{10}$]-DPMA 838.5</td>
<td>-0.01</td>
<td>1.01</td>
<td>0.37</td>
<td>0.63</td>
</tr>
<tr>
<td>E1250$^b$</td>
<td>Infusion</td>
<td>DPHM 3826.8 [${}^2$H$<em>{10}$]-DPHM 3468.2 DPMA 1468.8 [${}^2$H$</em>{10}$]-DPMA 650.5</td>
<td>-0.10</td>
<td>1.10</td>
<td>0.51</td>
<td>0.49</td>
</tr>
<tr>
<td>E2164$^b$</td>
<td>Infusion</td>
<td>DPHM 6348.4 [${}^2$H$<em>{10}$]-DPHM 5966.3 DPMA 1422.6 [${}^2$H$</em>{10}$]-DPMA 801.3</td>
<td>-0.06</td>
<td>1.06</td>
<td>0.40</td>
<td>0.60</td>
</tr>
<tr>
<td>Mean $\pm$S.D.</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

$^a$ in these animals $[^2]H_{10}$-DPHM was given via the umbilical vein and DPHM via the lateral tarsal vein.

$^b$ in these animals DPHM was given via the umbilical vein and $[^2]H_{10}$-DPHM via the lateral tarsal vein.

* - not significantly different from zero (p > 0.05); ** - not significantly different from 1 (p > 0.05).
3.2.3 Study C: Hepatic Uptake of DPHM in Adult Non-Pregnant Sheep

The average body weight of sheep utilized during the DPHM hepatic first-pass uptake experiments was 70.1 ± 10.5 kg.

Figure 3.4 shows the typical femoral arterial plasma concentration vs. time profiles of the parent drug and metabolite after separate but simultaneous administration of DPHM and \([^2\text{H}_{10}]\)-DPHM via intravenous and portal venous routes in two sheep. In E1154, DPHM was given via the portal venous route and \([^2\text{H}_{10}]\)-DPHM via the intravenous route. In E102, the order of administration was reversed, i.e., DPHM was administered intravenously and \([^2\text{H}_{10}]\)-DPHM via the portal route. The average maximal plasma concentrations \(C_{\text{max}}\) of the metabolite generated from the form of drug administered via the portal venous route were significantly higher compared to the \(C_{\text{max}}\) of the metabolite generated from the form of the drug given intravenously (447.8 ± 175.9 vs. 90.7 ± 75.8 ng/ml; paired t-test, p < 0.005). The times of occurrence of plasma \(C_{\text{max}}\) of the metabolite \(t_{\text{max}}\) after portal venous administration ranged from 5-20 min compared to 10-90 min after intravenous administration. Table 3.4 presents the calculated femoral arterial AUC’s of the parent drug and metabolite, hepatic first-pass extraction ratios and the fraction of intravenously administered dose metabolized in the liver. The AUC of the form of parent drug administered via the portal venous route was much smaller compared to that of the form administered intravenously (894.8 ± 767.2 vs. 13286.1 ± 5042.8 ng.min/ml; paired t-test, p < 0.005). The AUC of the metabolite generated from the form of parent drug administered via the portal venous route, however, was significantly larger compared to that generated from the form administered via the intravenous route (65042.8 ± 47634.9
vs. 24218.7 ± 27973.8 ng.min/ml; paired t-test, p < 0.01). The hepatic first-pass extraction ratio of the drug was high and ranged from 90.4 – 99% (mean 94.2 ± 3.7%; Table 3.4). The fraction of the i.v. parent drug dose metabolized in the liver ranged from 18.2 – 50.4% (mean 32.5 ± 14.0%; Table 3.4). Thus, the fraction of the i.v. dose metabolized/eliminated by extrahepatic tissues will be 49.6 – 81.8 % (mean 67.5 ± 14.0%).

Table 3.4 – Femoral arterial AUC’s of the parent drug (DPHM or $[^{2}H_{10}]$-DPHM) and the metabolite (DPMA or $[^{2}H_{10}]$-DPMA), parent drug hepatic first-pass extraction ratio, and the fraction of intravenously administered parent drug dose metabolized in the liver during hepatic first-pass uptake studies.

<table>
<thead>
<tr>
<th>Ewe #</th>
<th>AUC (ng.min/ml)</th>
<th>Hepatic First-Pass Extraction Ratio ($E_H$)</th>
<th>Fraction of i.v. Dose Metabolized in the Liver ($f_H$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Parent (i.v.)$^a$</td>
<td>Parent (p.v.)$^a$</td>
<td>Metabolite (i.v.)$^a$</td>
</tr>
<tr>
<td>1158$^b$</td>
<td>20437.5</td>
<td>1952.8</td>
<td>73888.5</td>
</tr>
<tr>
<td>1154</td>
<td>14728.5</td>
<td>1411.5</td>
<td>6811.4</td>
</tr>
<tr>
<td>139$^b$</td>
<td>14140.9</td>
<td>595.3</td>
<td>11518.8</td>
</tr>
<tr>
<td>102$^b$</td>
<td>9812.6</td>
<td>437.8</td>
<td>16360.9</td>
</tr>
<tr>
<td>989</td>
<td>7311.1</td>
<td>76.6</td>
<td>12513.7</td>
</tr>
<tr>
<td>Mean</td>
<td>13286.1</td>
<td>894.8</td>
<td>24218.7</td>
</tr>
<tr>
<td>± S.D.</td>
<td>± 5042.8</td>
<td>± 767.2$^*$</td>
<td>± 27973.8</td>
</tr>
</tbody>
</table>

$a$ - i.v. and p.v. refer to intravenous and portal venous administration of the parent drug.

$b$ - In these ewes, $[^{2}H_{10}]$-DPHM was given via the portal venous route and DPHM via the femoral venous route. In the other animals, the route of administration for the two forms of drug was reversed.

*- significantly lower than the parent drug AUC after intravenous administration (p < 0.005)

**- significantly larger than metabolite AUC after intravenous administration of the drug (p < 0.01).
Table 3.5 presents the estimates of systemic clearance, total hepatic blood flow and percentage of intravenously administered dose that is eventually delivered to the 'hepato-portal' system (gut and liver). The average percentage of i.v. dose delivered to the 'hepato-portal' system was not significantly different from 100% (unpaired t-test, p > 0.3).

Table 3.5 - Diphenhydramine systemic clearances, estimated hepatic blood flows and percentage of intravenously administered dose eventually delivered to the "hepato-portal system" in five adult sheep.

<table>
<thead>
<tr>
<th>Ewe #</th>
<th>Systemic Clearance (ml/min/kg)</th>
<th>Estimated Total Hepatic Blood Flow (ml/min/kg)</th>
<th>% of i.v. Dose Delivered to the Gut and Livera</th>
</tr>
</thead>
<tbody>
<tr>
<td>1158b</td>
<td>45.1</td>
<td>41.4</td>
<td>91.8</td>
</tr>
<tr>
<td>1154</td>
<td>54.1</td>
<td>59.5</td>
<td>109.9</td>
</tr>
<tr>
<td>139b</td>
<td>56.8</td>
<td>59.0</td>
<td>103.8</td>
</tr>
<tr>
<td>102b</td>
<td>85.1</td>
<td>74.0</td>
<td>86.9</td>
</tr>
<tr>
<td>989</td>
<td>78.5</td>
<td>78.9</td>
<td>100.5</td>
</tr>
<tr>
<td>Mean ± S.D.</td>
<td>63.9 ± 17.0</td>
<td>62.6 ± 14.7</td>
<td>98.6 ± 9.2</td>
</tr>
</tbody>
</table>

a - i.v. refers to intravenous administration of the parent drug.
b - In these ewes, [2H10]-DPHM was given via the portal venous route and DPHM via the femoral venous route. In the other animals, the route of administration for the two forms of drug was reversed.
Figure 3.4 – Representative femoral arterial plasma concentration vs. time profiles of the parent drug (DPHM and $^2$H$_{10}$-DPHM; A and C), and metabolite (DPMA and $^2$H$_{10}$-DPMA; B & D) in E1154 (upper panel) and E102 (lower panel). E1154 received 50 mg DPHM via the portal venous route and an equimolar dose of $^2$H$_{10}$-DPHM via the femoral venous route. The routes of administration of DPHM and $^2$H$_{10}$-DPHM were reversed in E102.
3.2.4 Study D: Gut Uptake of DPHM from the Systemic Circulation in Adult Non-Pregnant Sheep

The average body weight of sheep utilized for DPHM gut uptake studies was 64.0 ± 7.9 kg.

Figure 3.5 shows the average concentration vs. time profiles of DPHM in femoral arterial and portal venous plasma in four sheep during a 6 h DPHM infusion. Based on the previously mentioned criteria, DPHM plasma concentrations were at steady-state during the 2-6 h infusion period in both femoral arterial as well as portal venous plasma. The steady-state femoral arterial and portal venous plasma concentrations, systemic clearances, and the estimates of gut extraction ratio of DPHM in four sheep are presented in Table 3.6.

Figure 3.5 – Average femoral arterial and portal venous plasma concentration vs. time profiles of DPHM in four sheep during 6 h DPHM infusion.
The portal venous plasma concentrations of DPHM were significantly lower compared to its femoral arterial plasma concentrations throughout the experimental period in all animals (paired t-test, p < 0.005 in all cases). The gut extraction ratio of the drug in individual animals ranged from 46.3 – 53.4% (mean 49.0 ± 3.0%).

Table 3.6 – Steady-state femoral arterial and portal venous plasma concentrations, systemic clearance, and the gut extraction ratio of DPHM in four sheep during gut uptake experiments.

<table>
<thead>
<tr>
<th>Ewe #</th>
<th>Steady-State Plasma Concentration (ng/ml)</th>
<th>Systemic Clearance (ml/min/kg)</th>
<th>Gut Extraction (Eₐ)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Femoral Arterial</td>
<td>Portal Venous</td>
<td></td>
</tr>
<tr>
<td>E0224</td>
<td>296.7</td>
<td>148.2</td>
<td>40.3</td>
</tr>
<tr>
<td>E4140</td>
<td>197.3</td>
<td>105.9</td>
<td>50.0</td>
</tr>
<tr>
<td>E1225A</td>
<td>451.7</td>
<td>210.5</td>
<td>20.3</td>
</tr>
<tr>
<td>E6216</td>
<td>323.3</td>
<td>173.7</td>
<td>35.1</td>
</tr>
<tr>
<td>Mean ± S.D.</td>
<td>317.2 ± 104.8</td>
<td>159.6 ± 44.0*</td>
<td>36.4 ± 12.4</td>
</tr>
</tbody>
</table>

* - significantly lower compared to femoral arterial plasma concentrations.

3.2.5 Study E: Contribution of DPMA Formation to DPHM Non-Placental Clearance in Maternal and Fetal Sheep

In these studies, the average maternal body weight was 82.4 ± 14.1 kg and estimated fetal body weights on the day of maternal and fetal DPHM (or [³H₁₀]-DPHM) infusion were 3.16 ± 0.41 and 2.92 ± 0.22 kg, respectively. During the maternal experiments, the control period fetal femoral arterial pH, Po₂, Pco₂, O₂-saturation, and hemoglobin, glucose and lactate concentrations were 7.371 ± 0.017, 20.6 ± 1.8 mm Hg, 50.2 ± 3.3 mm Hg, 42.6 ±
7.0%, 11.3 ± 0.6 g/dl, 0.80 ± 0.21 mM and 1.15 ± 0.18 mM, respectively. Likewise during fetal administration, the control values for these variables were 7.344 ± 0.031, 24.8 ± 5.6 mm Hg, 53.1 ± 2.9 mm Hg, 55.0 ± 8.4%, 10.3 ± 1.3 g/dl, 0.78 ± 0.29 mM and 1.56 ± 0.65 mM, respectively. There were no consistent changes in any of these variables during the course of maternal or fetal administration experiments.

3.2.5.1 Maternal-Fetal Steady-State Plasma Drug Concentrations and Unbound Fractions, and Placental and Non-placental Clearance Estimates

Table 3.7 presents gestational age of the animals on the day of experiment, and maternal and fetal DPHM clearance data (net, total, placental and non-placental clearances).

The mean gestational age on the day of maternal and fetal experiments was 132.2 ± 5.9 and 129.4 ± 3.8 days, respectively, and these were not statistically different (paired t-test, \( p > 0.05 \)). The average maternal and fetal steady-state plasma DPHM (or \(^{2}H_{10}\)-DPHM) concentrations in these animals after maternal drug administration (\( C_{m} \) and \( C_{f} \), respectively) were 220.9 ± 40.3 (range 179.1 - 268.1) and 51.5 ± 45.4 (range 3.5 - 124.1) ng/ml, respectively, whereas those after fetal drug infusion were 32.7 ± 6.5 (\( C_{m}^{f} \): range 24.5 - 40.2) and 231.9 ± 91.9 (\( C_{f}^{f} \): range 132.5 - 374.7) ng/ml, respectively. The steady-state maternal (on the day of maternal experiment) and fetal (on the day of fetal experiment) plasma DPHM unbound fractions were 0.135 ± 0.069 (range 0.032 - 0.211) and 0.347 ± 0.114 (range 0.242 - 0.527), respectively. The mean maternal plasma unbound fraction was significantly lower compared to the mean fetal plasma unbound fraction (unpaired t-test, \( p < 0.005 \)). All fetal weight-normalized clearances (net, total body, placental and non-placental clearances) were significantly higher compared to the
corresponding maternal clearances (unpaired t-test, \( p < 0.02 \) in all cases). However, the contribution of \( \text{CL}_{\text{foc}} \) to \( \text{CL}_{\text{f(net)}} \) (41.8 ± 12.9\%) was significantly lower compared to that of \( \text{CL}_{\text{mo}} \) to \( \text{CL}_{\text{m(net)}} \) (97.8 ± 1.7 \%) (unpaired t-test, \( p < 0.001 \)).

### 3.2.5.2 Maternal-Fetal Arterial Plasma AUC Ratios of the Parent Drug, the Preformed Metabolite, and the \textit{in vivo} Generated Metabolite

Table 3.8 presents the different AUC ratios for the parent drug, the preformed metabolite and the \textit{in vivo} formed metabolite in maternal and the fetal arterial plasma. The FA/MA ratio of the formed metabolite AUC’s after maternal drug administration (2.97 ± 0.82) was significantly higher compared to the FA/MA ratio of the preformed metabolite AUC’s after maternal metabolite administration (0.41 ± 0.21; paired t-test, \( p < 0.005 \)). Although, the MA/FA ratio of the formed metabolite AUC’s after fetal drug administration (0.25 ± 0.35) was higher compared to the MA/FA ratio of the preformed metabolite AUC’s after fetal metabolite administration (0.02 ± 0.02) in all the individual animals, the difference between the means was not statistically significant. Also, the formed metabolite to parent drug AUC ratio in FA after maternal drug administration (21.5 ± 26.4) was higher compared to the corresponding ratio after fetal drug administration (2.32 ± 1.16) in all the individual animals; however, the difference between means was only near statistical significance (paired t-test, \( p = 0.08 \)). Similarly, the formed metabolite to parent drug AUC ratio in MA after fetal drug administration (2.69 ± 3.87) was higher compared to the corresponding ratio after maternal drug administration (1.13 ± 1.44) in all the individual animals; however, again the difference between means was not statistically significant (paired t-test, \( p > 0.05 \)).
Table 3.7 - Gestational age and weight-normalized estimates of net (CL_{m(net)} and CL_{f(net)}), total (CL_{mm} and CL_{ff}), placental (CL_{mf} and CL_{fm}), and non-placental (CL_{mo} and CL_{fo}) DPHM clearances in the mother and the fetus, respectively, obtained using a 2-compartment model of the maternal-fetal unit.

<table>
<thead>
<tr>
<th>Ewe No.</th>
<th>Gestational Age (days)</th>
<th>Clearance Parameter (ml/min/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Maternal Experiment</td>
<td>Fetal Experiment</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E1225A</td>
<td>135</td>
<td>129</td>
</tr>
<tr>
<td>E2174d</td>
<td>140</td>
<td>135</td>
</tr>
<tr>
<td>E303Yc</td>
<td>124</td>
<td>131</td>
</tr>
<tr>
<td>E4227d</td>
<td>131</td>
<td>126</td>
</tr>
<tr>
<td>E4230d</td>
<td>131</td>
<td>126</td>
</tr>
<tr>
<td>Mean</td>
<td>132.2</td>
<td>129.4</td>
</tr>
<tr>
<td>± S.D.</td>
<td>± 5.9</td>
<td>± 3.8</td>
</tr>
</tbody>
</table>

a – clearance values are in ml/min/kg maternal weight; b – clearance values are in ml/min/kg estimated fetal weight.

c – Administration: [^{2}H_{10}]-DPHM (parent drug), DPMA (preformed metabolite); d – DPHM (parent drug), [^{2}H_{10}]-DPMA (preformed metabolite).

* – significantly higher compared to the corresponding maternal value (p<0.05).

CL_{m(net)}: Maternal net clearance; CL_{mm}: Maternal total clearance; CL_{mf}: Maternal placental clearance; CL_{mo}: Maternal non-placental clearance; CL_{f(net)}: Fetal net clearance; CL_{ff}: Fetal total clearance; CL_{fm}: Fetal placental clearance; CL_{fo}: Fetal non-placental clearance.
Table 3.8 – Arterial plasma AUC ratios of the parent drug, the preformed metabolite and the *in vivo* formed metabolite in the mother and the fetus.

<table>
<thead>
<tr>
<th>Ratio</th>
<th>Ewe No.</th>
<th>Mean ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1225&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2174&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>0.06</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>3.78</td>
<td>2.08</td>
</tr>
<tr>
<td></td>
<td>0.43</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>67.2</td>
<td>4.6</td>
</tr>
</tbody>
</table>

**Maternal Administration**

**Fetal Administration**

<p>| | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.02</td>
<td>0.02</td>
<td>0.01</td>
<td>0.01</td>
<td>0.05</td>
<td>0.02 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>0.07</td>
<td>0.08</td>
<td>0.15</td>
<td>0.06</td>
<td>0.87</td>
<td>0.25 ± 0.35</td>
</tr>
<tr>
<td></td>
<td>1.10</td>
<td>0.79</td>
<td>1.22</td>
<td>0.74</td>
<td>9.61</td>
<td>2.69 ± 3.87</td>
</tr>
<tr>
<td></td>
<td>4.11</td>
<td>1.49</td>
<td>2.86</td>
<td>1.39</td>
<td>1.72</td>
<td>2.32 ± 1.16</td>
</tr>
</tbody>
</table>

<sup>a</sup> – Administration: [<sup>1</sup>H<sub>10</sub>]-DPHM (parent drug), DPMA (preformed metabolite); <sup>b</sup> – DPHM (parent drug), [<sup>2</sup>H<sub>10</sub>]-DPMA (preformed metabolite).
3.2.5.3 Comparative Maternal-Fetal Pharmacokinetics of the Parent Drug, the Preformed Metabolite, and the in vivo Generated Metabolite

Figure 3.6 shows the representative plasma concentration vs. time profiles of the parent drug, the preformed metabolite, and the in vivo generated metabolite in maternal and fetal arterial plasma during separate maternal (Figure 3.6A) and fetal (Figure 3.6B) administration experiments in E303Y.

The peak plasma concentrations (C\text{max}) of the preformed DPMA in MA and FA after maternal metabolite administration were 602.9 ± 105.9 and 17.3 ± 5.5 ng/ml and occurred at 5 (first sampling time) and 336 ± 50 min (t\text{max}) after injection, respectively. Similarly, the C\text{max}'s of the preformed metabolite in MA and FA after fetal metabolite administration were 17.0 ± 9.3 and 1579.9 ± 274.3 ng/ml corresponding to a t\text{max} of 276 ± 54 and 5 min, respectively. The C\text{max}'s of the in vivo generated metabolite in MA and FA after maternal drug administration were 201.5 ± 281.4 ng/ml (at 389 ± 51 min) and 138.9 ± 115.8 (at 518 ± 137 min), respectively. The C\text{max}'s of the in vivo generated metabolite in MA and FA after fetal drug administration were 79.8 ± 130.3 and 130.8 ± 37.5 ng/ml at t\text{max}'s of 371 ± 12 and 430 ± 50 min, respectively.

Tables 3.9 and 3.10 present the comparative pharmacokinetic parameters of the parent drug, the preformed DPMA metabolite, and the in vivo formed DPMA metabolite in the ewe and the fetus, respectively. The total body clearance (CL\text{ib} = total i.v. dose/AUC) and steady-state volume of distribution (Vd\text{ss}) of the parent drug were significantly higher in the fetus compared to the ewe (unpaired t-test, p < 0.01 in both cases).
Figure 3.6 – Representative plasma concentration vs. time profiles of the parent drug, the preformed metabolite and the in vivo generated metabolite in maternal and fetal plasma (E303Y). Figures 3.6A and 3.6B are the data from separate maternal and fetal administration experiments, respectively. In both experiments, unlabeled DPMA was administered as the preformed metabolite in combination with $[^2\text{H}_{10}]$-DPHM. The $[^2\text{H}_{10}]$-DPMA is thus the in vivo generated metabolite.
However, the elimination half-life ($t_{1/2\beta}$) and mean residence time (MRT) of the parent drug in the mother and the fetus were not significantly different (unpaired t-test, $p > 0.05$ in both cases). The total body clearance of the preformed metabolite in the fetus was not significantly different from that in the mother (unpaired t-test, $p > 0.05$). The fetal $V_d^{ss}$, $t_{1/2\beta}$ and MRT of the preformed metabolite were, however, significantly higher compared to those in the mother (unpaired t-test, $p < 0.005$ in all cases). Also, in both the fetus and the mother, the $CL_{ib}$ and $V_d^{ss}$ of the parent drug were significantly higher compared to those of the preformed metabolite; the preformed metabolite $t_{1/2\beta}$ and MRT were, however, longer than those of the parent drug. In both the ewe and the fetus, the MRT of the in vivo formed metabolite was significantly longer compared to that of the preformed metabolite (paired t-test, $p < 0.05$). The percent of total parent drug dose converted to the DPMA metabolite in vivo in the mother ($1.72 \pm 2.01\%$) tended to be greater compared to the fetus ($0.32 \pm 0.11\%$) (but not statistically different, unpaired t-test, $p = 0.09$). However, the formation clearance of the metabolite as a percent fraction of non-placental clearance in the ewe ($1.78 \pm 2.12\%$) and the fetus ($0.87 \pm 0.56\%$) was not statistically different (unpaired t-test, $p > 0.05$).

### 3.2.5.4 Renal Elimination of the Parent Drug, the Preformed Metabolite, and the in vivo Generated Metabolite in the Mother and the Fetus

Figure 3.7 shows the representative cumulative excretion profiles of the parent drug, the preformed metabolite and the in vivo generated metabolite in maternal and fetal urine during separate maternal (Figure 3.7A) and fetal (Figure 3.7B) administration experiments in E303Y.
Figure 3.7 – Representative cumulative renal excretion profiles of the parent drug, the preformed metabolite and the in vivo generated metabolite in maternal and fetal urine (E303Y). Figures 3.7A and 3.7B are the data from separate maternal and fetal administration experiments, respectively. In both experiments, unlabeled DPMA was administered as the preformed metabolite in combination with $^{2}$H$_{10}$-DPHM. The $^{2}$H$_{10}$-DPMA is thus the in vivo generated metabolite. Figure 3.7B shows that even after fetal administration, negligible amounts of the preformed as well as the in vivo generated metabolite are excreted in fetal urine in comparison to maternal urine.
Table 3.9 - Pharmacokinetic parameters of the parent drug, the preformed metabolite, and the in vivo formed metabolite in the mother.

<table>
<thead>
<tr>
<th>Pharmacokinetic Parameter</th>
<th>E1225A&lt;sup&gt;c&lt;/sup&gt;</th>
<th>E2174&lt;sup&gt;d&lt;/sup&gt;</th>
<th>E303Y&lt;sup&gt;c&lt;/sup&gt;</th>
<th>E4227&lt;sup&gt;d&lt;/sup&gt;</th>
<th>E4230&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Mean ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Parent Drug</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CL&lt;sub&gt;tb&lt;/sub&gt; (ml/min/kg)</td>
<td>54.1</td>
<td>24.9</td>
<td>56.6</td>
<td>31.1</td>
<td>38.0</td>
<td>40.9 ± 14.0</td>
</tr>
<tr>
<td>V&lt;sub&gt;ds&lt;/sub&gt; (L/kg)</td>
<td>3.6</td>
<td>1.1</td>
<td>2.5</td>
<td>1.0</td>
<td>2.3</td>
<td>2.1 ± 1.1</td>
</tr>
<tr>
<td>t&lt;sub&gt;1/2b&lt;/sub&gt; (min)</td>
<td>49.0</td>
<td>56.5</td>
<td>33.7</td>
<td>83.1</td>
<td>63.6</td>
<td>57.2 ± 18.2</td>
</tr>
<tr>
<td>MRT (min)</td>
<td>67.1</td>
<td>42.7</td>
<td>43.7</td>
<td>89.3</td>
<td>61.0</td>
<td>60.8 ± 19.1</td>
</tr>
<tr>
<td><strong>Preformed Metabolite</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CL&lt;sub&gt;tb&lt;/sub&gt; (ml/min/kg)</td>
<td>0.86</td>
<td>0.43</td>
<td>0.46</td>
<td>0.48</td>
<td>0.52</td>
<td>0.55 ± 0.18*</td>
</tr>
<tr>
<td>V&lt;sub&gt;ds&lt;/sub&gt; (L/kg)</td>
<td>0.11</td>
<td>0.08</td>
<td>0.13</td>
<td>0.09</td>
<td>0.09</td>
<td>0.10 ± 0.02*</td>
</tr>
<tr>
<td>t&lt;sub&gt;1/2b&lt;/sub&gt; (min)</td>
<td>102.9</td>
<td>138.0</td>
<td>339.9</td>
<td>150.4</td>
<td>143.4</td>
<td>174.9 ± 94.0**</td>
</tr>
<tr>
<td>MRT (min)</td>
<td>130.1</td>
<td>187.4</td>
<td>282.7</td>
<td>185.1</td>
<td>175.3</td>
<td>192.1 ± 55.7**</td>
</tr>
<tr>
<td><strong>In vivo Formed Metabolite</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F&lt;sub&gt;m&lt;/sub&gt; (%)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.72</td>
<td>0.64</td>
<td>0.84</td>
<td>1.10</td>
<td>5.30</td>
<td>1.72 ± 2.01</td>
</tr>
<tr>
<td>F&lt;sub&gt;m'&lt;/sub&gt; (%)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.72</td>
<td>0.66</td>
<td>0.86</td>
<td>1.11</td>
<td>5.56</td>
<td>1.78 ± 2.12</td>
</tr>
<tr>
<td>MRT (min)</td>
<td>220.3</td>
<td>243.3</td>
<td>368.3</td>
<td>281.0</td>
<td>378.3</td>
<td>298.2 ± 71.9***</td>
</tr>
</tbody>
</table>

<sup>a</sup> - In vivo formation clearance of the metabolite as a fraction of total body clearance or fraction of the parent drug dose converted to the metabolite in vivo.  
<sup>b</sup> - In vivo formation clearance of the metabolite as a fraction of non-placental clearance.  
<sup>c</sup> - Administration: [<sup>2</sup>H<sub>10</sub>]-DPHM (parent drug), DPMA (preformed metabolite);  
<sup>d</sup> - DPHM (parent drug), [<sup>2</sup>H<sub>10</sub>]-DPMA (preformed metabolite).  
* - significantly lower than the corresponding parameter for the parent drug; ** - significantly longer than the corresponding parameter of the parent drug; *** - significantly longer than the corresponding parameter of the preformed metabolite (p < 0.05 in all cases).  
CL<sub>tb</sub> - total body clearance; V<sub>ds</sub> - steady-state volume of distribution; t<sub>1/2b</sub> - terminal elimination half-life; MRT - mean residence time.
Table 3.10 - Pharmacokinetic parameters of the parent drug, the preformed metabolite, and the in vivo formed metabolite in the fetus.

<table>
<thead>
<tr>
<th>Pharmacokinetic Parameter</th>
<th>E1225A&lt;sup&gt;c&lt;/sup&gt;</th>
<th>E2174&lt;sup&gt;d&lt;/sup&gt;</th>
<th>E303Y&lt;sup&gt;c&lt;/sup&gt;</th>
<th>E4227&lt;sup&gt;d&lt;/sup&gt;</th>
<th>E4230&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Mean ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Parent Drug</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CL&lt;sub&gt;tb&lt;/sub&gt; (ml/min/kg)</td>
<td>465.0</td>
<td>304.6</td>
<td>294.1</td>
<td>128.7</td>
<td>235.6</td>
<td>285.6 ± 122.2</td>
</tr>
<tr>
<td>V&lt;sub&gt;dss&lt;/sub&gt; (L/kg)</td>
<td>18.1</td>
<td>10.5</td>
<td>13.4</td>
<td>10.6</td>
<td>13.0</td>
<td>13.1 ± 3.1</td>
</tr>
<tr>
<td>t&lt;sub&gt;1/2β&lt;/sub&gt; (min)</td>
<td>14.4</td>
<td>19.1</td>
<td>21.5</td>
<td>65.4</td>
<td>44.9</td>
<td>33.1 ± 21.6</td>
</tr>
<tr>
<td>MRT (min)</td>
<td>38.9</td>
<td>34.5</td>
<td>45.7</td>
<td>82.0</td>
<td>55.3</td>
<td>51.3 ± 18.9</td>
</tr>
<tr>
<td><strong>Preformed Metabolite</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CL&lt;sub&gt;tb&lt;/sub&gt; (ml/min/kg)</td>
<td>0.50</td>
<td>0.38</td>
<td>0.38</td>
<td>0.19</td>
<td>0.40</td>
<td>0.37 ± 0.11*</td>
</tr>
<tr>
<td>V&lt;sub&gt;dss&lt;/sub&gt; (L/kg)</td>
<td>0.43</td>
<td>0.40</td>
<td>0.48</td>
<td>0.32</td>
<td>0.37</td>
<td>0.40 ± 0.06*</td>
</tr>
<tr>
<td>t&lt;sub&gt;1/2β&lt;/sub&gt; (min)</td>
<td>630.4</td>
<td>762.3</td>
<td>895.3</td>
<td>1180.1</td>
<td>861.9</td>
<td>866.0 ± 203.6**</td>
</tr>
<tr>
<td>MRT (min)</td>
<td>868.4</td>
<td>1049.6</td>
<td>1237.3</td>
<td>1662.5</td>
<td>937.4</td>
<td>1151.1 ± 318.1**</td>
</tr>
<tr>
<td><strong>In vivo Formed Metabolite</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F&lt;sub&gt;m&lt;/sub&gt; (%)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.46</td>
<td>0.20</td>
<td>0.39</td>
<td>0.22</td>
<td>0.30</td>
<td>0.32 ± 0.11</td>
</tr>
<tr>
<td>F&lt;sub&gt;m'&lt;/sub&gt; (%)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.80</td>
<td>0.33</td>
<td>0.83</td>
<td>0.58</td>
<td>0.81</td>
<td>0.87 ± 0.56</td>
</tr>
<tr>
<td>MRT (min)</td>
<td>1008.3</td>
<td>1140.7</td>
<td>1407.1</td>
<td>2103.5</td>
<td>1349.3</td>
<td>1401.8 ± 423.7***</td>
</tr>
</tbody>
</table>

<sup>a</sup> - In vivo formation clearance of the metabolite as a fraction of total body clearance or fraction of the parent drug dose converted to the metabolite in vivo.  
<sup>b</sup> - In vivo formation clearance of the metabolite as a fraction of non-placental clearance.  
<sup>c</sup> - Administration: [²H₁₀]-DPHM (parent drug), DPMA (preformed metabolite);  
<sup>d</sup> - DPHM (parent drug), [²H₁₀]-DPMA (preformed metabolite).

* - significantly lower than the corresponding parameter for the parent drug; ** - significantly longer than the corresponding parameter of the parent drug; *** - significantly longer than the corresponding parameter of the preformed metabolite (p < 0.05 in all cases).

CL<sub>tb</sub> - total body clearance; V<sub>dss</sub> - steady-state volume of distribution; t<sub>1/2β</sub> - terminal elimination half-life; MRT - mean residence time.
Table 3.11 - Pharmacokinetic parameters of the renal elimination of the parent drug, the preformed metabolite, and the *in vivo* formed metabolite in maternal and fetal sheep.

<table>
<thead>
<tr>
<th>Pharmacokinetic Parameter</th>
<th>E1225A&lt;sup&gt;c&lt;/sup&gt;</th>
<th>E2174&lt;sup&gt;d&lt;/sup&gt;</th>
<th>E303Y&lt;sup&gt;c&lt;/sup&gt;</th>
<th>E4227&lt;sup&gt;d&lt;/sup&gt;</th>
<th>E4230&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Mean ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Maternal</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parent Drug</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CL&lt;sub&gt;r&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.07</td>
<td>0.03</td>
<td>0.13</td>
<td>0.01</td>
<td>0.15</td>
<td>0.08 ± 0.06</td>
</tr>
<tr>
<td>% dose excreted in maternal urine</td>
<td>0.14</td>
<td>0.11</td>
<td>0.22</td>
<td>0.09</td>
<td>0.38</td>
<td>0.19 ± 0.12</td>
</tr>
<tr>
<td>Preformed Metabolite</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CL&lt;sub&gt;r&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.79</td>
<td>0.36</td>
<td>0.37</td>
<td>0.46</td>
<td>0.48</td>
<td>0.49 ± 0.17</td>
</tr>
<tr>
<td>% dose excreted in maternal urine</td>
<td>91.4</td>
<td>83.5</td>
<td>79.0</td>
<td>94.7</td>
<td>91.5</td>
<td>88.0 ± 6.5</td>
</tr>
<tr>
<td><em>in vivo</em> Formed Metabolite</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CL&lt;sub&gt;r&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.86</td>
<td>0.43</td>
<td>0.51</td>
<td>0.35</td>
<td>0.48</td>
<td>0.53 ± 0.19</td>
</tr>
<tr>
<td>Amount excreted in maternal urine (as % of parent drug dose)</td>
<td>0.72</td>
<td>0.64</td>
<td>0.93</td>
<td>0.80</td>
<td>4.92</td>
<td>1.60 ± 1.86</td>
</tr>
<tr>
<td><strong>Fetal</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parent Drug</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CL&lt;sub&gt;r&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.00</td>
<td>N/A&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.20</td>
<td>0.13</td>
<td>N/A&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.44 ± 0.49</td>
</tr>
<tr>
<td>% dose excreted in fetal urine</td>
<td>0.22</td>
<td>N/A&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.07</td>
<td>0.10</td>
<td>N/A&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.13 ± 0.08</td>
</tr>
<tr>
<td>Preformed Metabolite</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CL&lt;sub&gt;r&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.005</td>
<td>N/A&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.002</td>
<td>0.014</td>
<td>N/A&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.007 ± 0.006*</td>
</tr>
<tr>
<td>% dose excreted in fetal urine</td>
<td>0.78</td>
<td>N/A&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.40</td>
<td>4.18</td>
<td>N/A&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.79 ± 2.08</td>
</tr>
<tr>
<td>% dose excreted in maternal urine</td>
<td>88.5</td>
<td>92.48</td>
<td>82.1</td>
<td>96.3</td>
<td>101.4</td>
<td>92.1 ± 7.4</td>
</tr>
<tr>
<td><em>in vivo</em> Formed Metabolite</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CL&lt;sub&gt;r&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.007</td>
<td>N/A&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.004</td>
<td>0.011</td>
<td>N/A&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.007 ± 0.004*</td>
</tr>
<tr>
<td>Amount excreted in fetal urine (as % of parent drug dose)</td>
<td>0.004</td>
<td>N/A&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.002</td>
<td>0.005</td>
<td>N/A&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.004 ± 0.002</td>
</tr>
</tbody>
</table>

<sup>a</sup> - Renal clearance in ml/min/kg.

<sup>b</sup> - Samples not available.

<sup>c</sup> - Administration: [3H<sub>10</sub>]-DPH (parent drug), DPMA (preformed metabolite);
<sup>d</sup> - DPHM (parent drug), [3H<sub>10</sub>]-DPMA (preformed metabolite).

* - significantly lower than the maternal renal clearance of the same compound (p < 0.05).
Table 3.11 presents the comparative pharmacokinetic parameters of the renal elimination of the parent drug, the preformed metabolite and the metabolite generated in vivo in the mother and the fetus. Fetal renal clearance (CL$_r$) of the parent drug was higher (but not significantly so) compared to that of the mother in the three animals where fetal urine was collected. In contrast, maternal renal clearance of the preformed as well as the in vivo formed metabolite was significantly greater than that of the fetus in these three animals (unpaired t-test, p < 0.05). However, there was no significant difference between CL$_r$ of the preformed and the in vivo generated metabolite in the mother or the fetus (paired t-test, p > 0.05). Renal clearance of the preformed metabolite accounted for 88.8 ± 6.5 % of its total body clearance in the mother, and only 3.0 ± 3.8 % in the fetus. Consequently, a significantly greater percentage of the maternal i.v. dose of the preformed metabolite was excreted in maternal urine (88.0 ± 6.5 %) as compared to the excretion of the fetal dose into fetal urine (1.79 ± 2.08%). Instead the majority (92.1 ± 7.4%) of the fetal i.v. dose of the preformed metabolite was eventually recovered in maternal urine (Table 3.11).

3.2.6 Study F: Disposition of the DPHM-N-oxide Metabolite in the Maternal-Fetal Unit

As mentioned earlier, maternal and fetal plasma and urine concentrations of DPHMNOX (or [2H$_0$]-DPHMNOX) were measured in four out of five animals (E1225A, E2174, E303Y and E4230) employed above in study E. Figure 3.8 shows the representative maternal and fetal plasma concentration vs. time profiles of the N-oxide metabolite in relation to those of the parent drug in E4230 after maternal (Figure 3.8A) and fetal (Figure 3.8B) drug administration. Table 3.12 presents the maternal and fetal arterial plasma AUC's of the N-oxide metabolite and also the corresponding FA/MA AUC ratios during maternal as well as fetal drug administration.
Figure 3.8 – Representative maternal and fetal arterial plasma profiles of the parent drug and the N-oxide metabolite in E4230 after A) 6 h maternal DPHM infusion (670 µg/min), B) 6 h fetal DPHM infusion (170 µg/min).
Renal clearances of the N-oxide metabolite, and the percentages of the total administered parent drug dose excreted in maternal and fetal urine as the N-oxide metabolite are presented in Table 3.13.

Table 3.12 – Maternal and fetal plasma AUC's of the DPHM-N-oxide (or [\(^{2}\text{H}_{10}\)]-DPHM-N-oxide) metabolite in four pregnant sheep during separate maternal and fetal 6 h DPHM (or [\(^{2}\text{H}_{10}\)]-DPHM) infusion.

<table>
<thead>
<tr>
<th>Ewe #</th>
<th>Arterial Plasma AUC (ng.min/ml)</th>
<th>FA/MA Ratio of the Metabolite</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Maternal Plasma (MA)</td>
<td>Fetal Plasma (FA)</td>
</tr>
<tr>
<td>Maternal Administration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E1225A(^a)</td>
<td>1762.2</td>
<td>3069.2</td>
</tr>
<tr>
<td>E2174(^b)</td>
<td>2107.0</td>
<td>3073.3</td>
</tr>
<tr>
<td>E303Y(^a)</td>
<td>2885.4</td>
<td>5767.3</td>
</tr>
<tr>
<td>E4230(^b)</td>
<td>16150.7</td>
<td>11137.5</td>
</tr>
<tr>
<td>Mean</td>
<td>5726.3</td>
<td>5761.8</td>
</tr>
<tr>
<td>± S.D.</td>
<td>± 6965.4</td>
<td>± 3802.5</td>
</tr>
<tr>
<td>Fetal Administration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E1225A(^a)</td>
<td>384.5</td>
<td>8112.9</td>
</tr>
<tr>
<td>E2174(^b)</td>
<td>795.0</td>
<td>15356.2</td>
</tr>
<tr>
<td>E303Y(^a)</td>
<td>616.5</td>
<td>20447.5</td>
</tr>
<tr>
<td>E4230(^b)</td>
<td>1846.1</td>
<td>34494.6</td>
</tr>
<tr>
<td>Mean</td>
<td>910.5</td>
<td>19602.8</td>
</tr>
<tr>
<td>± S.D.</td>
<td>± 646.0</td>
<td>± 11143.5</td>
</tr>
</tbody>
</table>

\(^a\) – in these ewes [\(^{2}\text{H}_{10}\)]-DPHM was infused.
\(^b\) – in these ewes unlabeled DPHM was infused.
* - significantly greater than the corresponding ratio after maternal administration (p < 0.05).
Table 3.13 – Pharmacokinetics of the renal elimination of the DPHM-N-oxide metabolite in the mother and the fetus.

<table>
<thead>
<tr>
<th>Ewe #</th>
<th>Maternal</th>
<th>Fetal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Renal Clearance (ml/min/kg)</td>
<td>% Dose Excreted as the N-oxide Metabolite</td>
</tr>
<tr>
<td>E1225A</td>
<td>5.1</td>
<td>0.31</td>
</tr>
<tr>
<td>E2174</td>
<td>1.4</td>
<td>0.14</td>
</tr>
<tr>
<td>E303Y</td>
<td>2.3</td>
<td>0.16</td>
</tr>
<tr>
<td>E4230</td>
<td>0.74</td>
<td>0.34 ± 0.28</td>
</tr>
</tbody>
</table>

Mean ± S.D. 2.6 ± 1.7

- in these ewes, [²H₁₀]-DPHM was infused.
- in these ewes, unlabeled DPHM was infused.
- maternal renal clearances were obtained from the data during maternal drug administration, whereas fetal renal clearances were obtained from the data during fetal drug administration.
- samples not available.

3.3 Discussion

3.3.1 Study A: Paired Maternal (DPHM) and Fetal ([²H₁₀]-DPHM) Infusions for the Determination of Placental and Non-Placental Clearances

This study utilized simultaneous administration of unlabeled (DPHM) and deuterium labeled DPHM ([²H₁₀]-DPHM) for the determination of maternal and fetal placental and non-placental clearances in a single experiment. The primary reason for employing this methodology in this study was to eliminate the potential confounding effects of fetal growth and maturation that may occur over the period between time-separated maternal and fetal drug infusions. This approach also reduces the overall duration of the experiment. This is an important factor in studies involving chronically-instrumented pregnant animals where there is a finite time window available for each preparation, and hence shorter experiments
allow for additional studies to be conducted on the same animal. However, it is important that the labeled and unlabeled forms of the drug be biologically equivalent (Baillie, 1981). If this is not so, the labeled drug could display different dispositional characteristics compared to the unlabeled drug, and thus be of limited use in pharmacokinetic studies (Baillie, 1981). Bioequivalence of DPHM and $^{2}$H$_{10}$-DPHM in terms of their pharmacokinetic characteristics and also in their metabolism to DPMA and $^{2}$H$_{10}$-DPMA metabolites has previously been demonstrated in this lab (Tonn, 1995).

3.3.1.1 DPHM and $^{2}$H$_{10}$-DPHM Plasma Concentrations in the Ewe and Fetus

The average total fetal plasma concentration of diphenhydramine achieved in the current experiments (i.e., DPHM + $^{2}$H$_{10}$-DPHM = ~289 ng/ml) lies between the two plasma concentrations achieved in the time-separated maternal (~36 ng/ml) and fetal infusions (~450 ng/ml) in an earlier study in this lab (Yoo et al., 1993). In the ewe, the total drug concentration in the current study (305 ng/ml) exceeds the levels achieved in the previous maternal (212 ng/ml) and fetal (31 ng/ml) infusions.

3.3.1.2 Placental and Non-Placental Clearances of DPHM in Fetal and Maternal Sheep

The maternal and fetal clearance values obtained for DPHM in the current study are similar to those determined previously (Yoo et al., 1993). In particular, it has been confirmed that fetal placental clearance of the drug is much higher than the maternal placental clearance. Similarly, the weight-normalized fetal non-placental clearance is also greater than that in the ewe. In the current study, CL$_{fm}$ was 5.4 fold higher than CL$_{mf}$, whereas previous estimates were 3.7 times higher based upon total drug concentrations,
and 1.6 times higher based upon unbound drug concentrations (Yoo et al., 1993). The fetal placental and non-placental clearance values for DPHM are the highest of any drug yet examined in pregnant sheep, and this is also the case with the CLfm-CLmf difference. Given the overall agreement between the results of two studies, we conclude that this latter difference is not an artefactual result of the time-separated maternal and fetal infusions in the previous studies that have employed the 2-compartment open model.

3.3.1.3 Maternal and Fetal Plasma Concentrations of DPMA and [²H₁₀]-DPMA

In humans, monkeys, and dogs, DPHM appears to be metabolized via two sequential N-demethylation steps followed by deamination to DPMA (see Chapter 1, Section 1.4.3). This DPMA metabolite and its conjugates are the major urinary metabolites of DPHM in these species (Chang et al., 1974; Drach and Howell; 1968, Drach et al., 1970; Glazko et al., 1974). DPMA is also present in the urine and plasma of non-pregnant ewes following DPHM administration (Tonn et al., 1995). In the present study, DPMA and [²H₁₀]-DPMA were detected in both maternal and fetal plasma during and following the simultaneous infusions of DPHM and [²H₁₀]-DPHM to the ewe and fetus, respectively. The consistently higher concentrations of the labeled metabolite in fetal plasma compared to those in the mother during the fetal [²H₁₀]-DPHM infusion provide strong evidence for its formation in the fetus. The presence of DPMA in the fetus and [²H₁₀]-DPMA in the ewe could be the result of two processes: 1) placental transfer of DPHM to the fetus and [²H₁₀]-DPHM to the ewe, with subsequent formation of the unlabeled and labeled metabolites in fetal and maternal compartments, respectively; and 2) fetal to maternal transfer of [²H₁₀]-DPMA and maternal to fetal transfer of DPMA. However, it seems unlikely that the latter process could be of much importance, since the minimal umbilical extraction ratio of [²H₁₀]-DPMA
and the long fetal elimination half-lives of the labeled and unlabeled metabolite (~15 h) suggest its limited transfer across the ovine placenta. This is perhaps due to a greater polarity of the metabolite compared to DPHM and its high degree of plasma protein binding (~99%) (Tonn, 1995). However, a larger maternal plasma ratio for $\frac{AUC_{\text{DPMA}}}{AUC_{\text{DPHCM}}} (0.96\pm 0.26)$ compared to $\frac{AUC_{\text{DPHCM}}}{AUC_{\text{DPHCM}}} (0.62\pm 0.15)$ suggests that at least a portion of the labeled metabolite that is formed in the fetus is transferred to the mother. A long half-life of the metabolite in the fetus also suggests that the elimination pathways for this metabolite may not be fully developed in the fetus as compared to the ewe. Our subsequent studies, involving fetal bolus administration of DPMA, have in fact indicated that virtually all of the administered dose ultimately appears in maternal urine over the ensuing 96 h (see Section 3.2.5.4 in this Chapter). This confirms that DPMA can cross the sheep placenta, albeit at a very slow rate. It also suggests that both the fetus and the ewe have no detectable ability to secondarily metabolize DPMA (see Section 3.3.4 later in this chapter for more details).

3.3.2 Studies A and B: Fetal Hepatic Uptake and Metabolism of DPHM

3.3.2.1 Evidence of Fetal Hepatic First-Pass DPHM Uptake from the Umbilical Vein

In a previous study (Tonn et al., 1996), the fetal hepatic first-pass uptake of DPHM following umbilical venous drug administration after both bolus and constant rate i.v. infusion was examined. However, no evidence of fetal hepatic first-pass DPHM uptake was obtained. In contrast, a substantial (>90%) hepatic presystemic elimination of the drug was observed after portal venous administration in adult sheep (see Section 3.2.3). However, the results from the fetal experiments did not completely rule out the
involvement of fetal liver in DPHM metabolism/DPMA formation because we subsequently detected the formation of DPMA from DPHM in fetal hepatic microsomes at rates similar to those in maternal liver microsomes (S. Kumar, G.R. Tonn, K.W. Riggs and D.W. Rurak, unpublished data). The data from Study A on DPMA/DPHM (8.20 ± 3.62) and [\(^{2}\text{H}_{10}]\)-DPMA/[\(^{2}\text{H}_{10}]\)-DPHM AUC (2.24 ± 1.19) ratios in the fetus (Table 3.2) indicate that more of the maternally-derived form of the drug (reaching the fetus via the umbilical vein and hence undergoing a 'partial' fetal hepatic first-pass) is converted to the metabolite than is the form administered directly to the fetus. These AUC ratios clearly demonstrate that the fetal liver is involved in the metabolism and non-placental clearance of the drug. Using equation 7 and the parent drug and metabolite AUC ratios from this study, the fetal hepatic first-pass extraction ratio of DPHM present in umbilical venous blood averaged 0.71 ± 0.16. However, using the DPHM, [\(^{2}\text{H}_{10}]\)-DPHM, DPMA and [\(^{2}\text{H}_{10}]\)-DPMA concentrations measured in samples from the previous umbilical hepatic first-pass experiments (Study B) and equation 10 (analogous to equation 7; sections 3.1.6.1 and 3.1.6.2), a mean value of 0.44 ± 0.14 was obtained. We feel that the higher estimate of this parameter obtained in the paired infusion study (Study A) is due to maternal to fetal transfer of a portion of the maternally formed DPMA to artefactually increase the fetal AUC_{DPMA} / AUC_{DPHM} ratio. In the fetal hepatic first-pass study (Study B), labeled and unlabeled DPHM were both administered to the fetus, so that maternal to fetal transfer of intact drug or metabolite was unlikely. Thus the mean value of 0.44 for the fetal hepatic DPHM extraction ratio estimated from direct fetal umbilical venous administration is likely more accurate.

The failure to detect a fetal hepatic first-pass effect in the previous study (Tonn et al., 1996), which measured only the concentrations of DPHM and [\(^{2}\text{H}_{10}]\)-DPHM, may have
resulted from the geometry and hemodynamics of the fetal circulation combined with a high placental permeability for the intact drug. In terms of the former factors, a greater portion of the umbilical and fetal hepatic venous return is preferentially distributed to the upper body, with only ~20% reaching the placenta in one circulation time (Edelstone and Rudolph, 1979; Reuss and Rudolph, 1980). In contrast, ~50% of inferior vena caval blood reaches the placenta (Edelstone and Rudolph, 1979; Reuss and Rudolph, 1980). This is likely a physiological mechanism for the fetus to preferentially supply highly oxygenated nutrient rich umbilical venous blood to vital organs such as the brain and heart, and channel the deoxygenated inferior vena caval blood to the placenta for reoxygenation. In Study A above, approximately 60% of the drug delivered to the placenta was extracted at this site. Thus, after one pass through the fetal circulation, the average systemic availability of the drug (DPHM or $[^2\text{H}_{10}]-\text{DPHM}$) injected via the umbilical vein will be ~50%. This is because on average ~50% of the drug injected via the umbilical vein is extracted in a single pass through the circulation (calculated as the fraction removed via hepatic first-pass extraction [~44%], plus the fraction extracted by the placenta [~6%], for a total of ~50%). Similarly, after a single pass through the fetal circulation, the systemic availability of the drug administered at the inferior vena cava (tarsal vein) will be ~70%, since ~30% of the drug will be extracted at the placenta. In addition to this factor, fetal circulatory transit times from the umbilical vein to the placenta (~5.1 sec) and from inferior vena cava to the placenta (~3.7 sec) are different (Power and Longo, 1975). Also, the transit time to the placenta for the ~50% of the umbilical blood flow that passes through the fetal liver is even longer (~9.8 sec, Power and Longo, 1975). Due to all the above described factors, the drug administered at the umbilical site likely experiences a 'fetal hepatic first-pass effect', while that administered at the tarsal venous site experiences a 'placental first-pass effect' (see Chapter 1,
Figure 1.3). Thus, even though fetal hepatic first-pass DPHM uptake from umbilical venous blood was present, the high placental extraction of drug administered via the tarsal vein may act to nullify the difference between systemic concentrations (AUC’s or $C_{ss}$) of the two forms of the parent drug. This would result in minimal and inconsistent differences in the systemic arterial levels and AUC’s of the two forms of drug and thus to an apparent lack of fetal hepatic DPHM elimination, as was concluded in the previous study (Tonn et al. 1996). However, if we assume that fetal hepatic DPHM elimination is via its metabolism in the fetal liver whereas placental elimination involves simple drug transfer to the maternal circulation, there should be differences in the fetal plasma concentrations of the DPHM metabolite (e.g., DPMA and $^{13}$C$_{10}$-DPMA) formed from the two forms of the drug. Also, these differences are more likely to be maintained over time because of the limited placental permeability of more polar and highly protein bound DPMA (see above) in contrast to the parent drug which can readily cross the placenta. In agreement with this, a consistent concentration difference between the labeled and unlabeled forms of DPMA in fetal arterial plasma (resulting from the hepatic first-pass uptake of umbilically administered drug and subsequent formation of higher amounts of metabolite from this form of the drug) was observed (Study B) (Figure 3.3).

The data on fetal plasma DPMA and $^{13}$C$_{10}$-DPMA concentrations allowed an indirect estimation of the fetal hepatic first-pass extraction of the parent drug after umbilical venous administration by using equation 10 (section 3.1.6.2). The estimate of 0.44 for fetal hepatic DPHM extraction is less than that determined in adult sheep (0.94; also see Section 3.2.3). However, ~50% of the umbilical venous return bypasses the fetal liver via the ductus venosus (Holzman, 1984), and thus drug present in this blood is not available for hepatic first-pass uptake. When the fetal hepatic extraction estimate is corrected for
this, it approaches the adult value (0.88 vs. 0.94). Consequently, it appears that the liver of the fetal lamb in late gestation is quite effective in metabolizing DPHM. Moreover, when this hepatic extraction estimate of 0.88 is multiplied by a published value for total hepatic blood flow in the fetal lamb (137 ml/min/kg fetal weight, Edelstone et al., 1978), the resulting estimate of fetal hepatic clearance is ~120 ml/min/kg. This can account for the entire CLf0 of DPHM (Table 3.1), suggesting that the fetal liver is the major organ responsible for fetal non-placental clearance of DPHM. Renal clearance of DPHM contributes only ~2% to CLf0 (Tonn, 1995), and previously we found that fetal pulmonary extraction of DPHM contributes another 8% (Rurak et al., 1991). Thus, the combined average clearances of the liver, kidney and lung are somewhat greater than our current estimate of CLf0. However, given that fetal hepatic extraction is estimated by an indirect method and is only an average estimate, and that the other data come from different studies, this difference is not unreasonably great. The metabolic fate of the DPHM taken up by the fetal liver was the subject of our subsequent studies (Studies E and F in this chapter).

There have been few other attempts to directly examine the role of the fetal liver in drug elimination/metabolism. In fact, there appears to be only one study where fetal hepatic extraction of propranolol was measured in utero (Mihaly et al., 1982b). This study employed the Fick method and quantified fetal sheep hepatic propranolol extraction by simultaneously measuring drug concentrations in umbilical, portal and right hepatic veins. An average drug extraction of 35 ± 12% was demonstrated from the umbilical venous blood supplying the fetal liver. In contrast, there was no apparent difference between drug concentrations in portal and right hepatic venous plasma. These data were explained by the fact that the left lobe of the fetal liver receives ~93% of its total blood supply from the
umbilical venous flow whereas this figure is only ~60% for the right lobe, with the rest being supplied by the portal vein (hepatic artery supplies only ~5% of the total fetal hepatic blood flow) (Edelstone et al., 1978; Holzman, 1984). Since the portal venous blood is relatively poorly oxygenated as compared to the umbilical venous blood, the right fetal hepatic lobe may also be less well oxygenated as compared to the left lobe. Thus, apparent differences in fetal hepatic propranolol extraction from the umbilical and portal venous blood were postulated to be due to the differences in oxygenation of the two fetal hepatic lobes (Mihaly et al., 1982b). Although direct evidence for this phenomenon is lacking and an accurate estimation of the fetal hepatic extraction of propranolol by the above approach would require additional sampling from the left fetal hepatic vein, these data in combination with our data on DPHM do indicate additional levels of complexity in the study of fetal hepatic disposition of xenobiotics. If indeed there are differences in the metabolic capacity of the right and left fetal hepatic lobes, the above estimate of a total fetal hepatic DPHM extraction of 88% (2 x 44%) may be an overestimate.

More recently, an isolated perfused fetal lamb liver preparation, with perfusion via the umbilical vein and simultaneous measurement of the ductus venosus shunt fraction (see Figure 1.3), has been utilized to study fetal hepatic drug disposition. Using this experimental system, significant uptake and metabolism of propranolol and p-nitrophenol by the late-gestation fetal lamb liver has been demonstrated (Ring et al., 1995; Ring et al., 1996). Also, under these in vitro conditions, where the level of oxygenation of the right and left lobes of the fetal liver was similar, the two lobes were found to be equally effective in propranolol uptake (Ring et al., 1998). Overall, significant fetal lamb hepatic uptake of DPHM appears to be in line with these studies of other compounds and indicates an important role of the fetal liver in drug elimination.
3.3.2.2 Impact of Fetal Hepatic Drug Uptake on the 2-Compartment Model Estimates of Maternal and Fetal Clearances

The placental clearance values calculated using the 2-compartment model are the "fundamental" clearances of the maternal-placental-fetal system and can be used to estimate the maximum possible rate of drug transfer across the placenta (under given conditions of blood flow and protein binding) assuming sink conditions on the other side. These clearance parameters are thus reflective of the true placental permeability of the drug in question. This is in contrast to the net rate of placental drug flux, which depends only upon the rate of non-placental drug elimination on the other side of the placenta.

It is important to realize that the proposed 2-compartment model incorporates both maternal as well as fetal drug elimination. Hence, after maternal or fetal drug administration, this system never reaches a state of equilibrium (defined as equal bidirectional drug fluxes and no net transfer of the drug across the placenta). It does, however, reach a steady-state where the rate of drug flux across the placenta becomes equal to the rate of drug elimination from the other side of the placenta. There could be 2 possible situations:

a) **Steady-state maternal drug administration:** In this case, there is net maternal-to-fetal drug transfer. The rate of this transfer is equal to the rate of drug elimination from the fetus via non-placental pathways, i.e.,

\[ \text{CL}_{\text{mf}} \times C_{\text{mss}} - \text{CL}_{\text{fm}} \times C_{\text{fss}} = \text{CL}_{\text{fo}} \times C_{\text{fss}} \]

b) **Steady-state fetal drug administration:** In this case, there is net fetal-to-maternal drug transfer and the rate of this transfer is equal to the rate of non-placental drug elimination from the mother, i.e.,

\[ \text{CL}_{\text{fm}} \times C_{\text{fss}}' - \text{CL}_{\text{mf}} \times C_{\text{mss}}' = \text{CL}_{\text{mo}} \times C_{\text{mss}}' \]
Hence, after maternal steady-state drug administration, the maternal-to-fetal drug flux always exceeds that in the opposite direction. The situation is reversed after fetal drug administration and fetal-to-maternal flux exceeds that in the opposite direction.

Now consider a hypothetical situation where equal steady-state drug concentrations are achieved in the mother and the fetus after separate drug administration. In this case, the maximal rate of maternal-to-fetal drug flux after maternal administration should be equal to the maximal rate of fetal-to-maternal drug flux after fetal administration. This is because of the presence of the same drug diffusion barrier in both directions (i.e., placenta) and similar rate of drug delivery to the placenta (due to relative equality between maternal and fetal placental blood flows and equal drug concentrations). By definition, the maximal placental drug flux rate is a product of placental clearance and steady-state drug concentration (maternal-to-fetal flux = $CL_{mf}C_m$, and fetal-to-maternal flux = $CL_{fm}C_f'$). Since we are assuming equal drug concentrations ($C_m$ and $C_f'$ after separate maternal and fetal drug administration, respectively), the placental clearance ($CL_{mf}$ and $CL_{fm}$) in both directions should be equal. Also, in linear pharmacokinetic systems, clearance is constant at all drug concentrations. Thus, $CL_{mf}$ and $CL_{fm}$ should be equal at all rates of drug infusion as long as the assumption of linearity holds. However, as noted in the Introduction (Chapter 1), with the exception of acetaminophen, all drugs studied in pregnant sheep have values of $CL_{fm}$ that are greater than $CL_{mf}$ (Table 1.1).

In the case of DPHM, we believe that the lower value of $CL_{mf}$ compared to $CL_{fm}$ is in part due to fetal hepatic first-pass uptake of the maternally administered form of the drug that reaches the fetus via the umbilical vein. As noted above, we feel that the value of 0.44 is a more accurate estimate of this fetal hepatic extraction of DPHM. Since ~44% of the
maternally-derived drug present in umbilical venous blood will not reach the fetal systemic arterial circulation, its steady-state concentration ($C_f$) and AUC in fetal arterial plasma will be reduced by approximately the same magnitude. Thus, the clearance estimates obtained by using equations 1-6 (section 3.1.6.1) will be biased by this reduction in $C_f$. When these clearance estimates are corrected for fetal hepatic first-pass extraction (using a systemic availability of 0.56 for maternally-derived DPHM), $CL_{mm}$ (39.4 vs 38.3 ml/min/kg, corrected vs. uncorrected), $CL_{ff}$ (332.1 vs. 324.2 ml/min/kg), $CL_{fm}$ (219.7 vs. 214.4 ml/min/kg), $CL_{mo}$ (36.1 vs. 36.6 ml/min/kg) and $CL_{fo}$ (112.4 vs. 109.8 ml/min/kg) are only minimally altered. However, the corrected estimate of $CL_{mf}$ (92.6 ml/min/kg) is substantially higher than the uncorrected value (50.3 ml/min/kg). It is still lower than the $CL_{fm}$ value (214.4 ml/min/kg). However, it is generally assumed that only the unbound drug can diffuse across the placenta, and we have previously reported that the free fraction of DPHM in fetal plasma (0.277) is significantly higher than that in maternal plasma (0.141, Yoo et al., 1993). When $CL_{mf}$ is corrected for the difference in maternal and fetal plasma unbound fractions of DPHM (i.e., by a factor of 1.96), the estimate of $CL_{mf}$ (181.9 ml/min/kg) approaches $CL_{fm}$. Thus, the apparent difference between $CL_{mf}$ and $CL_{fm}$ for DPHM, when the 2-compartment open model is employed to determine the clearance values, appears to be due to two factors: a difference in maternal and fetal plasma protein binding and a significant first-pass uptake of maternally derived drug by the fetal liver.

As noted in Chapter 1, the estimates of $CL_{mf}$ are lower than those for $CL_{fm}$ for all drugs studied using the 2-compartment open model, with the exception of acetaminophen. Some of these drugs (e.g., morphine) are not bound to any significant extent in maternal and fetal plasma, and for others (e.g., methadone) the placental clearance difference
remains even after the maternal and fetal differences in plasma protein binding of the drug are taken into account. Thus, the CL_{fm}-CL_{mf} clearance difference could largely be due to fetal hepatic first-pass uptake of maternally-derived drug for many of these drugs. The calculated clearance values could thus be in error, but this could only be determined by obtaining an estimate of fetal hepatic extraction from umbilical venous blood for each drug. However, from another viewpoint, the difference in maternal and fetal placental clearances of these drugs may provide evidence for significant fetal hepatic clearance of these compounds. In Figure 3.9, estimates of CL_{fo} for the drugs studied in pregnant sheep are plotted against their CL_{fm}-CL_{mf} difference. There is a highly significant linear relationship between the two variables, suggesting that with the exception of acetaminophen, fetal hepatic first-pass uptake is likely a significant factor in fetal non-placental elimination of these compounds. From this relationship, methadone and DPHM will be predicted to exhibit the most pronounced fetal hepatic first-pass effect and in agreement with this, these drugs also have the highest values for CL_{fo}. In the case of acetaminophen, the lack of evidence for any fetal first-pass effect from CL_{fm}-CL_{mf} difference is consistent with the low value of CL_{fo} for this drug.

The above finding of the involvement of fetal hepatic drug uptake in the underestimation of CL_{mf} is also significant in light of the fact that the relative magnitudes of CL_{mf} and CL_{fm} are often used as an indication of the active or passive transport of the drug across the placenta (Wang et al., 1986a; Pereira et al., 1994; Odinecs et al., 1996a; Tuntland et al., 1996; Tuntland et al., 1998). Our data indicate that this approach may not yield accurate conclusions if the fetal liver is significantly active in the uptake of the drug from the umbilical vein, as with DPHM.
Figure 3.9 - Relationship between fetal and maternal placental clearance difference ($\text{CL}_{\text{fm}} - \text{CL}_{\text{mf}}$, ml/min/kg) and fetal non-placental clearance ($\text{CL}_{\text{fo}}$, ml/min/kg) of drugs studied in pregnant sheep.

3.3.3 Studies C and D: Role of the Liver and Gut in Systemic DPHM Clearance in Adult Non-Pregnant Sheep

During our hepatic uptake studies (Study C), DPHM was extensively extracted across the sheep liver as evidenced by the much lower AUC's of the form of drug administered via the portal venous route, and an estimated mean hepatic first-pass extraction ratio of 94.2 ± 3.7% (Table 3.4). This is in line with the high systemic clearance of the drug in sheep (Table 3.5).

The principles of metabolite kinetics dictate that the overall shape of the metabolite plasma concentration vs. time profile is highly dependent on the route of drug
administration (Pang, 1981). In agreement with this, a significantly higher \(C_{\text{max}}\) of the form of metabolite generated from drug administered via the portal venous route (447.8 ± 175.9 ng/ml) was observed compared to that generated from the form of drug administered intravenously (90.7 ± 75.8 ng/ml). Also, the \(C_{\text{max}}\)'s of the former metabolite tended to occur at earlier sampling times compared to those of the latter. This is because portal venous administration results in an almost instantaneous metabolism of a large fraction of the administered dose (~94.2%, as indicated above), thus leading to an apparent "bolus" injection of the large amounts of formed metabolite into the systemic circulation. In contrast, the drug administered intravenously is more gradually metabolized, leading to a slower increase in plasma concentrations of the metabolite.

It has been demonstrated by computer simulations as well as theoretical analysis that the systemic arterial AUC's of the metabolite plasma profile after portal and intravenous administration of equal doses of the drug should be relatively equal (Pang, 1981; Houston and Taylor, 1984). This concept is valid if elimination of the drug by the kidney or other peripheral organs is < 10% of its total clearance and linear pharmacokinetics exist (Pang, 1981; Houston and Taylor, 1984). This is true in spite of the widely differing shapes of metabolite plasma profiles obtained after these routes of administration (see above). On the other hand, if renal or peripheral elimination of the drug is >10%, the AUC of the metabolite after p.v. administration becomes larger compared to that after i.v. administration (Pang, 1981; Houston and Taylor, 1984). The underlying reason for this theory is that if renal or peripheral elimination of the drug is negligible (<10%), the majority of the drug will be eliminated via hepatic metabolism. Since the fraction of drug metabolized via a particular metabolic pathway is constant in
a linear pharmacokinetic system, similar amounts of the metabolite will be eventually formed from equal doses of the drug administered via the p.v. and i.v. routes. This will in turn lead to similar systemic arterial AUC’s of the metabolite after these two routes of administration. However, if renal or peripheral elimination is significant (>10%), a larger fraction of the i.v. dose will be eliminated via these routes and less will be available for metabolism in the liver in comparison to the drug administered via the p.v. route. Thus, a larger amount of metabolite will be formed after p.v. administration resulting in a higher AUC compared to that after i.v. administration.

In previous studies in adult sheep, negligible renal and biliary clearances of DPHM were observed (<0.5% of the total body clearance combined) (Tonn, 1995). Also, in earlier dose-ranging studies in adult sheep (Yoo et al., 1990), apparently linear DPHM pharmacokinetics were observed at i.v. bolus doses of 50-250 mg, which covers the dose range employed in this study. Based on this, and the principles of metabolite kinetics above, the AUC’s of the DPHM metabolites after i.v. and p.v. administration of the drug should be equal if there is no peripheral elimination of the drug by other organs. However, in our DPHM hepatic first-pass studies (Study C), the AUC of the metabolite after i.v. administration was only 32.5 ± 14.0% (range 18.2 – 50.4%) of the metabolite AUC generated after p.v. administration. This provides clear evidence for significant peripheral elimination of the drug. Also, from the data presented in Table 3.4, it is evident that almost the entire DPHM dose administered via the p.v. route is metabolized in the liver. This mainly results from the metabolism of a large fraction of the dose during its first-pass through the liver (94.2 ± 3.7%); plus at least some additional amount of the remaining drug is metabolized in the liver during subsequent passes. Based on this and relative metabolite AUC’s after p.v. and i.v. administration, it
appears that only $32.5 \pm 14.0\%$ of the intravenously administered drug is metabolized in
the liver; the rest is likely eliminated via peripheral mechanisms. This conclusion is
based on the assumption of sole hepatic formation of the metabolite; if the metabolite is
also formed in peripheral organs, the percent metabolized in the liver will be
overestimated using the above approach (equation 13). The reason for this is that due
to the much lower plasma parent drug concentrations after p.v. administration (Figure
3.4), peripheral metabolite formation will contribute more towards the AUC of the
metabolite after i.v. than after p.v. administration. It should be noted that the above
analysis is valid in spite of the fact that the DPMA metabolite is not the only contributor
to DPHM total body clearance; it in fact only accounts for a very small fraction of DPHM
elimination ($\sim1\%$, see above Study E). This is based on the principle that the fraction of
the dose metabolized via a particular metabolic pathway is constant in a linear
pharmacokinetic system (Houston and Taylor, 1984). Hence, the plasma concentration-
time profiles of any other metabolites will also exhibit a behavior similar to that of
DPMA.

In order to gain further insight into the possible sites of peripheral DPHM elimination,
additional pharmacokinetic analysis was performed on the data from the hepatic first-
pass uptake studies. Parent drug pharmacokinetic data after i.v. and p.v. administration
can be used to estimate total hepatic blood flow ($Q_H$) using the principles of a well-
stirred model of hepatic drug clearance (Wilkinson and Shand, 1975; Pang and Gillette,
1978). Thus, $Q_H$ in the five adult sheep was estimated to be $62.6 \pm 14.7 \text{ ml/min/kg}$
(Table 3.5). This value is in excellent agreement with the reported literature estimates
of $Q_H$ in adult non-pregnant sheep obtained using more direct methods (Katz and
Bergman, 1969: $56.6 \pm 18.0 \text{ ml/min/kg}$; Boxenbaum, 1980: $48.6 \text{ ml/min/kg}$). It should
be emphasized that the above estimation of $Q_H$ assumes lack of significant DPHM uptake by the gut. However, the extraction of DPHM across the liver is high (~94%). Thus, the presence of any gut uptake of the drug will not significantly alter the total DPHM extraction across the 'hepato-portal' system (liver + gut) (i.e., observed 94% vs. maximum possible 100%), and will not unduly influence the $Q_H$ estimates.

The estimated values of $Q_H$ can be used to calculate the amounts of intravenously administered drug eventually delivered to the 'hepato-portal' system using equation 15. Thus, in these five sheep, almost the entire intravenously administered dose (average 98.6 ± 9.2%, Table 3.5) was eventually delivered to the 'hepato-portal' system. Since the extraction of DPHM even across the hepatic component of the "hepato-portal" system is nearly complete (~94%), only a very small fraction of the drug delivered to this system can escape uptake/metabolism. Thus, the liver and/or gut are likely the main organs responsible for elimination of a major portion of the DPHM i.v. dose. Consequently, these data provide evidence for a lack of any significant first-pass uptake of the drug by the lung. Moreover, since the liver metabolizes only 32.5 ± 14.0% of the intravenously administered dose, the gut is likely responsible for the elimination of the remainder (67.5 ± 14.0%). Thus, the data from hepatic first-pass uptake studies provide strong evidence for significant uptake of DPHM by the gut.

This possible gut uptake of DPHM was confirmed in a more direct study (Study D) where steady-state extraction ratio of the drug across the gut was measured by simultaneous sampling of femoral arterial and portal venous blood. Significantly lower DPHM concentrations in portal venous plasma as compared to femoral arterial plasma provide direct evidence for gut uptake of the drug from the systemic circulation. The
steady-state gut extraction ratio of DPHM was 49.0 ± 3.0%. As discussed above, it appears that the gut and liver are responsible for almost the entire DPHM systemic clearance in sheep. Since, the gut and liver are anatomically arranged in series and the major contributor to hepatic blood flow is portal venous flow (~80%, Katz and Bergman, 1969), roughly they will account for 49.0 ± 3.0% and 51.0 ± 3.0% of DPHM systemic clearance, respectively. In actual fact, the contribution of the liver will be somewhat greater and that of the gut somewhat lower due to the presence of the hepatic arterial blood flow component, which bypasses the gut. This estimate of the gut contribution to DPHM systemic clearance is near the low end of the range predicted from our hepatic first-pass experiments (i.e., 49.6-81.8%; Study C), whereas, the estimated contribution of the liver appears to be near the high end of the predicted range (18.2 – 50.4%). This may be due to inter-animal variability and the small number of animals used in these two studies. Also, the steady-state systemic clearances of DPHM during the gut uptake study appear to be somewhat lower compared to those estimated during the hepatic first-pass experiments. This may also be related to a low n value. Thus, overall the combined data from both studies indicate that gut uptake of the drug may account for ~50-80% of the DPHM systemic clearance. The exact mechanism of this DPHM gut uptake is not known at present. It may involve simple binding of the drug to tissue components, its secretion into the lumen via specific transporters (e.g., P-glycoprotein), or metabolism via pathways other than the formation of DPMA.

It has been known for a number of years that gut uptake/metabolism is an important factor in determining the oral bioavailability of a number of drugs (Krishna and Klotz, 1994). However, detailed study of the underlying mechanisms of this gut uptake/metabolism is relatively more recent. A first-pass intestinal extraction of 43%
was demonstrated for midazolam after intraduodenal administration in humans (Paine et al., 1996), and this was subsequently attributed to CYP3A-mediated metabolism of the drug in the gut mucosa (Thummel et al., 1996; Wandel et al., 1998). Oral bioavailability of cyclosporine, verapamil and nifedipine in humans also appears to be related to the activity of CYP3A in the intestinal mucosa (Hebert et al., 1992; Fromm et al., 1996; Holtbecker et al., 1996). More recently P-glycoprotein present in the intestinal mucosal wall has also been demonstrated to be an important factor in determining the bioavailability of some compounds such as cyclosporine and paclitaxel (Lown et al., 1997; Sparreboom et al., 1997; Asperen et al., 1997). It has been suggested that P-glycoprotein present in the intestinal mucosa causes a polarized basolateral-to-apical transport of these drugs in the intestine, thereby resulting in their reduced absorption and bioavailability. Thus, the bioavailability of paclitaxel is increased in gene knockout mice deficient in P-glycoprotein (mdr1a -/-), and also in wild type mice when the drug is co-administered with a P-glycoprotein blocker (Sparreboom et al., 1997; Asperen et al., 1997). It should be noted that most of these studies have assessed the effect of gut uptake/metabolism/secretion on the bioavailability of the drug after oral administration. Apart from a few isolated papers (du Souich et al., 1995), there appear to be few systematic studies on the extent of gut uptake of drugs from the systemic circulation, as was observed in our study. Hence, the role of gut uptake in the systemic clearance of drugs has not been extensively studied. For midazolam, gut uptake of the drug from the systemic circulation was negligible and hence it was concluded that the intestinal CYP3A was not accessible to the drug in the peripheral systemic circulation (Paine et al., 1996). Other studies have assumed that the gut uptake of the drug from the systemic circulation is negligible (Hebert et al., 1992; Holtbecker et al., 1996). Our data with DPHM show that this assumption may not necessarily be true for all drugs. Thus,
pharmacokinetic analyses of the hepato-portal drug disposition based on this assumption may not be entirely accurate, as was previously argued by Lin et al., (1997).

3.3.4 Study E: Contribution of DPMA Formation to DPHM Non-Placental Clearance in Maternal and Fetal Sheep

After demonstration of a significant role of fetal and maternal liver in their respective non-placental DPHM clearance, the logical next step was to examine the relative maternal and fetal capacity for various metabolic pathways for DPHM. The major route of DPHM elimination in many species is its metabolism to DPMA which is subsequently conjugated to amino acids such as glycine and glutamine (Drach and Howell, 1968; Drach et al., 1970; Chang et al., 1974). This study (Study E) examined in detail the comparative kinetics of DPHM metabolism to DPMA in maternal and fetal sheep, and the resulting effects on fetal exposure to this metabolite. The above aim required knowledge of the distribution and elimination characteristics of the parent drug as well as the metabolite in the mother and the fetus. However, the kinetics of the drug metabolites after parent drug administration alone are complex; a number of phenomena such as metabolite formation, distribution and elimination occur concurrently and their kinetics are difficult to resolve. More specifically, after parent drug administration alone, the volume of distribution of the metabolite and the extent of its sequential metabolism is not known, and hence the total amount formed can not be estimated unless all the sequential metabolic pathways are accounted for. Also, the estimation of the total contribution of primary as well as secondary metabolic pathways is much more difficult in the fetus where the formed metabolites may be excreted into a multiple number of fluid compartments (e.g., amniotic and tracheal fluids, fetal urine) which cannot be cumulatively sampled. The metabolites formed in the fetus also cross
the placenta and mix with those formed in the maternal circulation. One approach to examine the kinetics of metabolite distribution and elimination, that could be useful in such situations, is the *i.v.* administration of the preformed (synthesized) metabolite (Kaplan *et al.*, 1970; Kaplan *et al.*, 1973; Boxenbaum and Riegelman, 1976; Patel *et al.*, 1978; Lai *et al.*, 1978; Cobby *et al.*, 1978). These data may then be combined with the drug and metabolite data after parent drug administration and the kinetics of metabolite formation can be estimated. However, this approach can drastically underestimate the amount of the drug converted to a particular metabolite if the metabolite undergoes rapid sequential metabolism in the liver after its formation and before its egress into the circulation (Pang and Gillette, 1979). In such situations, the preformed metabolite must be administered *via* the portal venous route in order to more closely simulate the secondary hepatic metabolism and subsequent systemic availability of the metabolite formed from the drug (Pang *et al.*, 1979). In our preliminary studies, we found a complete lack of any secondary metabolism of DPMA in either maternal or fetal sheep (also see below). Hence, in the current study, it was acceptable to administer the preformed metabolite *via* the *i.v.* route in both the mother and fetus. This considerably simplified our surgical procedure especially in the fetus where implanting and maintaining chronic portal venous catheters is a difficult task.

In the maternal-fetal system, maternal as well as fetal drug clearance is comprised of a placental and a non-placental component. A comparison of the exact capacity of a particular metabolic pathway in the mother and the fetus requires the estimation of their non-placental clearance components. This is because the role of a particular metabolite in total maternal and fetal drug clearance is confounded by the different and variable
contributions of the placental clearance component (~ 1-10% in the mother vs. ~ 40-75% in the fetus for DPHM; Yoo et al., 1993; also see Sections 3.2.1.1 and 3.2.5.1).

The estimation of maternal and fetal placental and non-placental clearances in turn requires separate maternal and fetal steady-state drug administration (Szeto et al., 1982a). This, combined with the essential study of maternal and fetal preformed metabolite kinetics (see above), meant that essentially four experiments had to be conducted in each pregnant sheep preparation in our study. Given the duration of each experiment (96 h), this was difficult to accomplish in the limited time window (~ 2 weeks) available for experimentation in these late-gestational chronically-catheterized animals before labor and delivery. Also, the rapid fetal growth and physiological alterations during this part of gestation may confound the results of experiments conducted over a prolonged period of time. As discussed previously, in such situations, the use of stable-isotope labeled compounds in combination with mass-spectrometric analytical techniques provides a useful solution for minimizing the inter-occasion variability in pharmacokinetics (Baillie, 1981; Browne, 1990). Hence, we utilized a protocol where either a combination of the unlabeled parent drug and the labeled metabolite (DPHM and [2H10]-DPMA) or the labeled drug and the unlabeled metabolite ([2H10]-DPHM and DPMA) was administered to the mother or the fetus in two separate experiments. The assumption made in this protocol is that the preformed metabolite does not alter the pharmacokinetics of the parent drug and vice versa. The parent drug clearances in these animals were similar to those obtained in our earlier studies where the preformed metabolite was not administered (Yoo et al., 1993; Table 3.1). Also, the amount of formed DPMA recovered in maternal urine after parent drug administration to the mother was similar in this and the previous study (Tonn, 1995). These observations appear to support the above assumptions.
We have demonstrated that plasma protein binding of the drug is an important factor determining the magnitude of placental as well as non-placental DPHM clearance in the maternal-fetal unit (see Chapter 4). Thus, a greater fetal plasma free fraction of the drug compared to the mother is at least partly responsible for fetal placental and non-placental clearances being higher than the corresponding maternal clearances.

The presence of drug metabolites in the fetal circulation could be the result of two processes: 1) formation of the metabolite in the mother and its subsequent placental transfer, and, 2) formation of the metabolite by the fetus itself. In our study, the evidence of in utero fetal formation of the DPMA metabolite is provided by the various AUC ratios presented in Table 3.8. If the fetal ability to form a particular metabolite is absent or negligible, the FA/MA AUC ratios of the preformed as well as the metabolite formed in the maternal circulation from the parent drug should be similar (because in this case the maternal-to-fetal placental transfer of the metabolite will be the determining factor of this ratio in both situations). A higher FA/MA ratio of the formed metabolite (2.97 ± 0.82) compared to the preformed metabolite (0.41 ± 0.21) after maternal parent drug and preformed metabolite administration suggests the additional fetal formation of this metabolite from the drug transferred to the fetal circulation via the placenta. Analogous to this, after fetal parent drug and preformed metabolite administration, a higher (but not statistically significant due to high inter-animal variability) MA/FA ratio of the in vivo generated metabolite (0.25 ± 0.35) compared to the preformed metabolite (0.02 ± 0.02), indicates additional maternal formation of this metabolite from the drug transferred in the fetal-to-maternal direction.
As discussed above, the fetal liver can metabolize a significant but variable (due to the high variability in ductus venosus shunt fraction) proportion of DPHM present in the umbilical vein in a first-pass manner before it reaches the fetal circulation (see Section 3.3.2). This leads to a greater metabolism and fetal formation of metabolites from the drug transferred to the fetus via the placenta and umbilical vein, as compared to when it is directly administered i.v. to the fetus (see Section 3.3.2 and Table 3.2). This factor, combined with some maternal-to-fetal placental transfer of the metabolite formed in the mother, leads to higher (but again not statistically significant due to high inter-animal variability) in vivo generated metabolite to parent drug AUC ratios in fetal arterial plasma after maternal drug administration compared to when the drug is given directly to the fetus (21.5 ± 26.4 vs. 2.32 ± 1.16; Table 3.8). These data also indicate that depending upon the extent of fetal hepatic first-pass metabolism of the drug present in the umbilical vein, fetal exposure to drug metabolites relative to parent drug concentrations after maternal drug administration may actually be higher than that predicted based on direct fetal i.v. administration. Similar to the FA ratios above, but to a lesser extent, the MA ratio of the in vivo generated metabolite to parent drug AUC’s after fetal administration was greater in all the individual animals compared to that after maternal drug administration (2.69 ± 3.87 vs. 1.13 ± 1.44; Table 3.8). However the only possible explanation for this phenomenon appears to be some fetal-to-maternal placental transfer of the metabolite formed in the fetal circulation after fetal drug administration, leading to an increase in the former ratio.

There are significant differences in the disposition of the parent drug and the preformed DPMA (or [²H₁₀]-DPMA) metabolite both in the mother and the fetus. In particular, the CLₜb and Vdₜₚ of the metabolite are much lower compared to the parent drug in both the mother and fetus. In fact, the Vdₜₚ of the preformed metabolite is only about 2- and 3-
times the blood volume in the mother and the fetus, respectively. This indicates very limited tissue distribution of this compound which could be partly related to its very high plasma protein binding in both maternal and fetal plasma (> 99%; Tonn, 1995) and its low lipophilicity (octanol/pH 7.4 phosphate buffer partition coefficient = 0.29; S. Kumar, K.W. Riggs and D.W. Rurak, unpublished data). Also, the $t_{1/2\beta}$ and MRT of the metabolite are longer compared to the parent drug in both the mother and particularly in the fetus, indicating its slow overall elimination from the maternal and fetal circulation. A very long fetal $t_{1/2\beta}$ and MRT of the metabolite compared to the mother suggests that although the fetal lamb has the ability to form DPMA from DPHM, the mechanisms involved in DPMA elimination are only partially developed as compared to the mother. The placental transfer of DPMA appears to be extremely slow and limited as compared to the parent drug, since relatively low $C_{\text{max}}$'s in FA and MA occur at least 3-5 h after maternal and fetal preformed metabolite i.v. bolus administration, respectively. Slow placental transfer of this polar and highly plasma protein bound metabolite is partly responsible for its slow elimination from the fetus (also see below). At comparable parent drug concentrations, the fetal in vivo generated metabolite exposure based on relative AUC's of the metabolite is approximately 4-fold greater compared to the mother. Thus, at least in this situation, fetal metabolism of the drug actually leads to a considerably increased fetal exposure to the metabolite due to its “trapping” in the fetal circulation.

The MRT of the in vivo formed metabolite is longer compared to that of the preformed metabolite in both the mother and the fetus, suggesting differences in the disposition of in vivo generated and exogenously administered metabolite. This is probably related to the fact that in vivo metabolites are generated within the cell (e.g., hepatocytes) and
they must diffuse out of the cell into the circulation in order to be eliminated. However, this is not true for the exogenously administered preformed metabolite. If significant diffusional barriers exist to the efflux of the in vivo formed metabolite from the cell (due to their polarity, molecular size or binding to cellular components), its disposition would be different from that of the preformed metabolite. A diffusional barrier has been shown to exist for the entry of enalaprilat (the polar dicarboxylic acid metabolite of angiotensin converting enzyme inhibitor, enalapril) into hepatocytes from the circulating perfusate and its subsequent biliary elimination in an isolated-perfused liver preparation (De Lannoy and Pang, 1986; Schwab et al., 1990). A similar barrier may exist for the egress of the polar DPMA metabolite into the maternal and fetal circulation after its formation in the hepatocytes, and this could be responsible for its longer residence time compared to the preformed metabolite. In such instances, it is important to utilize mass-balance based approaches (such as AUC's and equation 18) rather than the rate-constant based pharmacokinetic modeling in order to calculate the quantitative importance of a particular metabolic pathway in total drug clearance using the preformed metabolite administration. The latter approach will actually estimate the rate constant of metabolite efflux from the hepatocytes rather than the actual metabolite formation rate constant in such cases.

Using equation 18 (section 3.1.6.5) and after taking into account the differences in placental clearance contribution to maternal and fetal total body clearance of the drug, the contribution of DPMA formation to maternal and fetal (non-placental) DPHM clearance was not statistically different and was typically ~1% in all but one animal (Tables 3.9 and 3.10). In E4230, the maternal DPMA formation accounted for 5.56% of the maternal non-placental clearance. We have also observed this high inter-animal
variability in our similar studies in non-pregnant sheep (not presented in this thesis) and reasons for this are not clear. Overall these data indicate that although the DPMA formation pathway is almost equally functional in both the mother and fetus, this is not a major route of DPHM clearance in maternal or fetal sheep. This is in contrast to many other species where DPMA and its amino acid conjugates account for ~ 40-60% of total DPHM metabolites (Drach and Howell, 1968; Drach et al., 1970; Chang et al., 1974).

Significant differences also exist in the renal handling of the parent drug and the DPMA metabolite in the mother and the fetus. Renal elimination of the parent drug in the mother is negligible and accounts for <0.5% of the total dose and clearance. In contrast, the $\text{CL}_r$ of preformed DPMA accounts for $88.8 \pm 6.5\%$ (Tables 3.9 and 3.11) of its $\text{CL}_t$ in the mother, and correspondingly almost all of the $i.v.$ dose administered to the mother was ultimately recovered in maternal urine ($88.0 \pm 6.5\%$; Table 3.11). This indicates that DPMA is not secondarily metabolized in maternal sheep and the entire dose is excreted unchanged in urine. This is also a species difference in sheep compared to the monkey, dog and the human, where significant proportions of total DPMA are recovered as its glycine, glutamine or an unidentified conjugate in urine (Drach and Howell, 1968; Drach et al., 1970; Chang et al., 1974). Due to the lack of this sequential metabolism of DPMA, the amount of $in\,vivo$ formed DPMA recovered in maternal urine as a percentage of the parent drug dose was similar to the percent contribution of this pathway to maternal non-placental DPHM clearance ($1.60 \pm 1.86$ vs. $1.78 \pm 2.12\%$, respectively; Tables 3.9 and 3.11). Similar to the mother, the excretion of the unchanged DPHM in fetal urine also accounted for < 0.5% of the total fetal parent drug dose in the 3 animals where fetal urine was collected. In contrast to the mother,
however, only 1.79 ± 2.08 % of the total fetal *i.v.* dose of the preformed metabolite was recovered in fetal urine due to a very low fetal renal clearance of this metabolite (Table 3.11). This is presumably related to a lack of any significant renal tubular secretion of many organic acid compounds such as para-aminohippurate (Elbourne *et al.*, 1990), acetaminophen and morphine glucuronides (Wang *et al.*, 1985; Olsen *et al.*, 1988), valproic acid (see Chapter 5) and indomethacin (Krishna *et al.*, 1995) in the late-gestation fetal lamb. Due to this lack of renal elimination of the preformed DPMA metabolite in fetal urine, almost all of the fetal *i.v.* dose of this metabolite (92.1 ± 7.4%; Table 3.11) underwent fetal-to-maternal placental transfer and was eventually recovered in the maternal urine over the ensuing 96 h sampling period. The lack of any significant fetal renal elimination of this metabolite combined with its slow placental transfer leads to the observed prolonged fetal $t_{1/2}$ (and MRT) and exposure of the preformed as well as the *in vivo* generated metabolite (Table 3.10).

### 3.3.5 Study F: Disposition of the DPHM-N-oxide Metabolite in the Maternal-Fetal Unit

The N-oxide metabolite was detectable in both maternal and fetal plasma during maternal as well as fetal drug administration. In contrast to DPMA, the elimination of this metabolite was almost as rapid as that of the parent drug in the mother as well as the fetus (Figure 3.8). The much higher FA/MA AUC ratios of the metabolite after fetal drug administration as compared to those after maternal drug administration provide evidence of the fetal ability to form this metabolite as well (Table 3.12).

A fraction of the N-oxide metabolite was excreted unchanged in maternal as well as fetal urine. The renal clearance data presented in Table 3.13 suggest significant fetal ability to
excrete the N-oxide metabolite via this pathway in comparison to the mother. However, the percentage of administered DPHM dose excreted in maternal as well as fetal urine as the unchanged N-oxide metabolite is <1%. This is in contrast to other species such as the rat, rhesus monkey, and dog where ~10-20% of the total dose is recovered as this metabolite in urine (Drach and Howell, 1968; Drach et al., 1970). The extent and nature of sequential metabolism of the N-oxide metabolite is not known in maternal or fetal sheep or any other species so that the total contribution of this pathway to maternal and fetal clearance can not be determined.

In summary, we have demonstrated that the liver and gut are the major organs responsible for DPHM systemic clearance in adult sheep. Fetal liver during late-gestation also appears to exhibit a significant ability for DPHM uptake and appears to be the major site of fetal non-placental clearance of the drug. The role of the gut in fetal DPHM non-placental clearance remains to be investigated; however, it may be less significant compared to the adult because gut blood flow is only ~20% of the total hepatic blood flow in the fetus, as compared to ~80% in the adult. The fetal first-pass hepatic uptake of the placentally transferred drug from the umbilical vein also appears in part responsible for an underestimation of maternal-to-fetal placental clearance. This may also be the case for other drugs studied in pregnant sheep using the 2-compartment open model that demonstrate higher values of CLfm compared to CLmf. Conversely, the difference between fetal and maternal placental clearance for these drugs may provide evidence for fetal hepatic uptake/metabolism of these compounds.

We have also demonstrated that the use of stable-isotope labeled drug and metabolites in combination with mass-spectrometry provides a powerful tool for studying drug and
metabolite kinetics within the maternal-fetal unit. Using an approach based on the simultaneous administration of differently labeled parent drug and metabolite to the mother and the fetus, we have shown that the contribution of DPMA formation to DPHM non-placental elimination in the maternal and the fetal sheep is typically ~0.5-1%. Hence, this is a minor pathway in overall DPHM elimination in this species. However, the \textit{in vivo} functional capacity of this metabolic pathway appears to be similar in the mother and the late-gestational fetal lamb. The DPMA metabolite is not sequentially metabolized in fetal or adult sheep. It is eliminated solely \textit{via} the renal pathway in the mother and \textit{via} the placenta (and eventually in maternal urine) in the fetus. The impaired renal excretion and slow placental transfer of the DPMA metabolite is responsible for its long half-life in the fetal circulation. The renal elimination of another DPHM metabolite, DPHM-N-oxide, also accounts for <1\% of the total DPHM elimination in the mother as well as the fetus. The minor contribution of the DPMA pathway and DPHMNOX renal elimination in sheep DPHM clearance is in contrast to other species (dog, rhesus monkey, man) where these routes account for ~40-60\% and ~10-20\% of the total DPHM dose, respectively. Thus, the exact pathways of DPHM metabolism are likely different in sheep compared to other species. Also, the fate and mechanism of the extensive gut uptake of the drug in adult sheep is not known. Overall, the exact routes of a large portion of maternal and fetal sheep DPHM elimination remain to be investigated. However, the pathways that were examined in these studies appear to be almost equally functional \textit{in utero} in the late-gestational fetal lamb in comparison to the mother.
Chapter 4

Inter-relationships between Plasma Drug Protein Binding, Gestational Age, Umbilical Blood Flow, and Diphenhydramine Clearances in the Ovine Maternal-Placental-Fetal Unit

Earlier studies in this lab and those in Chapter 3 have utilized DPHM as a model high clearance drug, which undergoes rapid and extensive placental transfer, to examine different aspects of maternal-fetal drug disposition of this class of compounds. This includes the study of comparative maternal-fetal drug clearance (Yoo et al., 1993), \textit{in utero} fetal hepatic drug uptake and its relationship to fetal drug clearance (see chapter 3, study A), and \textit{in utero} functional capacity of fetal drug metabolism pathways compared to the mother (see chapter 3, study E). As part of these studies, we have determined DPHM placental and non-placental clearances in several pregnant sheep during the last two weeks of their gestation. In this chapter, we have retrospectively examined the overall inter-relationships between maternal and fetal plasma protein binding, umbilical blood flow, DPHM maternal and fetal clearances, and indices of placental drug transfer using pooled data from the above three studies. Our aim was to better understand the qualitative and quantitative importance of these factors in determining the kinetics of placental drug transport and the extent of fetal drug exposure.

Also, the last third of gestation is a very dynamic period in terms of fetal development and is associated with profound changes in numerous physiological variables that may alter maternal-fetal drug disposition. These include changes in fetal plasma protein concentrations and hence the extent of drug binding (\textit{e.g.} propranolol and methadone, Czuba \textit{et al.}, 1988; Szeto \textit{et al.}, 1982c), changes in the drug metabolism capacity of the
fetal liver (Wang et al., 1986a), development of fetal renal function and drug excretion, and alterations in fetal circulatory and hemodynamic processes (Battaglia and Meschia, 1988). However, little has been done experimentally to elucidate the quantitative influence of these gestational age-related factors on drug disposition within the maternal-placental-fetal unit and the resulting alterations in fetal drug exposure. Hence, we have also assessed the inter-relationships between the above variables as a function of advancing gestation during the last 2-week period of pregnancy in sheep.

4.1 Methods

4.1.1 Animals and Surgical Preparation

Data from a total of 18 pregnant sheep were employed in these studies. Surgical procedures for 10 out of these 18 animals are described in chapter 3 (studies A and E). The surgical preparation for the other 8 animals was similar and has been described earlier (Yoo et al., 1993).

4.1.2 Experimental Protocols

All experiments were conducted between 124-140 days gestation. A total of 31 experiments were carried out on 18 pregnant sheep. Each animal received one of the following:

1) a 90 min separate maternal and fetal steady-state DPHM infusion with an appropriate washout period in between (n=8, experiments from Yoo et al., 1993).
2) a 6 h separate maternal and fetal steady-state DPHM infusion with an appropriate washout period in between (n=3, experiments from study E of chapter 3).

3) a 6 h separate maternal and fetal steady-state $[^2\text{H}_{10}]$-DPHM infusion with an appropriate washout period in between (n=2, experiments from study E of chapter 3).

4) a 6 h simultaneous steady-state infusion of DPHM to the mother and $[^2\text{H}_{10}]$-DPHM to the fetus (n=5, experiments from study A of chapter 3).

Drug (DPHM or $[^2\text{H}_{10}]$-DPHM) was administered to the mother in each experiment as a 20 mg i.v. bolus loading dose over 1.0 min, followed immediately by an infusion at 670 $\mu$g/min via the maternal femoral vein. In fetal experiments, a 5.0 mg i.v. bolus loading dose of DPHM or $[^2\text{H}_{10}]$-DPHM was given via the fetal lateral tarsal vein over 1.0 min, followed by an infusion of the same compound at 170 $\mu$g/min. Maternal and fetal sample collection, processing and storage protocols for all these experiments have been described in Chapter 3 of this thesis and in the earlier paper (Yoo et al., 1993).

4.1.3 Physiological Recording and Monitoring Procedures

Fetal blood pH, $\text{Po}_2$, $\text{Pco}_2$, $\text{O}_2$-saturation, and hemoglobin, glucose and lactate concentrations were measured in all these animals by the procedures described in Chapter 2. All of these fetal blood gas and metabolite concentrations have been reported earlier and were within the normal range observed in our and other laboratories at this stage of gestation in fetal sheep (Yoo et al., 1993; Chapter 3).

4.1.4 Protein Binding of DPHM and $[^2\text{H}_{10}]$-DPHM in Fetal and Maternal Plasma
The plasma protein binding/unbound fraction of DPHM (or $[^{2}H_{10}]$-DPHM) was measured ex vivo in pooled fetal and maternal steady-state plasma samples using an equilibrium dialysis procedure as described by Yoo et al. (1993). Maternal plasma protein binding was measured in plasma samples obtained during maternal drug infusion, whereas fetal plasma protein binding was measured in plasma samples obtained during fetal drug infusion. Maternal and fetal plasma unbound concentrations during fetal and maternal drug infusion, respectively, were below the limit of quantitation in many animals and were not used for the sake of uniformity.

4.1.5 Drug Analysis

The concentrations of DPHM in all biological fluids collected were measured using either a gas chromatographic-nitrogen phosphorus detection method (Yoo et al., 1986b; studies of Yoo et al., 1993) or by a GC-MS assay (studies in Chapter 3) capable of measuring both DPHM and $[^{2}H_{10}]$-DPHM simultaneously (Tonn et al., 1993). Both these assays have been shown to be comparable to each other with a similar limit of quantitation (2.0 ng/ml; Tonn et al., 1993).

4.1.6 Pharmacokinetic Analysis

The maternal and fetal steady-state arterial plasma DPHM and $[^{2}H_{10}]$-DPHM concentration data were treated according to a 2-compartment open model in order to calculate the placental and non-placental clearances of DPHM (or $[^{2}H_{10}]$-DPHM when present) in the ewe and the fetus, as described in Chapter 3.
4.1.7 Statistical Analysis

All values are reported as mean ± S.D. The significance level was p<0.05 in all cases. Fetal weight *in utero* at the time of experimentation was estimated from the weight at birth and the time interval between the experiment and birth (Koong *et al.*, 1975). The clearance values obtained in the earlier study of Yoo *et al.*, (1993) were also recalculated in order to obtain weight-normalized estimates with respect to the estimated fetal weight by the method of Koong *et al.* (1975).

4.2 Results

The average maternal body weight was 76.9 ± 12.6 kg and the estimated fetal body weights on the day of maternal and fetal DPHM (or $[^2H_{10}]$-DPHM) infusion were 2.61 ± 0.61 and 2.56 ± 0.54 kg, respectively.

Table 4.1 presents the gestational age of the animals on the day of experiment, maternal and fetal steady-state plasma unbound fractions of the drug, and maternal and fetal clearance (total body, placental and non-placental clearances) data calculated using the 2-compartment pharmacokinetic model.

The mean gestational age on the day of maternal and fetal steady-state DPHM infusion experiments was 130.9 ± 4.1 and 130.4 ± 3.7 days, respectively, and these were not statistically different (paired t-test, p > 0.05). The average maternal and fetal steady-state plasma drug concentrations in these animals after maternal administration ($C_m$ and $C_f$,}
respectively) were 228.0 ± 56.1 (range 140.3 - 360.3) and 43.1 ± 31.2 (range 3.5 - 124.1) ng/ml, respectively, whereas those after fetal drug infusion were 35.3 ± 11.9 ($C_m'$: range 17.9 - 66.3) and 331.1 ± 172.4 ($C_f'$: range 132.5 - 697.9) ng/ml, respectively. The steady-state maternal and fetal plasma unbound fractions of the drug were 0.120 ± 0.069 (range 0.032 - 0.293) and 0.301 ± 0.094 (range 0.165 - 0.527), respectively. The average maternal plasma unbound fraction (M-UF) was significantly lower compared to the average fetal plasma unbound fraction (F-UF, unpaired t-test, $p < 0.0001$). Maternal and fetal steady-state unbound plasma drug concentrations were calculated by multiplying the appropriate total plasma concentration with the corresponding plasma unbound fraction. The mean steady-state unbound plasma concentrations thus obtained were: $C_m = 25.1 ± 11.4$ (range 8.6 - 45.6) ng/ml; $C_f = 12.0 ± 8.6$ (range 1.9 - 40.4) ng/ml; $C_m' = 3.9 ± 1.8$ (range 0.9 - 7.2) ng/ml; and $C_f' = 89.3 ± 32.0$ (range 46.1 - 166.2) ng/ml. All fetal weight-normalized clearances (total body, placental and non-placental clearance) were significantly higher compared to the corresponding maternal clearance parameters (unpaired t-test, $p < 0.0001$ in all cases), as reported previously (Yoo et al., 1993; Chapter 3). However, the contribution of $CL_{fo}$ to $CL_{ff}$ (39.5 ± 10.7%) was significantly lower compared to that of $CL_{mo}$ to $CL_{mm}$ (96.3 ± 2.8 %) (unpaired t-test, $p < 0.0001$).

4.2.1 Relationships of Maternal and Fetal DPHM Clearances with Gestational Age

Figure 4.1 depicts the alterations in maternal and fetal clearances with advancing gestation over the 2-week period during which our experiments were conducted. Fetal total body ($CL_{ff}$) and placental ($CL_{fm}$) clearances exhibit a highly significant negative linear relationship with gestational age (Figures 4.1A and 4.1B). The calculated regression
equations predict a fall of ~59% (from 374.3 to 153.2 ml/min/kg) and ~66% (from 247.0 to 83.1 ml/min/kg) for \( CL_{ff} \) and \( CL_{fm} \), respectively, from 125 to 136 d gestation. Although the \( CL_{fo} \) parameter also exhibits a decreasing trend with gestation, this relationship is only near statistical significance (Figure 4.1C). Also, the percent contribution of \( CL_{fo} \) to \( CL_{ff} \) did not change as a function of gestational age (\(%[CL_{fo}/CL_{ff}] vs. GA, r = 0.2892, p > 0.2, data not shown\)). In contrast to fetal clearances, none of the maternal clearance parameters show any relationship with gestational age (\( CL_{mm} vs. GA, r = 0.0113, p > 0.9; CL_{mf} vs. GA, r = -0.0021, p > 0.9; CL_{mo} vs. GA, r = -0.0041, p > 0.9; data not shown\)).

### 4.2.2 Plasma Protein Binding Effects on Maternal and Fetal DPHM Clearances

Figure 4.2 depicts the underlying relationships between maternal and fetal steady-state plasma unbound fraction and the corresponding clearance parameters. The \( CL_{ff} \) and \( CL_{fm} \) are highly correlated with \( F-UF \) (Figures 4.2A and 4.2B). In contrast to \( CL_{fm} \), there was no relationship between \( CL_{mf} \) and \( M-UF \) (Figure 4.2E). Also, \( CL_{fm} \) and \( CL_{mf} \) were not significantly related to the extent of drug protein binding on the other side of the placenta, i.e., maternal and fetal plasma protein binding, respectively (\textit{data not shown}).

Although the linear relationships between \( M-UF \) and \( CL_{mo} \), and between \( F-UF \) and \( CL_{fo} \) were statistically significant (\( M-UF vs. CL_{mo} \): \( CL_{mo} = 107.7* M-UF + 28.8, r = 0.7649, p < 0.0005 \); \( F-UF vs. CL_{fo} \): \( CL_{fo} = 234.7* F-UF + 31.6, r = 0.4749, p < 0.05 \), a better fit of the data (\( M-UF vs. CL_{mo}, r = 0.8090 \); \( F-UF vs. CL_{fo}, r = 0.4678 \); Figures 2C and 2F) was obtained using an equation of the form (see Discussion of this chapter for further details on this):
<table>
<thead>
<tr>
<th>Ewe</th>
<th>Gestational Age (days)</th>
<th>Plasma Unbound Fraction</th>
<th>Clearance (ml/min/kg)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Maternal Infusion</td>
<td>Fetal Infusion</td>
<td>Maternal</td>
</tr>
<tr>
<td>121</td>
<td>134</td>
<td>136</td>
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</tr>
<tr>
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<td>134</td>
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<tr>
<td>303Y</td>
<td>131</td>
<td>126</td>
<td>0.211</td>
</tr>
</tbody>
</table>

| Mean | 130.9       | 130.4       | 0.120       | 0.301       | 41.7       | 40.2       | 45.0       | 263.0       | 102.3       | 160.7       |
| S.D. | 4.1         | 3.7         | 0.069       | 0.094       | 9.6        | 9.4        | 30.3       | 111.8       | 46.4        | 80.7        |

CL<sub>mm</sub>: Maternal total clearance; CL<sub>mf</sub>: Maternal placental clearance; CL<sub>mo</sub>: Maternal non-placental clearance; CL<sub>f</sub>: Fetal total clearance; CL<sub>fo</sub>: Fetal placental clearance; CL<sub>fo</sub>: Fetal non-placental clearance; <sup>a</sup> - per kg maternal weight; <sup>b</sup> - per kg estimated fetal weight at the time of fetal experiment.
Figure 4.1 - Relationships between fetal DPHM clearances and gestational age (GA). A) $CL_f$ vs. GA; B) $CL_{fm}$ vs. GA; C) $CL_{fo}$ vs. GA. Scatter points are the actual data in 18 pregnant sheep. The regression lines (solid) and the 95% confidence intervals (dotted) are also shown. $CL_f$: Fetal total clearance; $CL_{fm}$: Fetal placental clearance; $CL_{fo}$: Fetal non-placental clearance.
Figure 4.2 - Relationships between fetal and maternal DPHM clearances and the corresponding plasma unbound fractions of the drug. A) CLf vs. F-UF; B) CLfm vs. F-UF; C) CLfo vs. F-UF; D) CLmm vs. M-UF; E) CLmf vs. M-UF; and F) CLmo vs. M-UF. The CLfo, CLmm and CLmo relationships with the corresponding plasma unbound fraction of the drug were analyzed according to the well-stirred model of organ clearance. UF: unbound fraction; F and M refer to the mother and the fetus, respectively; Clearances are as defined in Table 4.1.
\[ y = \frac{P_1 \cdot x}{P_2 + \frac{P_1}{P_2} x} \]  

(1)

where, \(P_1\) and \(P_2\) are the parameters required to describe the relationship. This equation is exactly analogous to the following relationship describing hepatic uptake of compounds based on the well-stirred model of the liver (Wilkinson and Shand, 1975):

\[ \frac{CL_H}{Q_H + f_{ub} \cdot CL_{int}} = \frac{Q_H \cdot f_{ub} \cdot CL_{int}^u}{Q_H + f_{ub} \cdot CL_{int}^u} \]  

(2)

where, \(Q_H\), \(CL_H\), \(CL_{int}^u\) and \(f_{ub}\) are the total hepatic blood flow, total hepatic clearance, hepatic intrinsic clearance of the unbound drug and the unbound fraction of the drug, respectively.

In our previous studies, we have shown that the liver (liver and gut in case of the mother) is the major organ of non-placental clearance both in the mother as well as the fetus (see Chapter 3). Thus, in the fetus, the terms \(P_1\), \(P_2\) and \(P_1/P_2\) of equation (1) correspond to \(Q_H \cdot CL_{int}^u\), \(Q_H\), and \(CL_{int}^u\), respectively. In the mother, these parameters will describe the combined blood flow and intrinsic clearance of the liver and gut. This analysis produced fits that were statistically at least as good (F-UF vs. CL \(_{fo}\); F-test on sum of squared residuals, \(p > 0.05\)) or significantly better (M-UF vs. CL \(_{mo}\), F-test on sum of squared residuals, \(p < 0.05\)) compared to the corresponding linear model fits. From the fitting of CL \(_{mo}\) vs. maternal plasma unbound fraction data to equation (1), DPHM CL \(_{int}^u\) and \(Q_H\) in pregnant adult sheep were estimated to be 1242.6 ± 176.8 ml/min/kg (mean ± standard error of estimate) and 60.2 ± 5.7 ml/min/kg, respectively. A similar hyperbolic relationship was apparent between CL \(_{mm}\) and M-UF as well (Figure 4.2D). Similarly, the treatment of
F-UF and CLₚ₀ data according to equation (1) produced the fetal CLᵤₑ and Qₑ estimates of 517.6 ± 251.2 ml/min/kg (mean ± standard error of estimate) and 318.7 ± 306.6 ml/min/kg, respectively.

### 4.2.3 Changes in Maternal and Fetal Plasma Protein Binding with Gestational Age

Figure 4.3 shows the changes in fetal and maternal steady-state plasma unbound fractions with increasing gestational age during the last two week period of gestation under study. F-UF falls significantly from 125 to 136 d gestation by ~46.9% (from 0.383 to 0.203 as predicted by the regression equation, Figure 4.3A). In contrast, the M-UF does not change significantly during this period of gestation (Figure 4.3B).

### 4.2.4 Inter-relationships between Maternal and Fetal Plasma DPHM Concentrations, Unbound Fractions and the 2-Compartment Model Clearance Estimates

Maternal plasma drug concentration (Cₑ) after maternal drug infusion exhibited a highly significant negative linear relationship with M-UF of the drug (Figure 4.4A). In contrast, fetal plasma concentration after maternal infusion (Cₑ) was not related significantly to either maternal (r = 0.1751, p = 0.5) or fetal (r = -0.3676 , p > 0.1) plasma unbound fraction (data not shown). Analogous to the maternal situation, the fetal plasma DPHM concentration after fetal drug infusion (Cₑ) was inversely related to F-UF (Figure 4.4B). Also, maternal plasma concentration after fetal infusion (Cₑ) was not related to F-UF (r = -0.0966, p > 0.5, data not shown) and its negative relationship with M-UF was only near statistical significance (r = 0.4612, p = 0.05, data not shown).
Figure 4.3 - Alterations in (A) fetal and (B) maternal steady-state plasma unbound fraction of DPHM (or [\(^2\text{H}_{10}\)-DPHM) with increasing gestational age. Actual experimental data (scatter points), regression line (solid) and 95% confidence interval (dotted) are depicted.
Figure 4.4 - Relationships between (A) steady-state unbound fraction and plasma concentration of the drug in the mother after maternal drug administration and (B) steady-state unbound fraction and plasma concentration of the drug in the fetus after fetal drug administration. Scatter points are the experimental data in different sheep. The regression line (solid) and the 95% confidence interval (dotted) are also shown.
In order to determine the influence of various 2-compartment clearance terms on maternal and fetal plasma drug concentrations after maternal or fetal drug administration, different concentration vs. clearance relationships were analyzed according to the simple steady-state clearance model of the form: \( CL = k_0/C_{ss} \). The majority of the inter-animal variability in \( C_m \) was reflected in the estimated value of total \( CL_{mo} \) (not weight-normalized, because the total clearance is the actual determinant of plasma concentrations) as demonstrated by an excellent fit of the concentration vs. clearance data to this model (Figure 4.5A). However, \( C_m \) was not significantly related to the other 3 clearance parameters (\( CL_{mf}, CL_{fo}, \) and \( CL_{fm} \)) of the 2-compartment model (data not shown). Similarly, most of the inter-animal variability in \( C_f \) was reflected in the final estimates of \( CL_{mf} \) (Figure 4.5B). In contrast, when the drug was administered to the fetus, the inter-animal variability in \( C_f' \) was due to relatively equal contributions from the final \( CL_{fo} \) and \( CL_{fm} \) estimates (Figures 4.5C and 4.5D, respectively). Also, in contrast to the situation with \( C_f \) above (Figure 4.5B), the \( C_m' \) concentration was not related to the magnitude of fetal-to-maternal placental clearance (\( CL_{fm} \), Figure 4.5E). Instead the inter-animal variation in \( C_m' \) was best explained by the differences in their maternal non-placental clearances (Figure 4.5F).

### 4.2.5 Relationships between the Indices of Fetal Drug Exposure/Placental Transfer, Gestational Age and Plasma Protein Binding

Steady-state fetal to maternal arterial plasma concentration ratio after maternal drug administration (\( C_f/C_m \) in this study) is commonly used as an index of the efficiency of placental transfer and fetal drug exposure (Rurak et al., 1991). In our experiments, the average \( C_f/C_m \) ratio based on total plasma drug concentrations was \( 0.20 \pm 0.14 \). The same ratio calculated using unbound drug concentrations was significantly higher (\( 0.50 \pm 0.30 \), paired t-test, \( p < 0.0001 \)). The total drug plasma \( C_f/C_m \) ratio was not significantly
correlated with gestational age, maternal or fetal non-placental clearance, or F-UF, and its positive relationship with M-UF was only near statistical significance ($r = 0.4029$, $p < 0.1$, data not shown). However, this $C_f/C_m$ ratio was negatively correlated with the fetal to maternal plasma unbound fraction ratio (Figure 4.6A). The mean $C_m'/C_f'$ ratios during fetal drug administration based on total and unbound plasma drug concentrations were $0.14 \pm 0.08$ and $0.05 \pm 0.02$, respectively, the latter being significantly lower than the former (paired t-test, $p < 0.0001$). The fetal steady-state plasma concentration of the drug ($C_f'$) in these animals at the same fetal DPHM infusion rate of 170 $\mu$g/min gradually increased with progressing gestation (Figure 4.6B). This increase in $C_f'$ was associated with a progressive fall in $C_m'/C_f'$ ratio over this period of gestation (Figure 4.6C) because $C_m'$ did not change significantly with increasing gestational age (data not shown). The $C_m'/C_f'$ ratio was positively correlated with total (not weight-normalized) $CL_f$ and $CL_{fm}$ (Figures 4.6D and 4.6E, respectively), whereas its inverse relationship with total $CL_{mo}$ was only near statistical significance ($r = -0.4138$, $p < 0.1$, data not shown). The $C_m'/C_f'$ ratio also exhibited a highly significant positive relationship with F-UF (Figure 4.6F) but not with M-UF (data not shown).
Figure 4.5 – Influence of various clearance parameters of the 2-compartment model on different maternal-fetal plasma concentrations. A) $CL_{mo}$ vs. $C_m$; B) $CL_{mf}$ vs. $C_f$; C) $CL_{lo}$ vs. $C_l$; D) $CL_{lm}$ vs. $C_l$; E) $CL_{lm}$ vs. $C_m$; and F) $CL_{mo}$ vs. $C_m'$. All relationships except B) and E) were analyzed according to the steady-state clearance model, $CL = k_e/C_{ss}$; the solid lines represent the best-fit lines determined by this model.
Figure 4.6 – Relationships between indices of placental drug transfer/fetal drug exposure and their determining factors. A) $C_f/C_m$ vs. F-UF/M-UF; B) $C_f$ vs. GA; C) $C_f'/C_m'$ vs. GA; D) $C_f/C_m$ vs. CL_f; E) $C_f'/C_m'$ vs. CL_{fm}; and F) $C_f/C_m'$ vs. F-UF.
4.3 Discussion

A number of factors such as the lipophilicity and plasma protein binding of the drug, placental blood flows (uterine and umbilical), the efficiency of maternal and fetal drug elimination/metabolism and the gestational age of the fetus have been postulated to affect the degree of placental drug transfer and the resulting fetal drug exposure (Hill and Abramson, 1988; Reynolds and Knott, 1989; Rurak et al., 1991). The quantitative (e.g., plasma protein binding) and in some cases even qualitative (e.g., placental blood flows) influence of many of these variables on the kinetics of placental drug transfer has not been extensively studied under controlled experimental conditions either in vitro or in vivo. Instead these factors are generally accepted as the most important determinants of placental drug transfer/fetal drug exposure based on the common principles of biological membrane transport. Our overall objective in this study was to examine in detail the qualitative and quantitative role of different factors affecting placental transfer and fetal exposure of DPHM during late gestation in chronically-instrumented pregnant sheep. We have utilized DPHM as a model high clearance drug that undergoes rapid and extensive placental transfer in pregnant sheep (Yoo et al., 1986a) for evaluating these principles.

The 2-compartment model is most commonly employed to describe the pharmacokinetics of drugs in the maternal-fetal unit (Szeto et al., 1982a & b; Wang et al., 1986a; Riggs et al., 1990; Yoo et al., 1993; Kumar et al., 1997). This model has 4 clearance parameters: the maternal (CL_{mf}) and fetal (CL_{fm}) placental clearances are related to the efficiency of bidirectional maternal and fetal placental transfer of the drug, whereas the corresponding non-placental clearances (CL_{m0} and CL_{f0}) describe the extent of maternal and fetal drug elimination via all other routes (e.g., drug metabolism, renal excretion, pulmonary
elimination etc.). In our studies with DPHM, the contribution of $CL_{f0}$ to net fetal clearance ($40.4 \pm 10.4\%$) was much lower compared to that of $CL_{mo}$ to total maternal clearance ($98.5 \pm 1.1\%$), emphasizing the greater importance of the placental route in overall fetal drug elimination.

4.3.1 Maternal and Fetal DPHM Clearances: Gestational Age, Plasma Protein Binding and Umbilical Blood Flow Effects

One of the first observations we made from our DPHM clearance data in the 18 pregnant sheep was a drastic fall ($\sim 66\%$) in $CL_{fm}$ and a decreasing trend in $CL_{f0}$ with increasing gestational age over the period of our studies (Figures 4.1B and 4.1C). This resulted in a $\sim 59\%$ decrease in $CL_f$ during the 12 day gestation period of 125 - 136 d (Figure 4.1A). The decreasing trend in $CL_{f0}$ with gestational age was also consistent with the fact that in spite of the large decrease in $CL_{fm}$ with advancing gestation, the percent contribution of $CL_{f0}$ to $CL_f$ did not increase with increasing gestational age. In contrast to fetal clearances, none of the maternal clearance parameters changed significantly with gestational age. Thus, our subsequent analysis has focused on evaluating the mechanisms of this gestational age associated fall in fetal DPHM clearances.

It is widely accepted that only the free or unbound (not bound to plasma proteins) form of the drug is able to cross biological membranes. Hence, we examined the steady-state unbound fractions of DPHM in maternal and fetal plasma and their relationship with maternal and fetal drug clearances. DPHM is moderately to extensively bound in both maternal (range 71 - 97\%) and fetal (range 47 - 84\%) plasma, with the average fetal unbound fraction being significantly higher. In earlier studies in pregnant sheep, it has been demonstrated that DPHM plasma protein binding is linear in the concentration range
observed in these studies (Yoo et al., 1993). Detailed binding studies of DPHM with isolated plasma proteins have not been conducted and hence the specific binding proteins are not known. However, the degree of DPHM binding to human serum albumin is low (Drach et al., 1970) and the unbound fraction of the drug does not correlate with serum albumin concentration in humans (Meredith et al., 1984). This suggests that the tertiary amine DPHM may bind to \( \alpha_1 \)-acid glycoprotein like many other lipophilic basic drugs (Wilkinson, 1987). A lower degree of fetal plasma protein binding compared to the mother has also been observed for a number of other basic drugs (e.g., propranolol, methadone and lidocaine) in sheep as well as in humans, and this may be related to lower fetal plasma \( \alpha_1 \)-acid glycoprotein concentrations (Hill et al., 1986; Hill and Abramson, 1988; Szeto et al., 1982c; Czuba et al., 1988).

Figure 4.2 shows that both in the mother as well as the fetus, the magnitude of total DPHM clearance is tightly coupled with the corresponding plasma unbound DPHM fraction. This indicates that plasma protein binding of the drug is an important determinant of its total clearance. The relationship of F-UF with CL\(_f\) appears to be linear, whereas that of M-UF with CL\(_mm\) is closer to a hyperbola (see below). The F-UF also correlates very closely with CL\(_fm\) indicating that fetal to maternal placental transfer of the drug is dependent on protein binding of the drug in fetal plasma, as has been suggested earlier (Reynolds and Knott, 1989; Hill and Abramson, 1988; Rurak et al., 1991). However, as expected, CL\(_fm\) exhibited no relationship with protein binding of the drug in the maternal plasma (M-UF vs. CL\(_fm\), \( r = -0.1416, p > 0.5; \textit{data not shown} \)). In contrast to CL\(_fm\), the CL\(_mf\) clearance was not significantly related to M-UF (Figure 4.2E). At first, this may seem somewhat difficult to understand but a very plausible explanation can be made for this phenomenon. We have shown earlier in Chapter 3 that the fetal liver can metabolize a significant but variable (due
to the high variability in ductus venosus shunt fraction) and "unquantifiable" proportion of DPHM present in the umbilical vein in a first-pass manner before it reaches the fetal circulation. This leads to a corresponding variable underestimation of the $CL_{mf}$ parameter if the data are treated according to a 2-compartment model (Kumar et al., 1997; also see Chapter 3). Thus, in these studies, $CL_{mf}$ can not be estimated correctly and we believe that this underlies the lack of any obvious relationship between $M$-UF and $CL_{mf}$. Otherwise there appears to be no reason to believe that placental clearance in one direction ($CL_{fm}$) is dependent on plasma unbound fraction of the drug and not in the other ($CL_{mf}$).

Since F-UF is an important factor determining the magnitude of $CL_{fm}$, an obvious question is if F-UF changes significantly during the gestational period under study and whether this change can explain the observed fall of ~66% in $CL_{fm}$ from 125-136 d. Figure 4.3A demonstrates that fetal plasma protein binding increases progressively during these 12 days of gestation leading to a highly significant fall of ~47% in unbound fraction of the drug. Although we did not measure total or specific plasma protein concentrations during the course of these studies, at least total plasma protein concentrations in fetal sheep are known to gradually increase over this period of gestation (Kwan et al., 1995) and this might be related to the above fall in fetal DPHM plasma unbound fraction with advancing gestation. The observed fall in $CL_{fm}$ of ~66% from 125-136 d gestation is somewhat greater compared to the ~47% decrease in fetal plasma DPHM unbound fraction. Since F-UF is linearly related to $CL_{fm}$ (Figure 4.2B), the changes in F-UF do not appear to account for the entire observed fall in $CL_{fm}$ with gestational age. This, combined with two additional observations, strongly suggests the involvement of another factor in this $CL_{fm}$-gestational age relationship. Firstly, Figure 4.7 shows a relationship between $CL_{fm}$ clearance of the unbound drug (calculated using the unbound drug concentrations) with
gestational age and this parameter also falls significantly by ~43% from 125-136 d. Since, as expected, CL_{fm} (unbound drug) bears no relationship with F-UF (r = 0.1852, p = 0.5; data not shown), there must be an additional factor involved in the change of this unbound placental drug clearance with increasing gestational age. Secondly, in Figure 4.8 we have plotted a relationship between gestational age (from 109 to 134 d) and acetaminophen CL_{fm} data in pregnant sheep obtained by Wang et al. (1986a). Similar to DPHM, a significant inverse relationship between acetaminophen CL_{fm} and gestational age is also evident, although the magnitude of the change is less pronounced. This indicates that the gestational age-related fall in fetal placental drug clearance may be a more general phenomenon. However, in both the ewe and the fetus, plasma protein binding of acetaminophen is low (~10%, Wang et al., 1986a) and would not be a limiting factor for the placental transfer of the drug. Thus, it appears that protein binding is not the only factor involved in DPHM and acetaminophen CL_{fm} alterations with gestational age. We feel that the other possible factor affecting CL_{fm} is umbilical blood flow (Q_{um}). It has been shown that weight-normalized Q_{um} in sheep gradually decreases from 103 to 141 d gestation (Hedriana et al., 1995). We have observed a similar phenomenon in our laboratory as well. Figure 4.9 presents the umbilical flow data that we have obtained from 11 other pregnant sheep in different experiments. The mean fetal femoral arterial blood pH, P_{O_2}, P_{CO_2}, and O_2-saturation in these animals at the time of Q_{um} measurement were 7.350 ± 0.047, 19.9 ± 4.8 mm Hg, 47.7 ± 2.4 mm Hg, and 56.4 ± 13.4%, respectively, and these are within the normal range observed for fetal sheep at this stage of gestation in ours as well as others’ laboratories. The data indicate a significant inverse relationship between Q_{um} and gestational age and predict a ~37% fall in Q_{um} over 125 to 136 d gestation. This may represent an inability of the fetal cardiovascular system to sustain
both fetal regional perfusion and $Q_{um}$ during the rapid phase of fetal growth during late gestation (~2% increase in weight/day, Kwan et al., 1995). There is no clear indication for a similar relationship in the human fetus (St. John Sutton et al., 1990 & 1991), although a fall in vascular $P_{O_2}$ in humans as well as sheep over the last ~½ of gestation (Soothill et al., 1986; Bell et al., 1986) points towards a fall in weight-normalized $Q_{um}$ in both species. In sheep, human and guinea pig, there is no evidence for a fall in either uterine or maternal placental blood flow per kg fetal weight during the last part of gestation (Rosenfeld et al. 1974; Thaler et al. 1990; Myers et al. 1982; Peeters et al. 1982). Thus, the decrease in $Q_{um}$ with gestational age will result in a fall in fetal-to-maternal placental
blood flow ratio. In sheep, the relationship between placental clearance of flow-limited compounds (ethanol and antipyrine) and uterine (Q\textsubscript{ut}) and umbilical (Q\textsubscript{um}) blood flow is described by the equation: $\text{CL} = 1/(1/0.91\ Q_{\text{ut}} + 1/0.83\ Q_{\text{um}})$ (Wilkening \textit{et al.}, 1982). This equation predicts that a fall in either Q\textsubscript{ut} or Q\textsubscript{um} will reduce the clearance of these compounds. At a constant Q\textsubscript{ut}, the Q\textsubscript{um} exhibits a curvilinear relationship with placental clearance and hence a fall in Q\textsubscript{um} will lead to a less than proportional decrease in placental clearance. As CL\textsubscript{fm} for DPHM is similar to the values for ethanol and antipyrine (98.6 ± 11.9 and 113.4 ± 13.5 ml/min/kg, respectively; Wilkening \textit{et al.}, 1982), the above relationship likely holds for DPHM and many other high placental clearance drugs as well.
Figure 4.9 - Weight-normalized umbilical blood flow in 11 pregnant sheep between 128-141 d gestation as a function of gestational age. Blood flow was measured by an electromagnetic flow transducer (n = 4), an ultrasonic flow probe (n = 5), or radioactive microspheres (n=2).

The combined fall in F-UF and $Q_{um}$ appears more than sufficient to explain the observed relationship between DPHM $CL_{fm}$ and gestational age. The $CL_{fm}$ of acetaminophen is much lower compared to that of DPHM, ethanol and antipyrine. However, in vitro studies with perfused human placenta have demonstrated that the placental transfer of a low placental permeability compound, cimetidine, was also significantly affected by alterations in the rate of umbilical perfusion (Bassily et al., 1995). This indicates that a gestational age-related fall in $Q_{um}$ is likely responsible for a decrease in acetaminophen $CL_{fm}$ with advancing gestation. Also, in contrast to DPHM, $Q_{um}$ is probably the only variable involved in acetaminophen $CL_{fm}$ vs. gestational age relationship because the plasma protein binding for this drug is not a limiting factor.
In contrast to the fetal situation, the M-UF of the drug did not change significantly over the gestational period under study in our experiments (Figure 4.3B). Concomitant with this, there was no relationship between any of the maternal clearance parameters with gestational age.

Figures 4.2C and 4.2F show the relationships between F-UF vs. CL\_fo, and M-UF vs. CL\_mo, respectively. The linear regression of both CL\_fo vs. F-UF and M-UF vs. CL\_mo was statistically significant. However, the y-axis zero intercepts of both these relationships were positive, being 31 and 72 % of the average CL\_fo and CL\_mo estimates, respectively. This may be explained by the fact that the relationship of plasma unbound fraction with drug clearance is in fact curvilinear, as described by the most common models of organ clearance (e.g., well-stirred, parallel tube and dispersion models of hepatic elimination; Wilkinson, 1987). Thus, it would be conceptually inaccurate to fit our unbound fraction vs. non-placental clearance data to a simple linear model, and perhaps that underlies the high positive y-intercepts obtained in our linear regression analyses. Hence, these relationships were analyzed according to the most commonly studied and accepted well-stirred model of organ drug clearance (Wilkinson and Shand, 1975; Wilkinson, 1987). The data were fitted to equation (1) which is analogous to the unbound fraction vs. clearance relationship of the well-stirred model of hepatic elimination (equation 2). We have shown earlier that hepatic uptake of DPHM is a major component of CL\_fo (see Chapter 3), and hence the parameter terms P1, P2 and P1/P2 of equation (1) correspond to fetal Q\_H*CL\_uint, Q\_H, and CL\_uint, respectively. In the mother, both the liver and gut are the major organs contributing to CL\_mo, and hence Q\_H, and CL\_uint correspond to the entire hepato-portal system. The CL\_uint and Q\_H in the mother were estimated to be 1242.6 ± 176.8 and 60.2 ±
5.7 ml/min/kg, respectively. The estimated $Q_H$ is very close to that reported for pregnant sheep (65 ml/min/kg; Katz and Bergman, 1969), supporting the validity of our analysis. From the fit of F-UF vs. $CL_{fo}$ data, the $CL_{int}^u$ and $Q_H$ in the fetus were estimated to be $517.6 \pm 251.2$ and $318.7 \pm 306.6$ ml/min/kg, respectively. The high standard error in the $CL_{int}^u$ and $Q_H$ estimates in the fetus is related to a wider variability in unbound fraction vs. $CL_{fo}$ relationship (Figure 4.2C). Also, the estimated mean value of $Q_H$ is higher compared to the average reported for fetal sheep at this stage of gestation (~137 ml/min/kg; Edelstone et al., 1979). Overall, from the above analysis it is clear that plasma protein binding is an important factor in the hepatic (hepatic and gut in case of the mother) uptake of DPHM both in the mother as well as the fetus. Since F-UF falls with gestational age, it may explain the decreasing trend in $CL_{fo}$ with gestational age (Figure 4.1C). Also, ~75% of the fetal hepatic blood supply comes from $Q_{um}$ (Edelstone et al., 1978), and the gestational age-related fall in $Q_{um}$ may also contribute to the observed negative trend in $CL_{fo}$ with gestational age via reductions in fetal hepatic blood flow.

4.3.2 Inter-relationships between Maternal and Fetal Plasma DPHM Concentrations, Unbound Fractions and the 2-Compartment Model Clearance Estimates

Although the 2-compartment model of the maternal-fetal unit is the simplest pharmacokinetic representation of this system, the exact relationships between the four fundamental clearance parameters of this model ($CL_{mo}$, $CL_{mf}$, $CL_{fo}$ and $CL_{fm}$) and various maternal-fetal plasma drug concentrations ($C_m$, $C_f$, $C_f'$, $C_m'$) are not directly obvious. Also, the influence of maternal and fetal plasma drug protein binding in determining these concentrations is generally speculative and has rarely been determined experimentally. In our experiments, both $C_m$ and $C_f'$ were negatively correlated with M-UF and F-UF,
respectively (Figure 4.4). This indicates that maternal and fetal plasma protein binding is an important determinant of these concentrations possibly because of its effects on maternal and fetal clearance (Figure 4.2, also see below). In contrast, the $C_f$ concentration was not related to either M-UF or F-UF. However, as discussed before, the $C_f$ is variably underestimated due to fetal hepatic first-pass uptake of the drug from the umbilical vein (Chapter 3) and that may underlie this lack of any relationship. This indicates that for many drugs the major determinant of $C_f$ after maternal drug administration may, in fact, be the extent of this fetal hepatic first-pass uptake/metabolism of the drug rather than maternal or fetal plasma protein binding. Analogous to $C_f$, the $C_m$ concentration bore no relationship with F-UF on the other side of the placenta. Instead its negative correlation with M-UF was near statistical significance $(r = 0.4612, p = 0.05, \text{data not shown})$ indicating that the latter could be a determinant of this concentration via its effects on maternal clearance of the drug (see below).

We have also determined which maternal or fetal plasma drug concentrations affect the magnitude of different clearance parameters of the 2-compartment model or vice versa. For this purpose, various clearance parameters were analyzed against different maternal and fetal steady-state concentrations using the simple steady-state clearance model, $CL = \frac{k_o}{C_{ss}}$. The estimated value of $k_o$ from this relationship represents the amount of drug eliminated via that clearance route per unit time. Figure 4.5A shows that the majority of the variability in $C_m$ was reflected in the final estimates of total $CL_{m0}$ (not weight-normalized), whereas $C_m$ was not related to any other clearance parameter. This indicates that $CL_{m0}$ is the major determinant of maternal steady-state concentration after maternal drug administration. This will be generally true for most high clearance drugs because the absolute magnitude of $CL_{m0}$ is much higher compared to any other clearance
parameter (for DPHM, $CL_{mo} = 3058.5 \pm 745.5$ ml/min; $CL_{mf} = 114.5 \pm 88.6$ ml/min; $CL_{f0} = 257.5 \pm 135.0$ ml/min; $CL_{fm} = 408.5 \pm 225.1$ ml/min). The estimated value of the $k_0$ coefficient was $659.9 \pm 1.8$ $\mu$g/min which is ~98% of the total maternal drug infusion rate (670 $\mu$g/min), indicating that most of the drug infused to the mother is eliminated via maternal non-placental routes. In spite of a variable underestimation of $C_f$, the majority of the variability in measured $C_f$ was carried over to the $CL_{mf}$ parameter (Figure 4.5B), indicating that this clearance parameter is also almost equally underestimated. When the drug was infused to the fetus, the variability in $C_f$ was reflected relatively equally in the final $CL_{f0}$ and $CL_{fm}$ estimates, indicating that both of these clearances are important determinants of $C_f$ (in contrast to the situation in the mother where only $CL_{mo}$ is important, see above). The $k_0$ coefficients of $CL_{f0}$ vs. $C_f$ and $CL_{fm}$ vs. $C_f$ relationships were $59.2 \pm 4.9$ and $95.6 \pm 3.8$ $\mu$g/min, respectively, which when added together approach the total fetal drug infusion rate of 170 $\mu$g/min. In contrast to the $CL_{mf}$ vs. $C_f$ relationship (see above), the variability in $C_m$ was not related to the magnitude of estimated $CL_{fm}$ (Figure 4.5E). This is understandable because, as discussed above, the major determinant of maternal plasma concentrations is expected to be $CL_{mo}$. Based on this, $CL_{mo}$ does in fact appear to explain the variation in $C_m$ among different animals quite well (Figure 4.5F).

### 4.3.3 Relationships between the Indices of Fetal Drug Exposure/Placental Transfer, Gestational Age and Plasma Protein Binding

After maternal drug administration, the steady-state fetal to maternal arterial plasma concentration ratio ($C_f/C_m$ in this study) is commonly used as an index of the efficiency of placental drug transfer and fetal exposure to the drug (Rurak et al., 1991). In our experiments, the $C_f/C_m$ ratio based on total drug concentrations ($0.20 \pm 0.14$) was
significantly lower compared to that based on unbound drug concentrations (0.50 ± 0.30), indicating that the magnitude of total drug \( C_f/C_m \) ratio is partly determined by the differences in maternal and fetal plasma protein binding. The unbound drug \( C_f/C_m \) ratio was significantly lower than unity (unpaired t-test, \( p < 0.0001 \)) indicating irreversible non-placental drug elimination by the fetus (Szeto et al. 1982a; Czuba et al., 1988). It has been postulated that fetal plasma protein binding (Rurak et al., 1991) and total fetal clearance are important factors in determining the \( C_f/C_m \) ratio at steady-state (\( C_f/C_m = \frac{CL_{mf}}{[CL_{fm} + CL_{fo}], Szeto et al., 1982b} \)). However, in our experiments, the total drug plasma \( C_f/C_m \) ratio neither exhibited any significant relationship with fetal placental, non-placental or total drug clearance, nor with F-UF and gestational age (data not shown). We feel that the lack of any relationship among these variables is also related to the errors in the measurement of "true \( C_f \)" due to fetal first-pass hepatic uptake of the drug present in the umbilical venous blood (Chapter 3). The positive relationship of \( C_f/C_m \) with M-UF approached statistical significance (\( r = 0.4029, p < 0.1, data not shown \)) in spite of the errors in \( C_f \), and this was mainly because of a strong negative correlation between \( C_m \) and M-UF (Figure 4.4A). However, this \( C_f/C_m \) ratio exhibited a significant inverse relationship with the ratio of fetal to maternal unbound fraction (Figure 4.6A). The most common interpretations of these data, assuming that unbound drug concentrations on the two sides of the placenta exist in direct equilibrium, are: i) as the F-UF relative to M-UF increases, the placental transfer of the drug is reduced due to a fall in the important driving force (difference in unbound \( C_m \) and \( C_f \) concentrations across the placenta) for drug transport, and ii) it is the balance of relative unbound fractions in the maternal and fetal plasma, rather than their individual plasma protein binding characteristics, that is more important in determining fetal drug exposure (Morgan et al., 1988; Nau et al., 1984). It must be emphasized, however, that in our data the above \( C_f/C_m \) vs. F-UF/M-UF relationship
reached statistical significance mainly because of two phenomena operating independent of each other, *i.e.*, i) a close inverse relationship of M-UF with Cₘ possibly via its effects on CLₘo (Figures 4.2F, 4.4A and 4.5A), and ii) a slight but non-significant decreasing trend of Cᵢ with increasing F-UF. There appears to be, however, no cross influence of M-UF on Cᵢ, or that of F-UF on Cₘ, which is to be expected if the above interpretations based on the "unbound drug equilibrium" assumption are true. However, in practice, the latter relationship is less likely because the fetal compartment is very small compared to the mother. Thus, the DPHM data appear to be in conflict with the unbound drug equilibrium hypothesis of placental transport. Any of these interpretations involving Cᵢ, however, may not be entirely accurate due to the confounding effects of the above described fetal hepatic first-pass uptake of the drug from the umbilical vein on Cᵢ.

To overcome this problem, we evaluated the factors affecting the analogous index of placental transfer in the other direction (fetus to mother) after fetal drug administration, *i.e.*, the Cₘ'/Cᵢ' ratio. The Cₘ'/Cᵢ' ratios for DPHM based on total and unbound drug concentrations were 0.14 ± 0.08 and 0.05 ± 0.02, respectively. The latter ratio was significantly lower than the former as well as compared to unity due to reasons similar to those described above for the Cᵢ/Cₘ ratio. In all our experiments, the fetal drug infusion rate was the same (170 µg/min). However, at this infusion rate, the Cᵢ' concentration increased significantly (by ~67%, *i.e.*, from 165 to 506 ng/ml, as predicted by the regression equation) with increasing gestational age over the 125 - 136 d period (Figure 4.6B) due to a fall in CLₘ and possibly CL₀ (Figure 4.1, also see discussion above). This gestational age related increase in Cᵢ' was mainly responsible for a progressive decrease of ~53% (*i.e.*, from 0.25 to 0.12, as predicted by regression equation) in the Cₘ'/Cᵢ' ratio over the same period of gestation (Figure 4.6C), as Cₘ' demonstrated no change during
this time period. On similar lines to the $C_f/C_m$ ratio above, it can be hypothesized that $CL_{fm}$, $CL_{mo}$ and $CL_{mf}$ will be the important factors determining the $C_m'/C_f'$ ratio, i.e., $C_m'/C_f' = CL_{fm}/(CL_{mo} + CL_{mf})$. However, the $C_m'/C_f'$ ratio did not show any relationship with $CL_{mf}$ (again this could be due to errors in $CL_{mf}$ estimates) and its inverse relationship with $CL_{mo}$ (as well as $CL_{mm}$) was only near statistical significance ($r = -0.4138$, $p < 0.1$; data not shown). Thus, $CL_{mo}$ (and $CL_{mm}$) does not appear to be an important variable determining the magnitude of the $C_m'/C_f'$ ratio. Total $CL_{fo}$, total $CL_{fm}$ and F-UF are the important factors determining $C_f'$, all being inversely related to the magnitude of $C_f'$ (Figures 4.5C, 4.5D and 4.4B; also see discussion above). An increase in any of these variables leads to a fall in $C_f'$ (Figures 4.5C, 4.5D and 4.4B) and hence to a significant increase in the $C_m'/C_f'$ ratio (Figures 4.6D, 4.6E, 4.6F), $C_m'$ being unaffected by any of these factors. In contrast, the $C_m'/C_f'$ ratio was not significantly related to M-UF. Thus, in this situation, $CL_{fo}$, $CL_{fm}$ and F-UF appear to be the most important factors determining the $C_m'/C_f'$ ratio mainly via their effects on $C_f'$.

From a number of observations, it appears that at least for fetal-to-maternal placental drug transfer, the factors operating on the opposite side of the placenta do not provide any significant driving force for placental passage of the drug and thus have a minimal, if any, effect on the kinetics of placental transport. These observations include: i) the $C_m'/C_f'$ ratio is not affected by $CL_{mo}$ and M-UF, and ii) the $C_m'$ concentration is not at all influenced by F-UF or $CL_{fm}$. This is in contrast to the generally accepted principles of placental transport where these variables are considered the predominant factors affecting the passage of the drug across the placenta, at least for highly diffusible compounds, such as DPHM, whose placental transfer is not permeability rate limited. It is not entirely clear if the same phenomenon occurs in the maternal-to-fetal direction of DPHM placental transfer, although
M-UF and CL_{mo} have no influence on C_f. This cannot be determined until an approach is developed for accurate estimation of C_f and subsequently CL_{mf}. However, the presence of this phenomenon in at least the fetal-to-maternal direction may indicate that the unbound concentrations of the drug on both sides of the placenta may not be in complete equilibrium with each other at the site of placental exchange, as is generally assumed. The steady-state concentrations of a number of highly diffusible markers, which are not plasma protein bound and have blood flow limited clearance (e.g., antipyrine, ethanol, D_2O), do not equilibrate completely between the maternal and fetal placental outflow vessels (uterine and umbilical veins, respectively) in the sheep and cow (Meschia et al., 1967; Wilkening et al., 1982; Reynolds et al., 1985). This has been attributed to the fact that significant inefficiencies exist within the placental vasculature of many animal species including sheep, and also the human. These include partial shunting of the uterine and umbilical blood flows to non-exchange areas of the placenta and to non-placental tissues, and unequal maternal-fetal perfusion in different regions of the placenta (Wilkening et al., 1982; Reynolds et al., 1985; Bassily et al., 1995). However, in spite of the presence of these placental inefficiencies, it appears that the parameters describing the kinetics of fetal-to-maternal DPHM placental transfer (e.g., C_m'/C_f' ratio) should be at least somewhat, if not proportionately, influenced by M-UF and the magnitude of maternal non-placental (and total) clearance, if an equilibrium existed between the unbound drug concentrations in the blood supplying the two sides of the placental exchange site. The fact that DPHM placental transport is tightly coupled to many variables operating only on one side of the placenta and to none on the other strongly suggests that the assumption of a complete equilibrium between the unbound drug concentrations on the two sides of placenta may not be entirely accurate. This may explain the discrepancies observed in our DPHM data from the general rules of placental transport. The possibility of this phenomenon can be
realized by considering the anatomical structure of the epitheliochorial sheep placenta, which has a number of tissue layers separating maternal and fetal blood flows. Also, the available evidence on the geometrical arrangement of maternal and fetal placental blood flows at the placental exchange site suggests a relatively less efficient concurrent (sheep and cow; Wilkening et al., 1982; Reynolds et al., 1985) and pool flow (human, Bassily et al., 1995) arrangement in many species. These factors along with a rapid transit time of the blood through the placental circulation (Power and Longo, 1975) may lead to incomplete equilibration of the unbound drug concentrations in maternal and fetal blood at the placental exchange site even for compounds with very high placental permeability. It remains to be determined if a similar phenomenon exists during drug passage through the hemochorial human placenta which has fewer anatomical tissue layers compared to sheep.

In summary, our data demonstrate that DPHM fetal total, placental and possibly non-placental clearances decrease during the last 2 weeks of gestation in sheep. An increase in fetal plasma protein binding of the drug and a decrease in weight-normalized umbilical blood flow with advancing gestation appear responsible for these clearance changes. A similar phenomenon is apparent in the literature data of acetaminophen, a drug with much lower placental clearance compared to DPHM, indicating that it is not only limited to high placental clearance drugs. Plasma protein binding also appears to be an important determinant of maternal and fetal non-placental DPHM clearances. Fetal placental clearance of the drug is highly dependent on the extent of plasma protein binding in the fetus but not in the mother. An analogous relationship between maternal placental clearance and maternal plasma protein binding could not be demonstrated probably because of errors in the estimation of this placental
clearance due to fetal hepatic first-pass uptake of the drug from the umbilical venous blood. The major determinant of plasma drug concentrations in the mother after maternal as well as fetal administration is maternal plasma protein binding and maternal non-placental clearance. In contrast, the major determinant of fetal plasma concentrations after maternal drug administration is the extent of fetal first-pass hepatic drug uptake from the umbilical vein. However, after fetal drug administration, the fetal plasma concentrations are related to the extent of fetal plasma protein binding, and fetal placental and non-placental clearances. The index of fetal-to-maternal placental drug transfer (steady-state $C_m/C_f$ ratio) was related to $F-UF$, and fetal placental and non-placental clearance; its magnitude decreased significantly with advancing gestation due to a fall in all these variables. This index, however, was not related to the magnitude of factors operating on the other side of the placenta such as maternal plasma protein binding and maternal non-placental clearance, as is generally postulated. These variables thus do not seem to provide any significant driving force for placental transfer of the drug and have a minimal effect on the kinetics of placental transport. This might indicate a lack of complete equilibration of unbound drug concentrations on the two sides of the placenta, leading to departures from ideal placental transport characteristics. Similar types of relationships, however, could not be described for maternal-to-fetal placental transfer and the fetal drug exposure index after maternal drug administration (steady-state $C_f/C_m$ ratio) because of variable underestimation of $C_f$ due to fetal hepatic first-pass uptake of the drug from the umbilical venous blood.
This chapter describes the detailed studies on the disposition of valproic acid (VPA) in the mother and the fetus. In addition, studies were also conducted in the immediate newborn period because: 1) fetal metabolic capacity for the drug could not be accurately determined, and 2) VPA is occasionally administered to human newborns to control seizures that are refractory to other anticonvulsants. The rationale for studying VPA was to examine a drug with different physicochemical and pharmacokinetic properties, as well as distinctly different metabolic pathways as compared to DPHM. The overall objectives of these studies were similar to those of DPHM studies. These include: 1) to examine the extent of and the factors influencing fetal drug exposure during steady-state maternal administration, and 2) to study the pharmacokinetics and metabolism of the drug in the mother, fetus and newborn.

5.1 Methods

5.1.1 Animals and Surgical Preparation

A total of 5 pregnant Dorset-Suffolk cross-bred ewes, with a maternal body weight of 77.5 ± 10.6 kg (mean ± S.D.), were employed for maternal and fetal VPA disposition studies. The ewes were surgically prepared between 121-125 d gestation (term ~145 d). The surgical procedures were similar to those described in Chapter 3 for DPHM studies. Polyvinyl catheters (catheter i.d. 1.02 mm and o.d. 2.16 mm) were implanted in both fetal
femoral arteries and lateral tarsal veins, one fetal carotid artery, and a maternal femoral artery and vein. Catheters were also implanted in the fetal trachea, amniotic cavity and in four animals in the fetal urinary bladder (via a suprapubic incision). The procedures for post-operative care and maintenance of these animals were also similar to those described in Chapter 3. 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 was inserted via the urethra of the ewe and attached to a sterile polyvinyl bag for cumulative maternal urine collection. Fetal urine flow rate was estimated using a computer controlled roller pump assembly developed in our laboratory. The fetal bladder catheter was allowed to drain by gravity into a sterile reservoir (10 ml syringe barrel) to which a disposable DTX transducer was connected. When the pressure in the reservoir increased above a preset level (usually 3 mm Hg) as a result of urine accumulation, the computer activated a roller pump (DIAS, Ex154, DIAS Inc. Kalamazoo, MI) which pumped a calibrated volume of urine from the reservoir back to the amniotic cavity (via the amniotic catheter). The cumulative volume pumped per min, which equals fetal urine production rate per min, was stored on diskette. During the experimental period and at specified sampling time intervals, fetal urine samples (~5 ml) were collected by attaching a sterile sample collection syringe to the amniotic catheter via a 3-way stop-cock.

For newborn lamb studies, five additional pregnant sheep were prepared surgically as above at 121-132 d gestation. Lambs were allowed to undergo spontaneous delivery at term (139-143 d), along with their intact catheters. Experiments on the lambs were started the day after birth. On the day of experiment, the lambs were placed in small pens, in full view of the mother, and were fed mother's colostrum at intervals. After
completion of the drug infusion period (6 h), the lambs were returned to their mother. Cumulative newborn lamb urine samples were collected in the following manner. A sterile bag was attached to the bladder catheter, the bag was then placed in a small plastic housing which was bandaged to the abdomen of the animal. This permitted free movement of the lambs within the pen and also allowed them to nurse on their mother ad libitum.

5.1.2 Experimental Protocols

5.1.2.1 Pregnant Sheep Experiments

All experiments on pregnant sheep were completed between 125-138 d gestation (term ~145 d). Two sets of experiments were carried out on all five pregnant sheep in a randomized manner and with an appropriate washout period in between.

1. **Maternal Administration:** A bolus loading dose of VPA (Sodium Valproate, Sigma Chemical Co., St. Louis, MO) equivalent to 20.1 mg VPA/kg maternal body weight was administered to the ewe via the maternal femoral venous catheter over 1 min; this was followed immediately by a 24 h continuous infusion of the drug at 138.3 μg/min/kg via the same route.

2. **Fetal Administration:** The fetal experimental protocol was similar to that for the maternal experiments described above, except that doses were administered via the fetal lateral tarsal vein and were reduced to ¼ of the maternal doses (i.e., 5.0 mg/kg bolus and 34.6 μg/min/kg infusion rate based on maternal body weight).
5.1.2.2 Newborn Lamb Experiments

As mentioned above, the newborn lamb experiments were begun the day after birth. Drug administration involved a 10 mg/kg bolus administered over 1 min via the lateral tarsal vein, followed immediately by a continuous 6 h infusion at 138.3 μg/min/kg via the same route.

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.

In all pregnant sheep experiments, serial blood samples were collected from the fetal (2 ml) and maternal (3.0 ml) femoral arterial catheters at 5 min, and 0.5, 1, 3, 6, 9, 12, 20, 22, and 24 h during the infusion, and at 0.5, 1, 3, 6, 9, 12, 24, 36, 48, 60, and 72 h post-infusion. Fetal femoral arterial samples (0.5 ml) were also collected at the same time intervals for blood gas analysis and measurement of glucose and lactate concentrations. All fetal blood removed for sampling during the experiment was replaced, at intervals, by an equal volume of blood obtained from the mother prior to the start of the experiment or from another ewe (after the first day). Cumulative samples of maternal urine were collected at 1, 3, 6, 9, 12, 20, 22, and 24 h during the infusion, and at 3, 6, 9, 12, 24, 36, 48, 60, and 72 h post-infusion. Serial samples of fetal urine were collected at the same time intervals as maternal urine. Serial samples of amniotic fluid (2 ml) and fetal tracheal fluid (2 ml) were also collected at the same time intervals as the MA and FA blood samples.
During the newborn lamb experiments, serial femoral arterial blood samples were collected at 5 min, and 0.5, 1, 2, 3, 4, 5, and 6 h during the infusion, and at 0.5, 1, 2, 4, 6, 18, 30, 42, 54, 66, 78, and 90 h post-infusion. Cumulative urine samples were also collected at 2, 4, and 6 h during the infusion, and at 2, 4, 6, 18, 30, 42, 54, 66, 78, and 90 h post-infusion.

Plasma was harvested from the blood samples as described in Chapter 3. All plasma, urine, amniotic fluid and tracheal fluid samples were placed into clean borosilicate test tubes with polytetrafluoroethylene (PTFE)-lined caps and were stored at -20°C until the time of analysis.

5.1.3 Physiological Recording and Monitoring Procedures

Fetal blood pH, Po\textsubscript{2}, Pco\textsubscript{2}, O\textsubscript{2}-saturation, and hemoglobin, glucose and lactate concentrations were measured before and during the VPA infusion experiments as described earlier in Chapter 2 (Section 2.2.4).

5.1.4 Plasma Protein Binding of VPA and its Metabolites in the Mother and the Fetus

The unbound plasma concentrations of VPA and its metabolites were measured ex vivo in all fetal, maternal, and newborn plasma samples by an ultrafiltration procedure using Centrifree® micropartition devices (Amicon, Inc., Danver, MA). Briefly, 0.5 – 0.75 ml of plasma was placed into Centrifree® micropartition devices and centrifuged at 1000 x g for 30 min at a temperature of 4°C (in order to minimize lipolysis and release of free fatty
acids). This procedure typically yielded an ultrafiltrate volume of 0.2 – 0.3 ml for analysis of VPA and its metabolites.

5.1.5 Preparation of Maternal and Fetal Liver Microsomes

Hepatic microsomes were prepared from six maternal and five fetal sheep (gestational age 120 – 142 d). Pregnant ewes were sacrificed by an overdose of pentobarbital, and a single slice of maternal liver and the whole fetal liver was quickly harvested. The livers were thoroughly washed free of blood with ice-cold 0.05M Tris, 1.15% KCl (pH 7.4) (Tris/KCl) buffer and were either processed immediately or were quickly frozen in liquid nitrogen and stored at –80°C until processing. Microsomes were prepared using standard differential ultracentrifugation techniques at a temperature of 4°C (Lu and Levin, 1972). Briefly, the livers (or a liver slice for maternal livers) were minced and then homogenized in ice-cold Tris/KCl buffer using a Potter-Elvehjem glass mortar and a motor driven pestle. The homogenate was centrifuged twice at 10,000 x g for 20 min. The supernatant was then centrifuged at 100,000 x g for 60 min. The resulting microsomal pellet was resuspended in 10mM EDTA, 1.15% KCl buffer (pH 7.4), and centrifuged again at 100,000 x g for 60 min. The microsomal pellet was then resuspended in 0.25 M sucrose and aliquots were stored in cryo vials at –80°C until use. Microsomal protein concentrations were measured using the method of Bradford (Bradford, 1976).

5.1.6 VPA Glucuronidation in Maternal and Fetal Liver Microsomes

VPA glucuronidation kinetics were determined in pooled maternal and fetal microsomal preparations. For this purpose, equal amounts of microsomal protein from each maternal
or fetal microsomal preparation were mixed. Incubations were optimized with respect to
the concentrations of microsomal protein, substrate (VPA), and cofactor (UDPGA), and
linearity of product formation with time. Final 1 ml incubations contained 3 mg microsomal
protein, a 9.7 mg/ml (15 mM) concentration of UDPGA, and 10 mM magnesium chloride in
150 mM tris-HCl buffer (pH 7.4). Triton X100 was also included at a concentration of
1μl/ml incubation. After a pre-incubation time of 3 min at 39°C (sheep body temperature),
the reaction was started by the addition of substrate (VPA), and the incubations proceeded
for 15 min thereafter. At the end of the incubation period, the reaction was stopped by the
addition of 100 μl acetonitrile, and immediate freezing of the samples at −20°C. Enzyme
kinetic experiments were conducted at substrate concentrations of 5, 10, 20, 30, 50, 75,
100, 150, 250, 500, 1000, 1500, and 2000 μg/ml (range 0.035 – 13.9 mM). The $K_m$ and
$V_{max}$ estimates were obtained by nonlinear least square fitting of the glucuronidation rate
vs. substrate concentration data.

5.1.7 Drug and Metabolite Assay

The concentrations of VPA and its metabolites in all biological fluids and plasma
ultrafiltrate were measured using a previously developed gas chromatographic-mass
spectrometric (GC-MS) analytical method (Yu et al., 1995; Chapter 2, section 2.5). The
concentrations of VPA glucuronide in maternal, fetal and newborn urine, as well as in
microsomal incubations, were measured using a base hydrolysis procedure as follows.
The 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 analysis method. The
difference of total and unconjugated (unhydrolyzed) VPA concentrations gave the
concentration of VPA glucuronide. 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). The hydrolysis with alkali however is capable of measuring total VPA-glucuronide in spite of these possible rearrangements (Dickinson et al., 1984).

5.1.8 Pharmacokinetic Analysis

The net maternal and fetal clearances of the unbound drug (CL\textsubscript{m(\text{net})} and CL\textsubscript{f(\text{net})}, respectively) were calculated as:

\[
CL^u_{m(\text{net})} = \frac{\text{Maternal Infusion Rate}}{\text{MA Steady - State Unbound Concentration}} \quad (1)
\]

and,

\[
CL^u_{f(\text{net})} = \frac{\text{Fetal Infusion Rate}}{\text{FA Steady - State Unbound Concentration}} \quad (2)
\]

The net maternal and fetal clearances of the total drug (CL\textsubscript{m(\text{net})} and CL\textsubscript{f(\text{net})}, respectively) were calculated in an analogous fashion to equations (1) and (2), except that total instead of unbound MA and FA concentrations were used.
The placental and non-placental clearances of VPA in the ewe and the fetus were calculated from the maternal and fetal steady-state total and unbound arterial plasma concentration data according to the 2-compartment model described earlier (Szeto et al., 1982a; for equations see Chapter 3).

For the analysis of VPA protein binding data in maternal, fetal and newborn plasma, the plasma protein bound concentrations of the drug were first calculated from a difference between the corresponding experimentally measured total and unbound concentrations. Plasma protein binding parameters were calculated separately for maternal, fetal and newborn plasma using the pooled bound and unbound plasma concentration data from all animals. Rosenthal plots (bound/unbound concentration vs. bound concentration) were first constructed in order to identify the multiplicity of the binding sites. The bound vs. unbound concentration data were then fitted to the appropriate binding model using the nonlinear least squares regression program ADAPT II (D'Argenio and Schumitzky, 1997) in order to estimate the binding parameters. The best binding model was identified based on the reduction in sum of squared residuals and Akaike's Information Criterion (AIC).

All other pharmacokinetic parameters were calculated by the equations described below (Wagner, 1993; Gibaldi and Perrier, 1982):

Mean residence time of VPA (total or unbound):

\[
MRT_{\text{total or unbound drug}} = \left( \frac{AUMC_{0-\infty}^{\text{total or unbound}}} {AUC_{0-\infty}^{\text{total or unbound}}} \right) - \frac{k_0 \cdot \tau^2} {2(k_0 \cdot \tau + D_{\text{bolus}})}
\]  

(3)
where, $k_0$, $\tau$, and $D_{bolus}$ are the infusion rate, infusion duration, and initial bolus dose of VPA, respectively. Plasma $AUMC^{0-\infty}$ and $AUC^{0-\infty}$ of the unbound and total drug were calculated by the linear trapezoidal rule.

Total body clearance ($CL_{tb}$) of VPA (total or unbound):

$$CL_{tb} = \frac{\text{Total i.v. Dose}}{AUC^{0-\infty}}$$  \hspace{1cm} (4)

Area weighted unbound fraction of the drug ($f_p$):

$$f_p = \frac{AUC^{0-\infty}_{\text{unbound drug}}}{AUC^{0-\infty}_{\text{total drug}}}$$  \hspace{1cm} (5)

Steady-state volume of distribution of the unbound drug ($Vd_{ss}^u$):

$$Vd_{ss}^u = (CL_{tb})_{\text{unbound drug}} \cdot MRT_{\text{unbound drug}}$$  \hspace{1cm} (6)

Steady-state volume of distribution of the total drug ($Vd_{ss}$):

$$Vd_{ss} = (CL_{tb})_{\text{total drug}} \cdot MRT_{\text{total drug}}$$  \hspace{1cm} (7)

Steady-state volume of distribution of the total drug corrected for the effects of saturable plasma protein binding ($Vd_{ss}'$):
Maternal and fetal terminal elimination half-life ($t_{1/2p}$) of the total and unbound drug was obtained from a 2-compartment model fitting of the data using nonlinear least-squares regression software WinNonlin (Scientific Consulting, Inc., Apex, NC). The terminal plasma $t_{1/2}$ of the total and unbound drug in the newborn lamb was approximated using a 1-compartment model. These model fittings were carried out using a weighting factor of 1/predicted ($y^2$).

Renal clearance values for the total and unbound VPA in the ewe and the newborn lamb were calculated by dividing the total amount of unconjugated VPA excreted in urine by the respective plasma AUC$_{0-\infty}^{0-\infty}$ of the total or unbound drug. Fetal renal clearance of total and unbound drug was calculated by dividing the urinary excretion rate determined at each sampling point by the corresponding total or free fetal plasma drug concentration of the drug at that time. Urinary excretion rate of the drug at each sampling point was determined from the concentration of the drug in fetal urine and the average urine flow rate during the 0.5 h preceding the sampling time point. Fetal renal clearances thus calculated at each sampling point were averaged to obtain a mean value for this parameter.

Newborn lamb unbound plasma concentration data were fit to a one-compartment model with nonlinear Michaelis-Menten elimination in order to obtain the \textit{in vivo} estimates of $K_m$ and $V_{max}$. Maternal unbound plasma concentration data were also fit to this one-compartment Michaelis-Menten model. However, for this fitting, the estimate of $K_m$ was fixed to that obtained \textit{in vitro} from VPA glucuronidation experiments in pooled maternal liver microsomes, and only the $V_{max}$ parameter was estimated by model fitting (see
Discussion for further details on this). These Michaelis-Menten pharmacokinetic estimations were performed using a weighted least squares algorithm within the pharmacokinetic modeling software ADAPT II (D'Argenio and Schumitzky, 1997). Unbound plasma concentration data were weighted with the inverse of the variances of the measured concentrations. A linear variance model was specified where the coefficients of variation varied linearly from 20% at the LOQ to 5% at the highest measurable concentration of the assay.

5.1.9 Statistical Analysis

All data are reported as mean ± S.D. The achievement of steady-state for total and unbound VPA concentrations in maternal and fetal plasma was established according to two criteria: i) the slope of the plasma concentration vs. time curve should not be significantly different from zero, and, ii) the coefficient of variation of the measured concentrations should be <10%. Steady-state maternal and fetal plasma concentrations of VPA metabolites (during both maternal and fetal infusion) were compared with each other using a paired t-test. Also, maternal and fetal $C_{\text{max}}$'s of various VPA metabolites were compared against each other using a paired t-test. Since maternal and fetal maximal plasma concentrations ($C_{\text{max}}$) of various VPA metabolites are not completely independent and are influenced by each other due to placental transfer of the metabolites, they were compared with newborn plasma $C_{\text{max}}$'s independently using unpaired t-tests. The significance level was $p<0.05$ in all cases. Fetal weight in utero at the time of experimentation was estimated from the weight at birth and the time interval between the experiment and birth (Koong et al., 1975).
5.2 Results

The average maternal body weight was 77.5 ± 10.6 kg and estimated fetal body weights on the day of maternal and fetal VPA infusion were 2.93 ± 0.21 and 2.98 ± 0.34 kg, respectively. The mean gestational age on the day of maternal and fetal experiments was 129.8 ± 4.3 and 130.2 ± 2.2 d, respectively, and these were not statistically different (paired t-test, p > 0.05). During maternal experiments, the control period fetal femoral arterial blood pH, Po2, Pco2, O2-saturation, and hemoglobin, glucose and lactate concentrations were 7.344 ± 0.047, 20.8 ± 5.9 mm Hg, 50.4 ± 1.8 mm Hg, 41.6 ± 15.4%, 12.6 ± 3.2 g/dl, 0.54 ± 0.08 mM and 0.91 ± 0.40 mM, respectively. Likewise during fetal administration, the control values for these variables were 7.313 ± 0.067, 20.0 ± 4.1 mm Hg, 49.9 ± 2.6 mm Hg, 38.0 ± 13.3%, 12.8 ± 3.6 g/dl, 0.95 ± 0.48 mM and 1.27 ± 0.80 mM, respectively. Apart from a small increase in fetal blood lactate concentrations during both maternal (1.40 ± 0.54 mM) and fetal (1.40 ± 0.25 mM) infusions, there were no systematic changes in any of these variables during the experimental period. Since appropriate control saline infusion experiments were not carried out, a more detailed analysis of these data was not possible.

5.2.1 Maternal-Fetal VPA Plasma Concentrations, Plasma Protein Binding and Indices of Fetal Drug Exposure

Figure 5.1A shows representative total as well as unbound maternal and fetal arterial plasma concentration-time profiles of VPA during and after a 24 h maternal VPA infusion. Figure 5.1B shows similar profiles during and after a 24 h fetal VPA infusion. Total as well as unbound VPA concentrations in maternal and fetal plasma were at steady-state during the 6-24 h infusion period according to the criteria described in the data analysis section.
Figure 5.1 – Representative concentration vs. time profiles of the total and unbound VPA in maternal and fetal plasma during and after a 24 h steady-state VPA infusion. A) maternal infusion, B) fetal infusion.
Table 5.1 presents the average as well as individual animal steady-state maternal and fetal femoral arterial total plasma concentrations of VPA during separate maternal and fetal 24 h infusion experiments. During maternal infusion, the fetal steady-state total plasma concentrations ($C_f$) were significantly lower compared to the corresponding maternal steady-state plasma concentrations ($C_m$, paired t-test, $p < 0.005$). Thus, the index of fetal drug exposure based on steady-state fetal-to-maternal total plasma concentration ratio ($C_f/C_m$, $0.70 \pm 0.10$) was significantly less than unity (unpaired t-test, $p < 0.001$). Similarly during fetal drug infusion, the maternal total plasma concentrations ($C_m'$) were significantly lower than the corresponding fetal plasma concentrations ($C_f'$) (paired t-test, $p < 0.005$), and the $C_m'/C_f'$ ratio ($0.60 \pm 0.08$) was significantly lower than unity (unpaired t-test, $p < 0.001$).

**Table 5.1 – Steady-state maternal and fetal total plasma concentrations of VPA during separate maternal and fetal 24 h VPA infusions in five pregnant sheep.**

<table>
<thead>
<tr>
<th>Ewe #</th>
<th>Maternal Administration ($\mu$g/ml)</th>
<th>Fetal Administration ($\mu$g/ml)</th>
<th>$C_f/C_m$ Ratio</th>
<th>$C_m'/C_f'$ Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$C_m$</td>
<td>$C_f$</td>
<td>$C_m'$</td>
<td>$C_f'$</td>
</tr>
<tr>
<td>E4241</td>
<td>101.0</td>
<td>79.5</td>
<td>33.0</td>
<td>51.7</td>
</tr>
<tr>
<td>E5108</td>
<td>55.2</td>
<td>36.2</td>
<td>20.3</td>
<td>37.6</td>
</tr>
<tr>
<td>E105x</td>
<td>208.3</td>
<td>173.0</td>
<td>68.6</td>
<td>102.1</td>
</tr>
<tr>
<td>E4133</td>
<td>79.9</td>
<td>49.0</td>
<td>28.7</td>
<td>58.5</td>
</tr>
<tr>
<td>E1226</td>
<td>88.3</td>
<td>55.3</td>
<td>28.7</td>
<td>43.7</td>
</tr>
<tr>
<td>Mean ± S.D.</td>
<td>106.5 ± 59.3</td>
<td>78.6 ± 55.0*</td>
<td>35.9 ± 18.9**</td>
<td>58.7 ± 25.5</td>
</tr>
</tbody>
</table>

* - significantly lower than $C_m$ ($p < 0.005$); ** - significantly lower than $C_f'$ ($p < 0.005$); * - significantly less than unity ($p < 0.001$).

$C_m$: Steady-state maternal plasma concentration during maternal drug infusion; $C_f$: Steady-state fetal plasma concentration during maternal drug infusion; $C_m'$: Steady-state maternal plasma concentration during fetal drug infusion; $C_f'$: Steady-state fetal plasma concentration during fetal drug infusion.
Table 5.2 presents the average as well as individual animal steady-state maternal and fetal plasma unbound concentrations of VPA during maternal and fetal VPA infusion experiments. Similar to the total concentrations above, the $C_u^f$ concentrations during maternal drug infusion were significantly lower compared to the $C_u^m$ concentrations (paired t-test, $p < 0.005$), and the $C_u^f / C_u^m$ ratio ($0.81 \pm 0.09$) was significantly less than unity (unpaired t-test, $p < 0.001$). Also, during fetal drug infusion, the $C_u^m'$ concentrations were lower than the $C_u^f'$ concentrations (paired t-test, $p < 0.005$), and the $C_u^m'/C_u^f'$ ratio ($0.38 \pm 0.13$) was significantly less than unity (unpaired t-test, $p < 0.001$).

Table 5.2 – Steady-state maternal and fetal unbound plasma concentrations of VPA during separate maternal and fetal 24 h VPA infusions in five pregnant sheep.

<table>
<thead>
<tr>
<th>Ewe #</th>
<th>Maternal Administration (µg/ml)</th>
<th>Fetal Administration (µg/ml)</th>
<th>$C_u^m$</th>
<th>$C_u^f$</th>
<th>$C_u^m'$</th>
<th>$C_u^f'$</th>
<th>$C_u^m'/C_u^m$ Ratio</th>
<th>$C_u^m'/C_u^f'$ Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>E4241</td>
<td>35.2</td>
<td>29.9</td>
<td>6.5</td>
<td>17.1</td>
<td>0.85</td>
<td>0.38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E5108</td>
<td>20.0</td>
<td>13.6</td>
<td>3.8</td>
<td>7.7</td>
<td>0.68</td>
<td>0.50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E105x</td>
<td>113.1</td>
<td>103.2</td>
<td>20.3</td>
<td>40.1</td>
<td>0.91</td>
<td>0.51</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E4133</td>
<td>16.8</td>
<td>13.4</td>
<td>3.7</td>
<td>19.3</td>
<td>0.80</td>
<td>0.19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E1226</td>
<td>23.3</td>
<td>18.5</td>
<td>2.9</td>
<td>9.4</td>
<td>0.79</td>
<td>0.30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± S.D.</td>
<td></td>
<td></td>
<td>41.7 ± 40.5</td>
<td>35.7 ± 38.3*</td>
<td>7.4 ± 7.3**</td>
<td>18.7 ± 12.9</td>
<td>0.81 ± 0.09#</td>
<td>0.38 ± 0.13#</td>
</tr>
</tbody>
</table>

*- significantly lower than $C_u^m$ ($p < 0.005$); ** - significantly lower than $C_u^f'$ ($p < 0.005$); # - significantly less than unity ($p < 0.001$).

$C_u^m$: Steady-state maternal plasma unbound concentration during maternal drug infusion; $C_u^f$: Steady-state fetal plasma unbound concentration during maternal drug infusion; $C_u^m'$: Steady-state maternal plasma unbound concentration during fetal drug infusion; $C_u^f'$: Steady-state fetal plasma unbound concentration during fetal drug infusion.
Plasma protein binding of VPA was saturable (or nonlinear) both in the mother and the fetus, and varied inversely with total VPA concentration. Table 5.3 presents the range of maternal and fetal plasma unbound fractions measured during the course of maternal and fetal VPA infusion experiments. VPA plasma unbound fraction in both maternal and fetal plasma at steady-state was constant during individual administration experiments because maternal and fetal plasma total as well as unbound plasma concentrations were relatively constant (Figure 5.1). Table 5.4 presents the average steady-state maternal and fetal plasma unbound fractions in individual animals during maternal and fetal drug infusions. The average maternal plasma unbound fraction of VPA during fetal drug infusion was significantly lower compared to that during maternal drug infusion (paired t-test, p < 0.005). However, the average fetal plasma unbound fractions during maternal and fetal infusion experiments were not significantly different (paired t-test, p > 0.05).

Table 5.3 – Range of unbound fractions of VPA in maternal and fetal plasma over the course of the entire VPA concentration vs. time profile.

<table>
<thead>
<tr>
<th>Ewe #</th>
<th>Maternal Administration</th>
<th>Fetal Administration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MA</td>
<td>FA</td>
</tr>
<tr>
<td>E4241</td>
<td>0.10 – 0.49</td>
<td>0.13 – 0.40</td>
</tr>
<tr>
<td>E5108</td>
<td>0.07 – 0.49</td>
<td>0.11 – 0.40</td>
</tr>
<tr>
<td>E105x</td>
<td>0.15 – 0.56</td>
<td>0.12 – 0.61</td>
</tr>
<tr>
<td>E4133</td>
<td>0.08 – 0.38</td>
<td>0.13 – 0.41</td>
</tr>
<tr>
<td>E1226</td>
<td>0.06 – 0.33</td>
<td>0.12 – 0.38</td>
</tr>
<tr>
<td>Mean (± S.D.)</td>
<td>0.09 (± 0.04)</td>
<td>0.12 (± 0.01)</td>
</tr>
</tbody>
</table>

MA: Maternal femoral arterial plasma; FA: Fetal femoral arterial plasma.
Table 5.4 - Steady-state maternal and fetal plasma unbound fractions of VPA during separate maternal and fetal 24 h VPA infusions in five pregnant sheep.

<table>
<thead>
<tr>
<th>Ewe #</th>
<th>Maternal Administration</th>
<th>Fetal Administration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MA</td>
<td>FA</td>
</tr>
<tr>
<td>E4241</td>
<td>0.35</td>
<td>0.38</td>
</tr>
<tr>
<td>E5108</td>
<td>0.36</td>
<td>0.38</td>
</tr>
<tr>
<td>E105x</td>
<td>0.54</td>
<td>0.60</td>
</tr>
<tr>
<td>E4133</td>
<td>0.21</td>
<td>0.27</td>
</tr>
<tr>
<td>E1226</td>
<td>0.26</td>
<td>0.34</td>
</tr>
</tbody>
</table>

Mean ± S.D. | 0.35 ± 0.13 | 0.39 ± 0.12 | 0.18 ± 0.08* | 0.29 ± 0.08 |

* - significantly lower compared to the corresponding value during maternal administration (p < 0.005).

MA: Maternal femoral arterial plasma; FA: Fetal femoral arterial plasma.

Figure 5.2 shows VPA binding characteristics in maternal and fetal plasma using pooled total, bound and unbound VPA concentration data from all experiments. Rosenthal (Figures 5.2A and 5.2D for maternal and fetal plasma, respectively) as well as unbound vs. total concentration (Figures 5.2B and 5.2E for maternal and fetal plasma, respectively) plots in both maternal and fetal plasma show a biphasic curvilinear relationship. The initial steep declining portion of the Rosenthal plots suggests the presence of a high affinity but low capacity (saturable) binding site, whereas the relatively flat portion of the curves suggests a linear (nonsaturable) or another saturable binding site with a low binding affinity. Statistically better fits (lower AIC and sum of squares, smaller CV's for fitted parameters) were obtained when the bound vs. unbound concentration data were fit to a 2-site binding model with one saturable and one non-saturable binding site (equation 9):
Figure 5.2 – Maternal and fetal plasma protein binding characteristics of VPA; pooled data from all maternal and fetal VPA infusion experiments. $C_t$, $C_b$ and $C_u$ are the maternal or fetal plasma concentrations of the total, bound and unbound VPA. A) & D) are the Rosenthal plots of the data in maternal and fetal plasma, respectively, demonstrating a biphasic relationship. $C_t$ vs. $C_u$ data in maternal (B) and fetal (E) plasma also exhibit a biphasic relationship, indicating VPA binding at two classes of binding sites. C) & F) show the relationship between $C_b$ vs. $C_u$ in maternal and fetal plasma, respectively; actual data (scatter points) and model predicted line obtained from fit of the data to a 2-site binding model are depicted.
\[ C_{\text{bound}} = \frac{B_{\text{max}1} \cdot C_{\text{unbound}}}{K_{d1} + C_{\text{unbound}}} + \frac{B_{\text{max}2}}{K_{d2}} \]  \hspace{1cm} (9)

where, \( C_{\text{bound}} \) and \( C_{\text{unbound}} \) are the corresponding bound and unbound plasma concentrations. \( B_{\text{max}1} \) and \( B_{\text{max}2} \) are the maximal VPA 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.

Figures 5.2C and 5.2F show the scatter plots of the pooled bound vs. unbound VPA concentration data in maternal and fetal plasma, respectively, from all the experiments. The model-predicted lines based on equation (9) are also depicted, and indicate excellent fit of the data to the proposed model. Table 5.5 shows the estimates of protein binding parameters of VPA in maternal and fetal plasma obtained from this model fitting.

**Table 5.5 – In vivo plasma protein binding parameters of VPA in maternal, fetal and newborn sheep obtained by fitting the data to a 1- or 2-site binding model.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Maternal(^a)</th>
<th>Fetal(^a)</th>
<th>Newborn(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( B_{\text{max}1} ) (CV%) (ng/ml)</td>
<td>62.8 (8.6)</td>
<td>65.0 (13.0)</td>
<td>71.8 (21.9)</td>
</tr>
<tr>
<td>( K_{d1} ) (CV%) (ng/ml)</td>
<td>7.6 (16.6)</td>
<td>13.0 (22.0)</td>
<td>13.3 (50.0)</td>
</tr>
<tr>
<td>( B_{\text{max}2}/K_{d2} ) (CV%)</td>
<td>0.34 (19.0)</td>
<td>0.17 (43.9)</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^a\) - fit of the data to a 2-site binding model.  
\(^b\) - fit of the data to a 1-site binding model.

\( B_{\text{max}1} \): Maximal VPA binding capacity of the plasma at the high affinity saturable binding site.  
\( K_{d1} \): Dissociation constant of VPA binding at the high affinity saturable binding site.  
\( B_{\text{max}2}/K_{d2} \): Maximal binding capacity/dissociation constant ratio at the low affinity non-saturable binding site.
5.2.2 Placental and Non-Placental Clearances of VPA in the Mother and the Fetus

Table 5.6 presents the gestational age of the animals on the day of experiment and weight-normalized maternal and fetal placental and non-placental clearances of VPA calculated using the total VPA plasma concentration data. Fetal total (CL\(_f\)) and placental (CL\(_fm\)) clearances are significantly greater than maternal total (CL\(_mm\)) and placental (CL\(_mf\)) clearances, respectively (unpaired t-test, p < 0.005 in both cases). Maternal non-placental clearance (CL\(_mo\)) was low, whereas a negative estimate of fetal non-placental clearance (CL\(_fo\)) was obtained consistently in all the animals. As described in detail in the Discussion section, these clearance estimates obtained using the total plasma concentrations of the drug are inaccurate because of the different steady-state maternal and fetal plasma VPA unbound fractions during maternal and fetal infusion experiments due to nonlinear plasma protein binding of the drug. Hence, the placental and non-placental clearances of the drug were calculated using unbound VPA plasma concentrations.

Table 5.7 shows the weight-normalized maternal and fetal net, total, placental and non-placental clearances of the unbound drug calculated using the measured steady-state unbound VPA concentrations in maternal and fetal plasma. Fetal net (CL\(_u\)\(_f\)(net)) and total (CL\(_u\)\(_f\)) clearances of the unbound drug were significantly greater compared to the corresponding maternal clearances (CL\(_u\)\(_m\)(net) and CL\(_u\)\(_mm\), respectively; unpaired t-test, p < 0.0005 in both cases). However, there was no significant difference between maternal and fetal placental clearances of the unbound drug (CL\(_u\)\(_mf\) and CL\(_u\)\(_fm\), respectively) (unpaired t-test, p > 0.05). Also, a positive estimate of CL\(_u\)\(_fo\) was
consistently obtained, and the average $CL_{ufo}^u$ was significantly greater than $CL_{umo}^u$ (unpaired t-test, $p < 0.05$).

The net, total, placental and non-placental clearances of VPA, based on total drug concentrations, were calculated after correcting for alterations in steady-state plasma protein binding of the drug between maternal and fetal administration experiments. Table 5.8 presents these corrected clearances of the total drug. Maternal clearances ($CL_{m(net)}$, $CL_{nm}$, $CL_{mf}$, and $CL_{mo}$) are essentially the product of steady-state maternal plasma unbound fraction during maternal infusion and the corresponding maternal clearance of the unbound drug, whereas fetal clearances ($CL_{f(net)}$, $CL_{ff}$, $CL_{fm}$, and $CL_{fo}$) are the product of steady-state fetal plasma unbound fraction during fetal drug infusion and the corresponding fetal clearance of the unbound drug. Similar to the unbound clearances above, $CL_{f(net)}$, $CL_{ff}$ and $CL_{fo}$ were significantly higher compared to $CL_{m(net)}$, $CL_{nm}$ and $CL_{mo}$, respectively (unpaired t-test, $p < 0.005$ in all cases); however, $CL_{mf}$ and $CL_{fm}$ were not significantly different (unpaired t-test, $p > 0.05$).

The average contribution of maternal non-placental clearance to net maternal drug elimination ($%CL_{mo}/CL_{m(net)}$) based either on total or unbound concentrations was $79.6 \pm 13.2 \%$ (range $66.3 - 97.4 \%$), with placental elimination accounting for the remainder ($20.4 \pm 13.2 \%$; range $2.6 - 33.7\%$). The contribution of fetal non-placental clearance to net fetal drug elimination ($%CL_{fo}/CL_{f(net)}$) was significantly lower compared to that in the mother ($41.7 \pm 19.6 \%$; range $14.8 - 67.2 \%$; unpaired t-test, $p < 0.005$). This amounts to a greater contribution of fetal placental clearance to net fetal drug elimination as compared to the mother ($58.3 \pm 19.6 \%$; range $32.8 - 85.2\%$; unpaired t-test, $p < 0.005$).
Table 5.6 – Weight-normalized estimates of total \((CL_{mm} \text{ and } CL_{mf})\), placental \((CL_{mf} \text{ and } CL_{fm})\), and non-placental \((CL_{mo} \text{ and } CL_{fo})\) VPA clearances of the total drug in the mother and the fetus, respectively. Clearances were calculated using the measured total plasma VPA concentrations and 2-compartment model of the maternal-fetal unit.

<table>
<thead>
<tr>
<th>Ewe No.</th>
<th>Gestational Age (days)</th>
<th>Clearance Parameter (ml/min/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Maternal Infusion</td>
<td>Fetal Infusion</td>
</tr>
<tr>
<td>4241</td>
<td>134</td>
<td>129</td>
</tr>
<tr>
<td>5108</td>
<td>130</td>
<td>134</td>
</tr>
<tr>
<td>105x</td>
<td>134</td>
<td>130</td>
</tr>
<tr>
<td>4133</td>
<td>125</td>
<td>129</td>
</tr>
<tr>
<td>1226</td>
<td>126</td>
<td>129</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
</tr>
<tr>
<td>± S.D.</td>
<td>± 4.3</td>
<td>± 2.2</td>
</tr>
</tbody>
</table>

\(a\) - clearance values are in ml/min/kg maternal weight; \(b\) - clearance values are in ml/min/kg fetal weight.

* - significantly higher compared to the corresponding maternal value (p < 0.005).

\(CL_{mm}\): Maternal total clearance; \(CL_{mf}\): Maternal placental clearance; \(CL_{mo}\): Maternal non-placental clearance; \(CL_{f}\): Fetal total clearance; \(CL_{fm}\): Fetal placental clearance; \(CL_{fo}\): Fetal non-placental clearance.
Table 5.7 – Weight-normalized estimates of net (CL\textsuperscript{u}_{m(net)}) and CL\textsuperscript{u}_{f(net)}, total (CL\textsuperscript{u}_{mm} and CL\textsuperscript{u}_{ff}), placental (CL\textsuperscript{u}_{mf} and CL\textsuperscript{u}_{fm}), and non-placental (CL\textsuperscript{u}_{mo} and CL\textsuperscript{u}_{fo}) VPA clearances of the unbound drug in the mother and the fetus, respectively. Clearances were calculated using the measured unbound plasma VPA concentrations and 2-compartment model of the maternal-fetal unit.

<table>
<thead>
<tr>
<th>Ewe No.</th>
<th>CL\textsuperscript{u}_{m(net)}\textsuperscript{a}</th>
<th>CL\textsuperscript{u}_{mm}\textsuperscript{a}</th>
<th>CL\textsuperscript{u}_{mf}\textsuperscript{b}</th>
<th>CL\textsuperscript{u}_{mo}\textsuperscript{a}</th>
<th>CL\textsuperscript{u}_{f(net)}\textsuperscript{b}</th>
<th>CL\textsuperscript{u}_{ff}\textsuperscript{b}</th>
<th>CL\textsuperscript{u}_{fm}\textsuperscript{b}</th>
<th>CL\textsuperscript{u}_{fo}\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>4241</td>
<td>3.9</td>
<td>5.8</td>
<td>80.2</td>
<td>3.3</td>
<td>64.1</td>
<td>94.6</td>
<td>70.1</td>
<td>24.5</td>
</tr>
<tr>
<td>5108</td>
<td>6.9</td>
<td>10.5</td>
<td>88.7</td>
<td>5.8</td>
<td>86.3</td>
<td>130.4</td>
<td>99.6</td>
<td>30.8</td>
</tr>
<tr>
<td>105X</td>
<td>1.2</td>
<td>2.3</td>
<td>46.6</td>
<td>0.8</td>
<td>27.5</td>
<td>51.1</td>
<td>36.7</td>
<td>14.4</td>
</tr>
<tr>
<td>4133</td>
<td>8.2</td>
<td>9.7</td>
<td>47.8</td>
<td>8.0</td>
<td>50.9</td>
<td>60.0</td>
<td>52.5</td>
<td>7.5</td>
</tr>
<tr>
<td>1226</td>
<td>5.9</td>
<td>7.8</td>
<td>81.9</td>
<td>4.0</td>
<td>78.1</td>
<td>103.1</td>
<td>50.6</td>
<td>52.5</td>
</tr>
<tr>
<td>Mean ± S.D.</td>
<td>5.2 ± 2.7</td>
<td>7.2 ± 3.3</td>
<td>69.0 ± 20.2</td>
<td>4.4 ± 2.7</td>
<td>61.4 ± 23.2**</td>
<td>87.8 ± 32.4**</td>
<td>61.9 ± 24.2</td>
<td>25.9 ± 17.3*</td>
</tr>
</tbody>
</table>

\textsuperscript{a} - clearance values are in ml/min/kg maternal weight; \textsuperscript{b} - clearance values are in ml/min/kg estimated fetal weight.

* - significantly higher compared to the corresponding maternal value (p < 0.05).

** - significantly higher compared to the corresponding maternal value (p < 0.0005).

CL\textsuperscript{u}_{m(net)}: Maternal net unbound clearance; CL\textsuperscript{u}_{mm}: Maternal total unbound clearance; CL\textsuperscript{u}_{mf}: Maternal placental unbound clearance; CL\textsuperscript{u}_{mo}: Maternal non-placental unbound clearance; CL\textsuperscript{u}_{f(net)}: Fetal net unbound clearance; CL\textsuperscript{u}_{ff}: Fetal total unbound clearance; CL\textsuperscript{u}_{fm}: Fetal placental unbound clearance; CL\textsuperscript{u}_{fo}: Fetal non-placental unbound clearance.
Table 5.8 – Weight-normalized estimates of net (CL_{m(net)} and CL_{f(net)}), total (CL_{mm} and CL_{ff}), placental (CL_{mf} and CL_{fm}), and non-placental (CL_{mo} and CL_{fo}) VPA clearances of the total drug in the mother and the fetus, respectively. Clearances were calculated after correcting for the changes in maternal and fetal plasma steady-state unbound fractions between maternal and fetal experiments. Maternal clearances (CL_{mm}, CL_{mf} and CL_{mo}) are presented with respect to steady-state maternal plasma unbound fraction during maternal administration, whereas, fetal clearances (CL_{ff}, CL_{fm} and CL_{fo}) are with respect to steady-state fetal unbound fractions during fetal administration.

<table>
<thead>
<tr>
<th>Ewe No.</th>
<th>Clearance Parameter (ml/min/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CL_{m(net)}^a</td>
</tr>
<tr>
<td>4241</td>
<td>1.4</td>
</tr>
<tr>
<td>5108</td>
<td>2.5</td>
</tr>
<tr>
<td>105x</td>
<td>0.7</td>
</tr>
<tr>
<td>4133</td>
<td>1.7</td>
</tr>
<tr>
<td>1226</td>
<td>1.6</td>
</tr>
<tr>
<td>Mean ±</td>
<td>1.6 ±</td>
</tr>
<tr>
<td>S.D.</td>
<td>0.7</td>
</tr>
</tbody>
</table>

^a - clearance values are in ml/min/kg maternal weight; ^b - clearance values are in ml/min/kg fetal weight.
* - significantly higher compared to the corresponding maternal value (p<0.005).

CL_{m(net)}: Maternal net clearance; CL_{mm}: Maternal total clearance; CL_{mf}: Maternal placental clearance; CL_{mo}: Maternal non-placental clearance; CL_{f(net)}: Fetal net clearance; CL_{ff}: Fetal total clearance; CL_{fm}: Fetal placental clearance; CL_{fo}: Fetal non-placental clearance.
5.2.3 Amniotic and Fetal Tracheal Fluid Concentrations of VPA during Maternal and Fetal VPA Infusions

VPA was detectable in amniotic and fetal tracheal fluids at the earliest sampling time, i.e., 5 min after the start of maternal or fetal VPA infusion. As with maternal and fetal plasma, the amniotic and tracheal fluid VPA concentrations were also at an apparent steady-state during the 6-24 h period of infusion (Figure 5.3). The apparent steady-state VPA concentrations in these fluids during separate maternal and fetal experiments are presented in Table 5.9. The tracheal fluid VPA concentrations are lower compared to the fetal plasma unbound concentrations during both fetal (statistically significant) and maternal (not significant) drug administration. Similarly, the amniotic fluid VPA concentrations are lower than the fetal plasma unbound VPA concentrations in all but one animal; however, the difference between means is not statistically significant. During the post-infusion period, the concentrations in these fluids appear to decline in parallel with maternal and fetal plasma concentrations (Figures 5.1 and 5.3).

<table>
<thead>
<tr>
<th>Ewe #</th>
<th>Amniotic Fluid Concentration (µg/ml)</th>
<th>Tracheal Fluid Concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Maternal Infusion</td>
<td>Fetal Infusion</td>
</tr>
<tr>
<td>E4241</td>
<td>12.0</td>
<td>9.8</td>
</tr>
<tr>
<td>E5108</td>
<td>5.6</td>
<td>3.4</td>
</tr>
<tr>
<td>E105x</td>
<td>35.9</td>
<td>13.3</td>
</tr>
<tr>
<td>E4133</td>
<td>5.4</td>
<td>15.5</td>
</tr>
<tr>
<td>E1226</td>
<td>27.8</td>
<td>12.8</td>
</tr>
<tr>
<td>Mean ± S.D.</td>
<td>17.3 ± 13.8</td>
<td>11.0 ± 4.7</td>
</tr>
</tbody>
</table>

-a – samples not available
Figure 5.3 – Representative amniotic and fetal tracheal fluid concentration vs. time profiles of VPA in E4241, showing apparent steady-state concentrations in these fluids during the 6-24h infusion period and subsequent rapid decline in concentrations during the post-infusion phase.

5.2.4 Overall Total Body Clearance, Terminal Elimination Half-Life, Mean Residence Time, and Steady-State Volume of Distribution of VPA in the Mother and the Fetus

Tables 5.10 and 5.11 present the above pharmacokinetic parameters of VPA in the mother and the fetus, respectively. The mean maternal total body clearance of the unbound drug (CL_{ub}; calculated as total dose/AUC of the unbound drug, 5.4 ± 2.7 ml/min/kg) was slightly but significantly greater than the net steady-state maternal unbound clearance presented in Table 5.7 (5.2 ± 2.7 ml/min/kg; paired t-test, p < 0.005).

However, the mean maternal total body clearance of the total drug (CL_{tb}; calculated as total dose/AUC of the total drug, 1.5 ± 0.6 ml/min/kg) was significantly lower than the
net steady-state maternal total clearance presented in Table 5.8 (1.6 ± 0.7 ml/min/kg; paired t-test, p < 0.05). In contrast, the fetal $\text{CL}_{\text{utb}}^u$ (62.1 ± 22.4 ml/min/kg) was not significantly different from steady-state $\text{CL}_{f(\text{net})}^u$ (61.4 ± 23.2 ml/min/kg; Tables 5.7 and 5.11), and the difference between fetal total drug $\text{CL}_{\text{tb}}$ and $\text{CL}_{f(\text{net})}$ (16.3 ± 3.8 vs. 16.7 ± 3.7 ml/min/kg; Tables 5.8 and 5.11) was only near statistical significance (paired t-test, $p = 0.05$). Like steady-state net fetal clearance (Tables 5.7 and 5.8), the fetal $\text{CL}_{\text{tb}}^u$ and $\text{CL}_{\text{utb}}$ were significantly higher than the corresponding maternal values (Tables 5.10 and 5.11; unpaired t-test, $p < 0.005$ in both cases). Fetal terminal $t_{\text{i}/2}$ of the unbound drug ($t_{\text{i}/2}^u$) was significantly shorter than the corresponding maternal $t_{\text{i}/2}^u$ (3.1 ± 1.3 vs. 5.5 ± 1.9 h; Tables 5.10 and 5.11; unpaired t-test, $p < 0.05$). However, there was no significant difference between maternal and fetal $t_{\text{i}/2}$'s based on total plasma drug concentrations ($t_{\text{i}/2\beta}$) (5.6 ± 1.4 vs. 4.6 ± 1.9 h; Tables 5.10 and 5.11). Maternal $t_{\text{i}/2}$'s based on maternal plasma unbound ($t_{\text{i}/2}^u$) and total drug ($t_{\text{i}/2\beta}$) concentrations were not statistically different (5.5 ± 1.9 vs. 5.6 ± 1.4 h; Table 5.10; paired t-test, $p > 0.05$). In contrast, fetal $t_{\text{i}/2}^u$ was significantly shorter than fetal $t_{\text{i}/2\beta}$ (3.1 ± 1.3 vs. 4.6 ± 1.9 h; Table 5.11; paired t-test, $p < 0.05$). There was no significant difference between MRT of the drug in maternal and fetal circulation based either on total or unbound drug concentrations (Tables 5.10 and 5.11; unpaired t-test, $p > 0.05$ in both cases). However, in both the mother and the fetus, the MRT of the unbound drug was significantly shorter compared to that for the total drug (Tables 5.10 and 5.11; paired t-test, $p < 0.05$ in both cases). All fetal steady-state volume of distribution parameters presented in Table 5.11 are significantly greater than the corresponding maternal volumes in Table 5.10 (unpaired t-test, $p < 0.0005$ in all cases).
Table 5.10 – Comparative pharmacokinetic parameters of the unbound and total VPA in the mother during maternal VPA infusion.

<table>
<thead>
<tr>
<th>Pharmacokinetic Parameter</th>
<th>E4241</th>
<th>E5108</th>
<th>E105x</th>
<th>E4133</th>
<th>E1226</th>
<th>Mean ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Unbound VPA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$CL_{ub}$ (ml/min/kg)</td>
<td>4.1</td>
<td>7.0</td>
<td>1.4</td>
<td>8.3</td>
<td>6.0</td>
<td>5.4 ± 2.7</td>
</tr>
<tr>
<td>$t_{1/2\beta}$ (h)</td>
<td>5.0</td>
<td>4.0</td>
<td>7.4</td>
<td>3.5</td>
<td>7.5</td>
<td>5.5 ± 1.9</td>
</tr>
<tr>
<td>MRT$^u$ (h)</td>
<td>2.9</td>
<td>3.1</td>
<td>5.8</td>
<td>1.5</td>
<td>2.3</td>
<td>3.1 ± 1.6</td>
</tr>
<tr>
<td>$Vd_{ss}^u$ (L/kg)</td>
<td>0.70</td>
<td>1.29</td>
<td>0.47</td>
<td>0.73</td>
<td>0.83</td>
<td>0.80 ± 0.30</td>
</tr>
<tr>
<td><strong>Total VPA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$CL_{tb}$ (ml/min/kg)</td>
<td>1.3</td>
<td>2.3</td>
<td>0.6</td>
<td>1.7</td>
<td>1.5</td>
<td>1.5 ± 0.6</td>
</tr>
<tr>
<td>$t_{1/2\beta}$ (h)</td>
<td>4.2</td>
<td>5.3</td>
<td>7.3</td>
<td>4.5</td>
<td>6.7</td>
<td>5.6 ± 1.4</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>4.4</td>
<td>4.6</td>
<td>8.4</td>
<td>2.4</td>
<td>3.6</td>
<td>4.7 ± 2.3</td>
</tr>
<tr>
<td>$f_p$</td>
<td>0.32</td>
<td>0.33</td>
<td>0.46</td>
<td>0.21</td>
<td>0.24</td>
<td>0.31 ± 0.10</td>
</tr>
<tr>
<td>$Vd_{ss}$ (L/kg)</td>
<td>0.34</td>
<td>0.63</td>
<td>0.32</td>
<td>0.24</td>
<td>0.32</td>
<td>0.37 ± 0.15</td>
</tr>
<tr>
<td>$Vd_{ss}'$ (L/kg)</td>
<td>0.22</td>
<td>0.42</td>
<td>0.22</td>
<td>0.15</td>
<td>0.20</td>
<td>0.24 ± 0.10</td>
</tr>
</tbody>
</table>

$CL_{ub}$ – total body clearance of the unbound drug; $t_{1/2\beta}$ – apparent elimination half-life of the unbound drug; MRT$^u$ – mean residence time of the unbound drug; $Vd_{ss}^u$ – steady-state volume of distribution of the unbound drug; $f_p$ – area weighted unbound fraction of the drug; $CL_{tb}$ – total body clearance of the total (unbound + bound) drug; $t_{1/2\beta}$ – apparent elimination half-life of the total drug; MRT – mean residence time of the total drug; $Vd_{ss}$ – steady-state volume of distribution of the total drug; $Vd_{ss}'$ – corrected steady-state volume of distribution of the total drug.
Table 5.11 – Comparative pharmacokinetic parameters of the unbound and total VPA in the fetus during fetal VPA infusion.

<table>
<thead>
<tr>
<th>Pharmacokinetic Parameter</th>
<th>E4241</th>
<th>E5108</th>
<th>E105x</th>
<th>E4133</th>
<th>E1226</th>
<th>Mean ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Unbound VPA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CL_{ub} (ml/min/kg)</td>
<td>62.4</td>
<td>86.1</td>
<td>30.3</td>
<td>52.1</td>
<td>79.8</td>
<td>62.1 ± 22.4</td>
</tr>
<tr>
<td>t_{1/2β} (h)</td>
<td>3.8</td>
<td>2.4</td>
<td>5.0</td>
<td>1.9</td>
<td>2.3</td>
<td>3.1 ± 1.3</td>
</tr>
<tr>
<td>MRT_{ub} (h)</td>
<td>1.8</td>
<td>1.0</td>
<td>4.2</td>
<td>1.7</td>
<td>1.5</td>
<td>2.0 ± 1.3</td>
</tr>
<tr>
<td>Vd_{ss}^{ub} (L/kg)</td>
<td>6.65</td>
<td>4.92</td>
<td>7.72</td>
<td>5.31</td>
<td>6.97</td>
<td>6.31 ± 1.17</td>
</tr>
<tr>
<td><strong>Total VPA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CL_{tb} (ml/min/kg)</td>
<td>20.4</td>
<td>17.5</td>
<td>10.1</td>
<td>16.5</td>
<td>16.9</td>
<td>16.3 ± 3.8</td>
</tr>
<tr>
<td>t_{1/2β} (h)</td>
<td>4.8</td>
<td>4.3</td>
<td>7.4</td>
<td>2.1</td>
<td>4.5</td>
<td>4.6 ± 1.9</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>3.2</td>
<td>2.0</td>
<td>6.3</td>
<td>1.9</td>
<td>1.8</td>
<td>3.0 ± 1.9</td>
</tr>
<tr>
<td>f_p</td>
<td>0.33</td>
<td>0.20</td>
<td>0.33</td>
<td>0.32</td>
<td>0.21</td>
<td>0.28 ± 0.07</td>
</tr>
<tr>
<td>Vd_{ss} (L/kg)</td>
<td>3.89</td>
<td>2.07</td>
<td>3.84</td>
<td>1.87</td>
<td>1.86</td>
<td>2.70 ± 1.06</td>
</tr>
<tr>
<td>Vd_{ss}′ (L/kg)</td>
<td>2.18</td>
<td>1.00</td>
<td>2.57</td>
<td>1.68</td>
<td>1.48</td>
<td>1.78 ± 0.61</td>
</tr>
</tbody>
</table>

CL_{ub}^{u} – total body clearance of the unbound drug; t_{1/2β}^{u} – apparent elimination half-life of the unbound drug; MRT_{ub}^{u} – mean residence time of the unbound drug; Vd_{ss}^{u} – steady-state volume of distribution of the unbound drug; f_p – area weighted unbound fraction of the drug; CL_{tb}^{u} – total body clearance of the total (unbound + bound) drug; t_{1/2β} – apparent elimination half-life of the total drug; MRT – mean residence time of the total drug; Vd_{ss} – steady-state volume of distribution of the total drug; Vd_{ss}′ – corrected steady-state volume of distribution of the total drug.
5.2.5 Pharmacokinetics and Plasma Protein Binding of VPA in Newborn Lambs

Figure 5.4A shows representative plasma concentration vs. time profiles of the total as well as unbound VPA in a newborn lamb. Unbound and total VPA concentrations did not reach steady-state in newborn plasma during the 6 h infusion period. VPA appeared to continuously accumulate in the newborn plasma throughout the infusion period and resulted in a plasma $C_{\text{max}}$ of 136.9 ± 30.6 µg/ml (range 107.5 – 173.3 µg/ml). This accumulation behavior of VPA in lambs is different from the VPA pharmacokinetic profile in maternal and fetal plasma during the initial 6 h of the 24 h infusion period after maternal or fetal dosing (Figures 5.4B and 5.4C). Also, in contrast to the mother and the fetus (Figure 5.1), a typical convexity was evident during the post-infusion portion of the unbound and total plasma concentration vs. time pharmacokinetic profile in the newborn lamb (Figure 5.4A). This convexity is characteristic of the Michaelis-Menten nonlinear (saturable) pharmacokinetic behavior (Gibaldi and Perrier, 1982; Wagner, 1993). Table 5.12 presents the pharmacokinetic parameters of unbound and total VPA in newborn lambs. The average total body unbound (CL$_{\text{ut}}$) as well as total drug (CL$_{\text{tb}}$) clearance in the newborn lamb was significantly lower than the corresponding values in the mother and the fetus (Tables 5.10, 5.11 and 5.12; unpaired t-test, p < 0.05 in all cases). The terminal $t_{1/2}$ and MRT of the unbound as well as total VPA were significantly longer in the newborn lamb compared to the corresponding values in the mother and the fetus (Tables 5.10, 5.11 and 5.12; unpaired t-test, p < 0.005 in all cases). All steady-state volume of distribution parameters ($V_{\text{dss}}$, $V_{\text{dss}}'$) in the newborn lamb were significantly lower compared to the fetus (Tables 5.11 and 5.12; unpaired t-test, p < 0.005 in all cases). However, these volume of distribution
parameters in the lambs were not significantly different from those of the mother (Tables 5.10 and 5.12 unpaired t-test, \( p > 0.05 \) in all cases).

Figures 5.5 and 5.6 depict the newborn plasma protein binding characteristics of VPA in pooled data from the four newborn lambs where plasma bound and unbound drug concentrations were available. Figure 5.5 shows the newborn plasma protein binding plots analogous to those depicted in Figure 5.2 for the mother and the fetus. In Figure 5.6, the pooled newborn plasma bound and unbound plasma concentration data were partitioned into those obtained on day 1 (0 – 24 h; Figures 5.6A, 5.6B and 5.6C) and those obtained after day 1 (24 h – 96 h; Figures 5.6D, 5.6E, 5.6F) of the newborn lamb experiments. In the data from day 1 (Figure 5.6A), the bound/unbound concentration ratio is positively related to the bound concentration (bound/unbound concentration ratio = 0.1543 * bound concentration + 0.0154; \( r = 0.6815, p < 0.0001 \)). Also, in contrast to the mother and the fetus (Figure 5.2), there does not appear to be a tight relationship between the total and unbound concentrations (Figure 5.6B) or between bound and unbound concentrations (Figure 5.6C). However, the behavior of bound and unbound VPA concentration data in newborn plasma samples obtained after day 1 (Figures 5.6D, 5.6E, 5.6F) appears to be similar to that of the mother (Figures 5.2A, 5.2B, and 5.2C) and the fetus (Figures 5.2D, 5.2E, and 5.2F). As in the mother and the fetus, a biphasic relationship appears to exist in the Rosenthal plot of the data (Figure 5.6D). However, enough data points were not available in each phase to adequately model the data according to a 2-site binding model, as in the mother and the fetus. Hence, these data were modeled according to a 1-saturable binding site model and the estimates of binding parameters are presented in Table 5.5. Fit of the data to this 1-site binding model resulted in a lower AIC as compared to the fit to a 2-site binding model.
Figure 5.4 – A) Representative plasma concentration vs. time profile of the total and unbound VPA in the newborn lamb NL0123z. B) Unbound and total VPA plasma concentrations in NL0123z during the 6 h infusion period, showing continuous accumulation of the drug in newborn plasma. C) Typical plasma concentration vs. time profiles of the total and unbound VPA in maternal and fetal plasma during the initial 6 h period of the 24 h maternal VPA infusion in E5108, showing lack of any drug accumulation. Profiles similar to C) were also observed in all animals after fetal VPA infusion.
Table 5.12 – Comparative pharmacokinetic parameters of the unbound and total VPA in newborn lambs.

<table>
<thead>
<tr>
<th>Pharmacokinetic Parameter</th>
<th>NL4241</th>
<th>NL4124</th>
<th>NL0123z</th>
<th>NL2243(1)</th>
<th>Mean ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Unbound VPA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\text{CL}^u_{tb}$ (ml/min/kg)</td>
<td>0.78</td>
<td>1.00</td>
<td>0.37</td>
<td>0.50</td>
<td>0.66 ± 0.28</td>
</tr>
<tr>
<td>$t_{1/2}^u$ (h)</td>
<td>12.9</td>
<td>9.7</td>
<td>12.2</td>
<td>13.4</td>
<td>12.1 ± 1.6</td>
</tr>
<tr>
<td>MRT$^u$ (h)</td>
<td>18.1</td>
<td>12.8</td>
<td>18.0</td>
<td>17.4</td>
<td>16.6 ± 2.5</td>
</tr>
<tr>
<td>$V_{dss}^u$ (L/kg)</td>
<td>0.84</td>
<td>0.77</td>
<td>0.40</td>
<td>0.53</td>
<td>0.63 ± 0.21</td>
</tr>
<tr>
<td><strong>Total VPA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\text{CL}_{tb}$ (ml/min/kg)</td>
<td>0.27</td>
<td>0.33</td>
<td>0.16</td>
<td>0.25</td>
<td>0.25 ± 0.07</td>
</tr>
<tr>
<td>$t_{1/2}$ (h)</td>
<td>20.5</td>
<td>16.5</td>
<td>20.6</td>
<td>16.8</td>
<td>18.6 ± 2.3</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>23.6</td>
<td>17.5</td>
<td>25.5</td>
<td>21.7</td>
<td>22.1 ± 3.4</td>
</tr>
<tr>
<td>$f_p$</td>
<td>0.35</td>
<td>0.33</td>
<td>0.44</td>
<td>0.49</td>
<td>0.40 ± 0.08</td>
</tr>
<tr>
<td>$V_{dss}$ (L/kg)</td>
<td>0.38</td>
<td>0.35</td>
<td>0.25</td>
<td>0.32</td>
<td>0.33 ± 0.06</td>
</tr>
<tr>
<td>$V_{dss}'$ (L/kg)</td>
<td>0.29</td>
<td>0.26</td>
<td>0.17</td>
<td>0.26</td>
<td>0.25 ± 0.05</td>
</tr>
</tbody>
</table>

$\text{CL}^u_{tb}$ - total body clearance of the unbound drug; $t_{1/2}^u$ - apparent elimination half-life of the unbound drug; MRT$^u$ - mean residence time of the unbound drug; $V_{dss}^u$ - steady-state volume of distribution of the unbound drug; $f_p$ - area weighted unbound fraction of the drug; $\text{CL}_{tb}$ - total body clearance of the total (unbound + bound) drug; $t_{1/2\beta}$ - apparent elimination half-life of the total drug; MRT - mean residence time of the total drug; $V_{dss}$ - steady-state volume of distribution of the total drug; $V_{dss}'$ - corrected steady-state volume of distribution of the total drug.
Figure 5.5 - Plasma protein binding characteristics of VPA in newborn lamb plasma; pooled data from four lambs. C_t, C_b and C_u are the plasma concentrations of the total, bound and unbound VPA, respectively. A) Rosenthal plot of the data. B) Relationship between C_t and C_u. C) Relationship between C_b and C_u. Anomalous VPA binding characteristics in the newborn plasma are evident when compared to maternal and fetal data in Figure 5.2.
Figure 5.6 – Partitioning of newborn lamb VPA plasma protein binding data into those obtained during day 1 of the experiment (A-C) and those obtained after day 1 (D-F). C_t, C_b and C_u are the plasma concentrations of the total, bound and unbound VPA. A) and D) are the Rosenthal plots. B) and E) are the relationships between C_t and C_u. C) and F) are the relationships between C_b and C_u. The data in F) were fitted to a 1-site binding model and the model-predicted line is also shown. Striking differences in VPA binding characteristics between the two groups of data are evident.
5.2.6 Kinetics of VPA-Glucuronide Formation in Maternal and Fetal Liver Microsomes

Figure 5.7A shows the saturation plot of VPA glucuronide formation rate with increasing substrate concentration in pooled maternal microsomal preparations. Eadie-Hofstee plots of these data (reaction velocity vs. reaction velocity/substrate concentration) are monophasic (Figure 5.7B) indicating the possible involvement of only one isoform of the UDP-glucuronosyltransferase enzyme in this reaction. Therefore, the glucuronide formation rate vs. substrate concentration data were fit to an equation of the form:

\[
V = \frac{V_{\text{max}}[S]}{K_m + [S]}
\]  

(10)

where, \( V_{\text{max}} \) and \( K_m \) are the \textit{in vitro} Michaelis-Menten parameters of glucuronidation formation, and \( v \) and \([S]\) are the reaction velocity and substrate concentration. The estimates of \( V_{\text{max}} \) and \( K_m \) for VPA glucuronidation in pooled maternal hepatic microsomes were 15.2 ± 0.6 nmol/min/mg microsomal protein (or 2.19 ± 0.08 μg/min/mg microsomal protein; mean ± standard error of estimate) and 2.52 ± 0.28 mM (or 363.4 ± 40.1 μg/ml), respectively. The VPA glucuronidation rates in pooled fetal liver microsomes were extremely low (at most only one fifth of the maternal microsomal glucuronidation rates at the two lowest substrate concentrations). These rates could not be measured accurately at a wide range of substrate concentrations due to the indirect hydrolyzed-unhydrolyzed difference-based nature of our assay method for VPA-glucuronide. Hence, fetal \textit{in vitro} glucuronidation kinetic parameters could not be calculated.
Figure 5.7 – *In vitro* glucuronidation of VPA in pooled maternal sheep liver microsomes. A) Saturation plot of reaction velocity vs. substrate concentration. B) Eadie-Hofstee plot showing monophasic nature of glucuronidation reaction in maternal liver microsomes. $v$ and $[S]$ are the reaction velocity and substrate concentration, respectively.
5.2.7 Comparative in vivo Michaelis-Menten Pharmacokinetic Parameters of VPA in the Mother and the Newborn

Due to the presence of a pronounced nonlinear Michaelis-Menten type plasma pharmacokinetic profile in the newborn lamb, these data were treated according to a 1-compartment model with nonlinear Michaelis-Menten elimination. For comparison purposes, Michaelis-Menten pharmacokinetic parameters (only $V_{\text{max}}$) were also estimated in the mother using the approach described in detail in the data analysis section. Representative fits of the one-compartment model with Michaelis-Menten elimination to unbound VPA plasma concentrations in a newborn lamb and a pregnant ewe are shown in Figure 5.8. The comparative maternal and newborn $V_{\text{max}}$ and $K_m$ data obtained from this model fitting, along with the coefficients of variation of each parameter estimate, are presented in Table 5.13. The average estimated body weight-normalized $V_{\text{max}}$ in the newborn lambs was significantly lower compared to that in maternal sheep ($123.5 \pm 82.0$ vs. $2207.1 \pm 1075.3$ μg/min/kg body weight; unpaired t-test, $p < 0.01$).

5.2.8 Pharmacokinetics of Renal Excretion of Unchanged VPA in the Mother, Fetus and the Newborn

Table 5.14 presents the renal clearance of unbound and total VPA in maternal and fetal sheep. Maternal renal clearance data are available for all five animals during maternal infusion experiments and for four animals during the fetal infusion experiments. In these four animals, the average renal clearance of the unbound drug during maternal experiments ($0.83 \pm 0.36$ ml/min/kg) was not significantly different compared to that during fetal experiments ($0.81 \pm 0.47$ ml/min/kg) (paired t-test, $p > 0.05$). However, the renal clearance of the total drug in these animals during maternal infusion ($0.26 \pm 0.09$
ml/min/kg) was significantly greater compared to the corresponding value during fetal experiments (0.12 ± 0.05 ml/min/kg) (paired t-test, p < 0.01).

Table 5.13 – *in vivo* Michaelis-Menten pharmacokinetic parameters of the unbound VPA in maternal and newborn sheep.

<table>
<thead>
<tr>
<th>Animal #</th>
<th>$V_{\text{max}}$ (CV%) ($\mu$g/min/kg)</th>
<th>$K_m$ (CV%) ($\mu$g/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Maternal Sheep</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E4241</td>
<td>1674.8 (1.3)</td>
<td>363.4$^a$</td>
</tr>
<tr>
<td>E5108</td>
<td>2805.5 (1.4)</td>
<td>363.4$^a$</td>
</tr>
<tr>
<td>E105x</td>
<td>634.6 (1.1)</td>
<td>363.4$^a$</td>
</tr>
<tr>
<td>E4133</td>
<td>3394.1 (1.5)</td>
<td>363.4$^a$</td>
</tr>
<tr>
<td>E1226</td>
<td>2526.4 (1.4)</td>
<td>363.4$^a$</td>
</tr>
<tr>
<td><strong>Mean ± S.D</strong></td>
<td>2207.1 ± 1075.3</td>
<td>363.4$^a$</td>
</tr>
</tbody>
</table>

| **Newborn Lambs**                                  |                                        |                          |
| NL4241      | 124.3 (34.6)                           | 126.3 (41.3)             |
| NL4124      | 58.6 (8.4)                             | 66.9 (12.2)              |
| NL0123z     | 72.2 (6.1)                             | 145.0 (7.8)              |
| NL2243(1)   | 239.0 (23.4)                           | 304.3 (26.0)             |
| **Mean ± S.D**                                     | 123.5 ± 82.0*                         | 160.6 ± 101.4            |

$^a$ – $K_m$ of *in vitro* VPA glucuronidation in pooled maternal liver microsomes.

$^*$ - significantly lower compared to maternal $V_{\text{max}}$ (p < 0.01).

Fetal renal clearance data are available for four animals during fetal drug infusion and for three animals during maternal infusion experiments. In the three animals, where data were available during both maternal and fetal infusions, the fetal renal clearances of the unbound as well as total drug appear to be similar during maternal and fetal experiments (unbound: 0.015 ± 0.004 vs. 0.018 ± 0.009 ml/min/kg and total: 0.004 ± 0.001 vs. 0.005 ± 0.002 ml/min/kg, during maternal and fetal drug infusions, respectively).
Figure 5.8 – Representative pharmacokinetic fitting of the newborn and maternal sheep unbound plasma concentration vs. time data to a one-compartment model with Michaelis-Menten elimination. Actual data (scatter) and model predicted profile (solid line) are shown. A) newborn lamb (NL4124), B) pregnant ewe (E5108).
Meaningful statistical analysis of these data, however, is not possible due to a low n value (n=3). During maternal as well as fetal experiments, fetal renal clearances of the unbound and total VPA were significantly lower compared to the corresponding maternal values (unpaired t-test, p < 0.01 in all cases). In four animals in which maternal urine was collected during maternal as well as fetal infusion experiments, a significantly smaller percentage of the total VPA dose was excreted unchanged in maternal urine during fetal experiments (19.9 ± 6.5 vs. 13.3 ± 5.2% during maternal and fetal experiments, respectively; paired t-test, p < 0.005).

Table 5.15 presents the renal clearance data of the unbound and total VPA in newborn lambs. Paired plasma and urine data were available in only 3 lambs and hence renal clearance could be calculated only in these 3 animals. Because of catheter failure, urine samples were not available in NL4124, whereas plasma samples could not be collected in NL2243(2). Similar to the fetus, renal clearance of the unbound as well as total VPA in newborn lambs was much lower compared to the corresponding values in the mother (unbound: 0.08 ± 0.06 vs. 0.94 ± 0.40 ml/min/kg; total: 0.03 ± 0.02 vs. 0.26 ± 0.08 ml/min/kg). Also, renal clearance of the unbound as well as total VPA in newborn lambs appears to be somewhat higher compared to the fetus (unbound: 0.08 ± 0.06 vs. 0.019 ± 0.008 ml/min/kg; total: 0.03 ± 0.02 vs. 0.005 ± 0.002 ml/min/kg). However, in both these cases, meaningful statistical comparisons can not be performed due to a low n value. The percentage of the total VPA dose excreted unchanged in newborn lamb urine was significantly lower than that excreted in maternal urine during maternal infusion experiments (unpaired t-test, p < 0.05); it was, however, not statistically different from that excreted in maternal urine during fetal infusion experiments (unpaired t-test, p > 0.05).
Table 5.14 – Pharmacokinetic parameters of renal elimination of VPA in maternal and fetal sheep.

<table>
<thead>
<tr>
<th>Ewe #</th>
<th>Maternal Administration</th>
<th>Fetal Administration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CL$^u_r$</td>
<td>CL$^u_r$</td>
</tr>
<tr>
<td>Maternal Parameters</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E4241</td>
<td>1.12</td>
<td>0.36</td>
</tr>
<tr>
<td>E5108</td>
<td>0.88</td>
<td>0.29</td>
</tr>
<tr>
<td>E105x</td>
<td>0.31</td>
<td>0.14</td>
</tr>
<tr>
<td>E4133</td>
<td>1.41</td>
<td>0.29</td>
</tr>
<tr>
<td>E1226</td>
<td>1.00</td>
<td>0.24</td>
</tr>
<tr>
<td>Mean ± S.D.</td>
<td>0.94 ± 0.40*</td>
<td>0.26 ± 0.08*</td>
</tr>
<tr>
<td>Fetal Parameters</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E4241</td>
<td>0.015</td>
<td>0.003</td>
</tr>
<tr>
<td>E5108</td>
<td>0.019</td>
<td>0.006</td>
</tr>
<tr>
<td>E105x</td>
<td>0.012</td>
<td>0.004</td>
</tr>
<tr>
<td>E4133</td>
<td>N/A$^a$</td>
<td>N/A$^a$</td>
</tr>
<tr>
<td>E1226</td>
<td>N/A$^a$</td>
<td>N/A$^a$</td>
</tr>
<tr>
<td>Mean ± S.D.</td>
<td>0.015 ± 0.004</td>
<td>0.004 ± 0.001</td>
</tr>
</tbody>
</table>

All clearances are expressed as ml/min/kg body weight.

$^a$ - Samples not available

* - significantly higher than the corresponding fetal value (p < 0.01).

** - significantly lower than the corresponding maternal value during maternal infusion (p < 0.01).

CL$^u_r$– Renal clearance of the unbound drug; CL$^r$ - Renal clearance of the total drug; F$^r$ - % fraction of the total dose excreted unchanged in urine.
Table 5.15 – Pharmacokinetic parameters of renal elimination of VPA in newborn lambs.

<table>
<thead>
<tr>
<th>Newborn Lamb #</th>
<th>( CL^u )</th>
<th>( CL_r )</th>
<th>( F_r ) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NL4241</td>
<td>0.15</td>
<td>0.05</td>
<td>19.2</td>
</tr>
<tr>
<td>NL4124(^b)</td>
<td>N/A(^a)</td>
<td>N/A(^a)</td>
<td>N/A(^a)</td>
</tr>
<tr>
<td>NL0123z</td>
<td>0.04</td>
<td>0.02</td>
<td>10.7</td>
</tr>
<tr>
<td>NL2243(1)</td>
<td>0.04</td>
<td>0.02</td>
<td>8.4</td>
</tr>
<tr>
<td>NL2243(2)(^c)</td>
<td>N/A(^a)</td>
<td>N/A(^a)</td>
<td>5.6</td>
</tr>
<tr>
<td>Mean ± S.D.</td>
<td>0.08 ± 0.06</td>
<td>0.03 ± 0.02</td>
<td>11.0 ± 5.8</td>
</tr>
</tbody>
</table>

All clearances are expressed as ml/min/kg body weight.
\(^a\) – data not available; \(^b\) – urine samples not available; \(^c\) – plasma samples not available

\( CL^u\) – Renal clearance of the unbound drug; \( CL_r\) - Renal clearance of the total drug; \( F_r\) - percent fraction of the total dose excreted unchanged in urine.

Figure 5.9 shows the representative cumulative urinary excretion vs. time plots for unchanged VPA and VPA-glucuronide in a pregnant ewe (E4241, after maternal administration) and a newborn lamb [NL2243(1)].
Figure 5.9 - Representative cumulative amount excreted vs. time plots of VPA and VPA-glucuronide in A) a pregnant ewe (E4241, after maternal administration) and B) a newborn lamb [NL2243(1)]. Profound differences in the kinetics of urinary excretion of these two compounds relative to the duration of VPA infusion in the ewe and the newborn lamb are evident.
5.2.9 Maternal and Fetal Plasma Concentrations of VPA Metabolites

A number of metabolites of VPA such as those formed via the fatty acid β-oxidation ((E)-2-ene, (E)-3-ene and 3-keto VPA), and cytochrome P450-mediated desaturation (4-ene VPA), and hydroxylation and subsequent oxidation (3-OH, 4-OH, 5-OH, and 4-keto VPA, 2-PSA, 2-PGA) were measured in maternal and fetal plasma during the course of these studies. Maternal and fetal plasma concentrations of 3-OH VPA and 2-PSA were generally below the limit of quantitation (LOQ) of the assay (see footnote \( ^b \) in Table 5.16 for exceptions). Also, the 2-PGA metabolite was below the LOQ in all the fetal plasma samples. During fetal infusion experiments, measurable concentrations of 2-PGA were detected in maternal plasma samples from only one animal (E105x). Average maternal and fetal plasma concentration vs. time profiles of these VPA metabolites are shown in Figures 5.10 - 5.13, with the upper and lower panels of each figure showing data from maternal and fetal infusion experiments, respectively. All VPA metabolites, except (E)-2-ene and 3-keto VPA, appeared to be at steady-state in maternal as well as fetal plasma during the 20-24 h period of infusion. The slopes of plasma concentration vs. time profiles of these metabolites were not significantly different from 0 (\( p > 0.05 \) in all cases) during the 20-24 h infusion period. Also, the CV's of the plasma concentrations ranged from < 10% (for (E)-3-ene, 4-ene, and 5-OH VPA, 2-PGA) to < 20% (for 4-OH and 4-keto VPA) for various metabolites during this period. After the infusion was stopped, the concentrations of many of these metabolites in maternal and fetal plasma declined relatively rapidly and were below the LOQ within 12-24 h in most of the animals (Figures 5.10 - 5.13). At the end of the infusion period, the plasma concentrations of (E)-2-ene and 3-keto VPA still appeared to be increasing and the maximal concentrations were typically observed a few hours after the end of VPA
infusion (Figure 5.10). In all animals, these two metabolites were detectable in maternal and fetal plasma for up to 36-72 h after the infusion was stopped (Figure 5.10). Tables 5.16 and 5.17 present the average maternal and fetal plasma concentrations of these metabolites during the final 4 h VPA infusion period (20-24 h) of maternal and fetal administration experiments, respectively. During maternal as well as fetal VPA infusion, the average fetal plasma concentrations of 3-keto VPA during the 20-24 h infusion period were significantly higher compared to the corresponding maternal plasma concentrations (Tables 5.16 and 5.17; paired t-test, p < 0.01 in both cases). During fetal but not maternal VPA infusion, fetal plasma concentrations of 5-OH VPA were also significantly higher relative to the corresponding maternal plasma concentrations (Table 5.17; paired t-test, p < 0.01). During maternal VPA infusion, the fetal plasma 5-OH VPA concentrations were higher in all but one animal and the difference between means was near statistical significance (0.73 ± 0.67 vs. 0.90 ± 0.81 μg/ml in maternal and fetal plasma, respectively; paired t-test, 1-tailed p < 0.1) (Table 5.16). In contrast to 3-keto and 5-OH VPA metabolites, fetal plasma concentrations of 4-ene and 4-keto VPA were significantly lower compared to the corresponding maternal plasma concentrations during both maternal as well as fetal infusion experiments (paired t-test, p < 0.05 in all cases) (Tables 5.16 and 5.17). The 20-24 h fetal and maternal plasma concentrations of (E)-2-ene VPA were not significantly different during maternal infusion (Table 5.16; paired t-test, p > 0.05), whereas during fetal VPA infusion, fetal plasma concentrations of this metabolite were significantly lower (Table 5.17; paired t-test, p < 0.05). The average fetal and maternal plasma concentrations of (E)-3-ene and 4-OH VPA metabolites during the 20-24 h period were not significantly different during maternal or fetal VPA infusion experiments (paired t-test, p ≥ 0.05 in all cases) (Tables 5.16 and 5.17).
Figure 5.10 – Average maternal and fetal plasma concentration vs. time profiles of the (E)-2-ene (A and C) and 3-keto (B and D) VPA metabolites in five pregnant sheep during and after a 24 h steady-state VPA infusion. Upper panel: maternal VPA infusion; lower panel: fetal VPA infusion.
Figure 5.11 – Average maternal and fetal plasma concentration vs. time profiles of the (E)-3-ene (A and C) and 4-ene (B and D) VPA metabolites in five pregnant sheep during and after a 24 h steady-state VPA infusion. Upper panel: maternal VPA infusion; lower panel: fetal VPA infusion.
Figure 5.12 – Average maternal and fetal plasma concentration vs. time profiles of the 4-OH (A and C) and 4-keto (B and D) VPA metabolites in five pregnant sheep during and after a 24 h steady-state VPA infusion. Upper panel: maternal VPA infusion; lower panel: fetal VPA infusion.
Figure 5.13 - A) and B) Average maternal and fetal plasma concentration vs. time profiles of 5-OH VPA in 5 pregnant sheep during maternal and fetal VPA infusions, respectively. C) Average maternal plasma concentration vs. time profile of 2-PGA during 24 h maternal steady-state VPA infusion. 2-PGA was below the LOQ in all fetal plasma samples and was measurable in maternal samples from only one ewe during fetal VPA infusion (see Tables 5.16 and 5.17 for details).
Table 5.16 – Steady-state plasma concentrations of various VPA metabolites in maternal and fetal plasma during the final 4 h period of maternal drug infusion.

<table>
<thead>
<tr>
<th>Metabolite a,b</th>
<th>E4241</th>
<th>E5108</th>
<th>E105x</th>
<th>E4133</th>
<th>E1226</th>
<th>Mean ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MA</td>
<td>FA</td>
<td>MA</td>
<td>FA</td>
<td>MA</td>
<td>FA</td>
</tr>
<tr>
<td>VPA</td>
<td>101.0</td>
<td>79.5</td>
<td>55.2</td>
<td>36.2</td>
<td>208.3</td>
<td>173.0</td>
</tr>
<tr>
<td>(E)-2-Ene d</td>
<td>0.54</td>
<td>0.49</td>
<td>0.57</td>
<td>0.40</td>
<td>0.49</td>
<td>0.92</td>
</tr>
<tr>
<td>(E)-3-Ene</td>
<td>0.10</td>
<td>0.23</td>
<td>0.08</td>
<td>0.06</td>
<td>0.16</td>
<td>0.17</td>
</tr>
<tr>
<td>3-Keto d</td>
<td>0.28</td>
<td>2.1</td>
<td>0.40</td>
<td>1.0</td>
<td>0.72</td>
<td>2.2</td>
</tr>
<tr>
<td>4-Ene</td>
<td>0.26</td>
<td>0.23</td>
<td>0.12</td>
<td>0.09</td>
<td>0.95</td>
<td>0.92</td>
</tr>
<tr>
<td>4-Keto</td>
<td>0.23</td>
<td>0.23</td>
<td>0.10</td>
<td>0.03</td>
<td>0.25</td>
<td>0.08</td>
</tr>
<tr>
<td>4-OH</td>
<td>3.2</td>
<td>1.6</td>
<td>3.1</td>
<td>1.4</td>
<td>7.7</td>
<td>4.2</td>
</tr>
<tr>
<td>5-OH</td>
<td>0.48</td>
<td>0.83</td>
<td>0.23</td>
<td>0.27</td>
<td>1.9</td>
<td>2.3</td>
</tr>
<tr>
<td>2-PGA</td>
<td>0.13</td>
<td>BLOQ c</td>
<td>0.08</td>
<td>BLOQ c</td>
<td>0.28</td>
<td>BLOQ c</td>
</tr>
</tbody>
</table>

a – All concentrations are presented as μg/ml plasma. MA and FA refer to maternal and fetal arterial plasma, respectively.
* = significantly higher than the corresponding concentration in maternal plasma (p < 0.01); ** = significantly lower than the corresponding concentration in maternal plasma (p < 0.05).
b – The 3-OH metabolites was below the LOQ in maternal and fetal plasma.
c – The 2-PSA metabolite was below the LOQ in all maternal and fetal plasma samples, except that in E4241 and E5108 average maternal plasma concentrations during the 20-24 h period of maternal VPA infusion were 0.05 and 0.02 μg/ml, respectively.
d – Below the limit of quantitation of the assay.
d – (E)-2-ene and 3-keto metabolites did not reach steady-state in fetal plasma during the 24 h infusion period and the average concentrations during the final 4 h infusion period are presented.
Table 5.17 – Steady-state plasma concentrations of various VPA metabolites in maternal and fetal plasma during the final 4 h period of fetal drug infusion.

<table>
<thead>
<tr>
<th>Metabolitea,b</th>
<th>E4241 MA</th>
<th>E4241 FA</th>
<th>E5108 MA</th>
<th>E5108 FA</th>
<th>E105x MA</th>
<th>E105x FA</th>
<th>E4133 MA</th>
<th>E4133 FA</th>
<th>E1226 MA</th>
<th>E1226 FA</th>
<th>Mean ± S.D. MA</th>
<th>Mean ± S.D. FA</th>
</tr>
</thead>
<tbody>
<tr>
<td>VPA</td>
<td>33.0</td>
<td>51.7</td>
<td>20.3</td>
<td>37.6</td>
<td>68.6</td>
<td>102.1</td>
<td>28.7</td>
<td>58.5</td>
<td>28.7</td>
<td>43.7</td>
<td>35.9 ± 18.9</td>
<td>58.7 ± 25.5</td>
</tr>
<tr>
<td>(E)-2-Ene²</td>
<td>0.37</td>
<td>0.24</td>
<td>0.55</td>
<td>0.41</td>
<td>0.59</td>
<td>0.55</td>
<td>0.49</td>
<td>0.41</td>
<td>0.67</td>
<td>0.39</td>
<td>0.53 ± 0.11</td>
<td>0.40 ± 0.11**</td>
</tr>
<tr>
<td>(E)-3-Ene</td>
<td>0.03</td>
<td>0.04</td>
<td>0.06</td>
<td>0.04</td>
<td>0.15</td>
<td>0.13</td>
<td>0.07</td>
<td>0.05</td>
<td>0.09</td>
<td>0.04</td>
<td>0.08 ± 0.04</td>
<td>0.06 ± 0.04</td>
</tr>
<tr>
<td>3-Keto²</td>
<td>0.15</td>
<td>0.96</td>
<td>0.48</td>
<td>1.5</td>
<td>0.26</td>
<td>1.5</td>
<td>0.17</td>
<td>1.6</td>
<td>0.14</td>
<td>0.83</td>
<td>0.24 ± 0.14</td>
<td>1.3 ± 0.4*</td>
</tr>
<tr>
<td>4-Ene</td>
<td>0.08</td>
<td>0.05</td>
<td>0.04</td>
<td>0.02</td>
<td>0.34</td>
<td>0.29</td>
<td>0.06</td>
<td>0.03</td>
<td>0.06</td>
<td>0.03</td>
<td>0.12 ± 0.13</td>
<td>0.08 ± 0.12**</td>
</tr>
<tr>
<td>4-Keto</td>
<td>0.06</td>
<td>0.03</td>
<td>0.03</td>
<td>BLOQ²</td>
<td>0.09</td>
<td>0.04</td>
<td>0.10</td>
<td>0.02</td>
<td>0.03</td>
<td>0.01</td>
<td>0.06 ± 0.03</td>
<td>0.03 ± 0.01**</td>
</tr>
<tr>
<td>4-OH</td>
<td>0.59</td>
<td>0.64</td>
<td>0.52</td>
<td>0.20</td>
<td>1.2</td>
<td>0.80</td>
<td>0.28</td>
<td>0.35</td>
<td>0.57</td>
<td>0.19</td>
<td>0.63 ± 0.34</td>
<td>0.44 ± 0.27</td>
</tr>
<tr>
<td>5-OH</td>
<td>0.06</td>
<td>0.15</td>
<td>0.07</td>
<td>0.21</td>
<td>0.52</td>
<td>0.72</td>
<td>0.12</td>
<td>0.36</td>
<td>0.07</td>
<td>0.14</td>
<td>0.17 ± 0.20</td>
<td>0.32 ± 0.24*</td>
</tr>
<tr>
<td>2-PGA</td>
<td>BLOQ²</td>
<td>BLOQ²</td>
<td>BLOQ²</td>
<td>BLOQ²</td>
<td>0.11</td>
<td>BLOQ²</td>
<td>BLOQ²</td>
<td>BLOQ²</td>
<td>BLOQ²</td>
<td>BLOQ²</td>
<td>0.11</td>
<td>BLOQ²</td>
</tr>
</tbody>
</table>

² – All concentrations are presented as µg/ml plasma. MA and FA refer to maternal and fetal arterial plasma, respectively.

* – significantly higher than the corresponding concentration in maternal plasma (p < 0.01); ** - significantly lower than the corresponding concentration in maternal plasma (p < 0.05).

² – The 3-OH and 2-PSA metabolites were below the limit of quantitation of the assay in maternal and fetal plasma.

² – Below the limit of quantitation of the assay.

² – (E)-2-ene and 3-keto metabolites did not reach steady-state in fetal plasma during the 24 h infusion period and the average concentrations during the final 4 h infusion period are presented.
5.2.10 Plasma Concentrations of VPA Metabolites in Newborn Lambs, and Fetal and Newborn VPA Metabolite Exposure Relative to the Mother

Plasma concentrations of all the above VPA metabolites were also measured in samples obtained from four newborn lambs. As with the fetus, the plasma concentrations of 2-PSA, 2-PGA and 3-OH VPA metabolites were below the LOQ in newborn lambs. Also, none of the metabolites appeared to be at steady-state during the course of VPA infusion in these experiments (Figures 5.14 and 5.15). Thus, it was necessary to compare the maximal plasma concentrations ($C_{\text{max}}$) of these metabolites in newborn plasma with those in maternal and fetal plasma in order to assess their relative metabolite exposure. Tables 5.18 and 5.19 present the $C_{\text{max}}$'s of VPA and its metabolites in maternal and fetal plasma during maternal and fetal infusion experiments, respectively. The maternal plasma $C_{\text{max}}$ of VPA after maternal administration occurred at 5 min, i.e., immediately after the i.v. bolus loading dose, whereas that after fetal administration occurred within 1-3 h. Similarly, the fetal plasma $C_{\text{max}}$ of VPA after fetal administration occurred at 5 min, whereas that after maternal administration occurred within 1-3 h. The maternal and fetal $C_{\text{max}}$'s of all metabolites, except (E)-2-ene and 3-keto VPA, occurred within the infusion period; the $C_{\text{max}}$'s of these two metabolites occurred within 1-12 h after the end of VPA infusion (Figures 5.10 – 5.13). Table 5.20 presents the maximal plasma concentrations ($C_{\text{max}}$) of VPA and various metabolites in newborn plasma and the corresponding times of their occurrence ($t_{\text{max}}$). The average plasma concentration vs. time profiles of various VPA metabolites in the newborn lamb are shown in Figures 5.14 and 5.15. The $C_{\text{max}}$'s of these metabolites occurred at times ranging from 0.5 h to 54 h after the end of VPA infusion. Low concentrations of many VPA metabolites ((E)-3-ene, 4-ene, 4-OH, 4-keto VPA) were detectable in the newborn plasma until 54-66 h after the end of infusion. This is in
contrast to pregnant sheep where maternal and fetal plasma concentrations of these metabolites declined to below LOQ levels within 12-24 h after the end of infusion period in most animals. Also, (E)-2-ene, 3-keto and 5-OH VPA metabolites were still detectable in newborn plasma in significant concentrations (12.7 ± 7.9%, 24.4 ± 16.0% and 7.0 ± 7.3 % of the respective C$_{max}$ for (E)-2-ene, 3-keto and 5-OH VPA) at the end of 96 h experimental protocol (Figures 5.14 and 5.15). Maternal, fetal and newborn plasma C$_{max}$'s of various VPA metabolites were compared separately against each other in order to assess the degree of fetal and newborn exposure to these metabolites relative to the mother. The maternal and fetal C$_{max}$'s of the (E)-2-ene VPA and (E)-3-ene metabolites during maternal infusion were not significantly different; however, during fetal infusion, the maternal C$_{max}$ of these metabolites was significantly higher (paired t-test, p < 0.05 in both cases). The C$_{max}$'s of (E)-2-ene and (E)-3-ene VPA in newborn lambs were significantly higher relative to both the mother and the fetus during maternal as well as fetal drug infusion (unpaired t-test, p < 0.01 in all cases for (E)-2-ene and p < 0.05 for (E)-3-ene). As with the average MA and FA concentrations during the 20-24 h period, the fetal C$_{max}$ of 3-keto VPA during both maternal and fetal VPA infusions was significantly higher than the maternal C$_{max}$ of this metabolite (paired t-test, p < 0.01 in both cases). However, the C$_{max}$ of 3-keto VPA in the newborn lamb was significantly higher compared to the maternal as well as fetal C$_{max}$ achieved during the course of the maternal or fetal VPA infusions (unpaired t-test, p < 0.001 in all cases). The fetal C$_{max}$ of 5-OH VPA was significantly higher compared to the mother during fetal drug infusion (paired t-test, p < 0.05). During maternal infusion, the fetal C$_{max}$ of this metabolite was higher in four out of five animals but the difference between means was not statistically significant (paired t-test, p = 0.12). The newborn C$_{max}$ of this metabolite was, however, significantly higher compared to that in the mother and the fetus during
maternal as well as fetal infusions (unpaired t-test, p < 0.05 in all cases). The fetal $C_{\text{max}}$ of 4-ene VPA was slightly but significantly lower compared to the mother during maternal as well as fetal drug infusion (paired t-test, p < 0.05 in both cases). The newborn $C_{\text{max}}$ of 4-ene VPA was not significantly different from that in the mother or the fetus during both pregnant sheep experiments (unpaired t-test, p > 0.05). The maternal $C_{\text{max}}$ of 4-keto VPA was significantly higher than the fetus during maternal as well as fetal infusion experiments. The newborn $C_{\text{max}}$ of this metabolite was not significantly different from the maternal and fetal $C_{\text{max}}$ during maternal infusion, and from the maternal $C_{\text{max}}$ during fetal infusion (unpaired t-test, p > 0.05 in all cases); it was, however, significantly higher compared to the fetal $C_{\text{max}}$ during fetal infusion (unpaired t-test, p < 0.05). The maternal $C_{\text{max}}$'s of the 4-OH VPA metabolite appear to be higher than the fetal $C_{\text{max}}$'s during maternal (paired t-test, p < 0.1) as well as fetal (paired t-test, p < 0.05) drug infusion. The newborn $C_{\text{max}}$ of 4-OH VPA metabolite was not statistically different relative to the maternal and fetal $C_{\text{max}}$ of this metabolite during maternal infusion experiments. During the fetal infusion experiments, both the maternal and fetal $C_{\text{max}}$'s of 4-OH VPA were significantly lower compared to the newborn lamb.
Table 5.18 – Maximal plasma concentrations (C\textsubscript{max}) of various VPA metabolites in maternal and fetal plasma during maternal drug infusion.

<table>
<thead>
<tr>
<th>Metabolite\textsuperscript{a,b}</th>
<th>E4241</th>
<th>E5108</th>
<th>E105x</th>
<th>E4133</th>
<th>E1226</th>
<th>Mean ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MA</td>
<td>FA</td>
<td>MA</td>
<td>FA</td>
<td>MA</td>
<td>FA</td>
</tr>
<tr>
<td>VPA</td>
<td>126.2</td>
<td>82.7</td>
<td>75.0</td>
<td>35.8</td>
<td>215.7</td>
<td>179.3</td>
</tr>
<tr>
<td>(E)-2-Ene</td>
<td>0.57</td>
<td>0.53</td>
<td>0.63</td>
<td>0.44</td>
<td>0.78</td>
<td>0.93</td>
</tr>
<tr>
<td>(E)-3-Ene</td>
<td>0.11</td>
<td>0.24</td>
<td>0.10</td>
<td>0.07</td>
<td>0.22</td>
<td>0.19</td>
</tr>
<tr>
<td>3-Keto</td>
<td>0.29</td>
<td>2.98</td>
<td>0.43</td>
<td>1.44</td>
<td>0.96</td>
<td>3.49</td>
</tr>
<tr>
<td>4-Ene</td>
<td>0.28</td>
<td>0.24</td>
<td>0.14</td>
<td>0.09</td>
<td>1.05</td>
<td>0.93</td>
</tr>
<tr>
<td>4-Keto</td>
<td>0.25</td>
<td>0.24</td>
<td>0.10</td>
<td>0.03</td>
<td>0.30</td>
<td>0.09</td>
</tr>
<tr>
<td>4-OH</td>
<td>4.39</td>
<td>1.87</td>
<td>3.16</td>
<td>1.61</td>
<td>8.62</td>
<td>5.48</td>
</tr>
<tr>
<td>5-OH</td>
<td>0.53</td>
<td>0.96</td>
<td>0.23</td>
<td>0.35</td>
<td>2.38</td>
<td>2.49</td>
</tr>
<tr>
<td>2-PGA</td>
<td>0.14</td>
<td>BLOQ\textsuperscript{c}</td>
<td>0.08</td>
<td>BLOQ\textsuperscript{c}</td>
<td>0.35</td>
<td>BLOQ\textsuperscript{c}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} – All concentrations are presented as μg/ml plasma.

\textsuperscript{*} – significantly higher than the corresponding concentration in maternal plasma (p < 0.01); \textsuperscript{**} - significantly lower than the corresponding concentration in maternal plasma (p < 0.05).

\textsuperscript{b} – The 3-OH metabolite was below the LOQ in maternal and fetal plasma.

\textsuperscript{c} – The 2-PSA metabolite was below the LOQ in all maternal and fetal plasma samples, except that in E4241 and E5108, the maternal plasma C\textsubscript{max}’s were 0.05 and 0.03 μg/ml, respectively.

\textsuperscript{c} – Below the limit of quantitation of the assay.
Table 5.19 – Maximal plasma concentrations ($C_{\text{max}}$) of various VPA metabolites in maternal and fetal plasma during fetal drug infusion.

<table>
<thead>
<tr>
<th>Metabolite$^{a,b}$</th>
<th>E4241 MA</th>
<th>E5108 MA</th>
<th>E105x MA</th>
<th>E4133 MA</th>
<th>E1226 MA</th>
<th>Mean $\pm$ S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VPA</td>
<td>35.0</td>
<td>114.7</td>
<td>22.2</td>
<td>113.3</td>
<td>75.6</td>
<td>210.4</td>
</tr>
<tr>
<td>(E)-2-Ene</td>
<td>0.41</td>
<td>0.3</td>
<td>0.58</td>
<td>0.42</td>
<td>0.72</td>
<td>0.68</td>
</tr>
<tr>
<td>(E)-3-Ene</td>
<td>0.06</td>
<td>0.05</td>
<td>0.07</td>
<td>0.04</td>
<td>0.16</td>
<td>0.15</td>
</tr>
<tr>
<td>3-Keto</td>
<td>0.18</td>
<td>1.22</td>
<td>0.5</td>
<td>1.81</td>
<td>0.36</td>
<td>2.28</td>
</tr>
<tr>
<td>4-Ene</td>
<td>0.09</td>
<td>0.07</td>
<td>0.04</td>
<td>0.03</td>
<td>0.45</td>
<td>0.37</td>
</tr>
<tr>
<td>4-Keto</td>
<td>0.06</td>
<td>0.04</td>
<td>0.031</td>
<td>BLOQ$^c$</td>
<td>0.11</td>
<td>0.038</td>
</tr>
<tr>
<td>4-OH</td>
<td>0.78</td>
<td>0.65</td>
<td>0.55</td>
<td>0.31</td>
<td>1.52</td>
<td>1.35</td>
</tr>
<tr>
<td>5-OH</td>
<td>0.07</td>
<td>0.17</td>
<td>0.09</td>
<td>0.32</td>
<td>0.61</td>
<td>0.83</td>
</tr>
<tr>
<td>2-PGA</td>
<td>BLOQ$^c$</td>
<td>BLOQ$^c$</td>
<td>BLOQ$^c$</td>
<td>BLOQ$^c$</td>
<td>0.14</td>
<td>BLOQ$^c$</td>
</tr>
</tbody>
</table>

$^a$ All concentrations are presented as $\mu$g/ml plasma.

$^b$ – significantly higher than the corresponding concentration in maternal plasma ($p < 0.05$); ** - significantly lower than the corresponding concentration in maternal plasma ($p < 0.05$).

$^b$ – The 3-OH and 2-PSA metabolites were below the limit of quantitation of the assay in maternal and fetal plasma.

$^c$ – Below the limit of quantitation of the assay.
Table 5.20 – Maximal plasma concentrations ($C_{\text{max}}$) of VPA and its metabolites, and the corresponding times of their occurrence ($t_{\text{max}}$) in four newborn lambs after a 6 h VPA infusion.

<table>
<thead>
<tr>
<th>Metabolite$^a$</th>
<th>NL4241</th>
<th>NL4124</th>
<th>NL0123z</th>
<th>NL2243(1)</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$C_{\text{max}}$ (µg/ml)</td>
<td>$t_{\text{max}}$ (h)</td>
<td>$C_{\text{max}}$ (µg/ml)</td>
<td>$t_{\text{max}}$ (h)</td>
<td>$C_{\text{max}}$ (µg/ml)</td>
</tr>
<tr>
<td>VPA</td>
<td>107.5</td>
<td>6.0</td>
<td>173.3</td>
<td>6.0</td>
<td>150.7</td>
</tr>
<tr>
<td>(E)-2-Enone</td>
<td>1.1</td>
<td>36.0</td>
<td>2.5</td>
<td>24.0</td>
<td>1.5</td>
</tr>
<tr>
<td>(E)-3-Enone</td>
<td>0.19</td>
<td>24.0</td>
<td>0.59</td>
<td>18.0</td>
<td>0.23</td>
</tr>
<tr>
<td>3-Keto</td>
<td>5.1</td>
<td>60.0</td>
<td>7.0</td>
<td>36.0</td>
<td>6.2</td>
</tr>
<tr>
<td>4-Enone</td>
<td>0.15</td>
<td>36.0</td>
<td>0.24</td>
<td>10.0</td>
<td>0.12</td>
</tr>
<tr>
<td>4-Keto</td>
<td>0.08</td>
<td>6.5</td>
<td>0.24</td>
<td>10.0</td>
<td>0.04</td>
</tr>
<tr>
<td>4-OH VPA</td>
<td>3.0</td>
<td>10.0</td>
<td>9.1</td>
<td>10.0</td>
<td>1.9</td>
</tr>
<tr>
<td>5-OH VPA</td>
<td>2.0</td>
<td>24.0</td>
<td>2.2</td>
<td>10.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>

$^a$ – 3-OH VPA, 2-PSA and 2-PGA metabolites were below the limit of quantitation of the assay in newborn lamb plasma.

$^b$ – $t_{\text{max}}$ data are presented as median.

* – significantly higher than maternal and fetal $C_{\text{max}}$ of this metabolite during maternal as well as fetal infusion (p < 0.05); ** – significantly higher than maternal and fetal $C_{\text{max}}$ of this metabolite during fetal infusion (p < 0.05).

Plasma samples could not be obtained in the fifth lamb, NL2243(2), due to catheter failure.
Figure 5.14 - Average plasma concentration vs. time profiles of the VPA metabolites formed via the β-oxidation pathway in four newborn lambs during and after a 6 h VPA infusion. A) (E)-2-ene VPA, B) 3-keto VPA, C) (E)-3-ene VPA.
Figure 5.15 - Average plasma concentration vs. time profiles of the VPA metabolites formed via microsomal oxidation pathways in four newborn lambs during and after a 6 h VPA infusion. A) 4-ene VPA, B) 4-keto VPA, C) 4-OH VPA, D) 5-OH VPA.
5.2.11 Excretion of Unchanged VPA and its Metabolites in Maternal and Fetal Urine

Tables 5.21 and 5.22 present the percentage of total VPA dose excreted as unchanged VPA and various VPA metabolites in maternal urine during maternal and fetal VPA infusion, respectively. Maternal urine could not be quantitatively collected from E4133 during fetal VPA infusion because of catheter failure. In the four animals where maternal urine was collected both during maternal and fetal infusion experiments, the percentage of total dose excreted collectively via the routes presented in Tables 5.21 and 5.22 was not statistically different during the two experimental periods (105.3 ± 3.7 vs. 83.1 ± 24.5% during maternal and fetal experiments, respectively; paired t-test, p > 0.05). In these four animals, the percentage of dose recovered as unchanged VPA, however, was greater during the maternal infusion period (19.9 ± 6.6 vs. 13.3 ± 5.2% during maternal and fetal experiments, respectively; paired t-test, p < 0.005). The percentage of total dose recovered as 4-ene VPA and 2-PGA metabolites was also greater during maternal experiments (paired t-test, p < 0.05 in both cases). All other pathways accounted for a similar fraction of the dose during both maternal and fetal VPA infusion. Unchanged VPA and VPA-glucuronide were also detectable in fetal urine samples (Figure 5.16); the concentrations of glucuronide being less than or approximately equal to those of the unconjugated VPA (Figure 5.16). Similarly, all other VPA metabolites were also detectable in fetal urine samples, albeit at much lower concentrations compared to maternal urine. Cumulative collection of fetal urine was not performed and hence the total amounts of these compounds excreted in fetal urine as a fraction of the total dose could not be estimated.
Figure 5.16 – Average concentrations of VPA before and after base-hydrolysis in fetal urine samples obtained from four pregnant sheep at different times during and after steady-state VPA infusion experiments. A) maternal infusion experiments, B) fetal infusion experiments.
5.2.12 Excretion of Unchanged VPA and its Metabolites in the Newborn Lamb Urine

Cumulative urine samples were collected in four newborn lambs. Urine could not be collected in NL4124 due to catheter failure. Table 5.23 presents the percent fractions of total VPA dose recovered as unchanged VPA and various VPA metabolites in these four lambs. The percentages of different compounds in lamb urine were statistically compared with those in maternal urine after maternal VPA infusion (Table 5.21). The percentage of total dose excreted in newborn urine as unchanged VPA, VPA-glucuronide and 4-keto VPA was significantly smaller compared to the mother (unpaired t-test, p < 0.05 for unchanged VPA, and p < 0.005 for VPA-glucuronide and 4-keto VPA). In contrast, a significant larger percentage of the dose was excreted in newborn urine as (E)-2-ene (unpaired t-test, p < 0.005), (E)-3-ene (unpaired t-test, p < 0.05), 3-keto (unpaired t-test, p < 0.005), 3-OH (unpaired t-test, p < 0.005), 4-OH (unpaired t-test, p < 0.05) and 5-OH VPA (unpaired t-test, p < 0.005) metabolites. There was no significant difference between the percent fractions of total dose excreted as 4-ene VPA, 2-PSA and 2-PGA metabolites in newborn and maternal urine (unpaired t-test, p > 0.05 in all cases).
Table 5.21 – Recovery of unchanged VPA and its metabolites as a percentage of the total dose in maternal urine after maternal steady-state VPA infusion.

<table>
<thead>
<tr>
<th>Drug or Metabolite</th>
<th>Percent of Administered Dose in Maternal Urine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E4241</td>
</tr>
<tr>
<td>Unchanged VPA</td>
<td>27.3</td>
</tr>
<tr>
<td>VPA – Glucuronide</td>
<td>68.5</td>
</tr>
<tr>
<td>(E)-2-En VPA</td>
<td>0.016</td>
</tr>
<tr>
<td>(E)-3-En VPA</td>
<td>0.003</td>
</tr>
<tr>
<td>3-Keto VPA</td>
<td>1.41</td>
</tr>
<tr>
<td>4-En VPA</td>
<td>0.020</td>
</tr>
<tr>
<td>4-Keto VPA</td>
<td>0.56</td>
</tr>
<tr>
<td>3-OH VPA</td>
<td>0.24</td>
</tr>
<tr>
<td>4-OH VPA</td>
<td>0.95</td>
</tr>
<tr>
<td>5-OH VPA</td>
<td>0.46</td>
</tr>
<tr>
<td>2-PSA</td>
<td>0.11</td>
</tr>
<tr>
<td>2-PGA</td>
<td>0.80</td>
</tr>
<tr>
<td>Total</td>
<td>100.4</td>
</tr>
</tbody>
</table>
Table 5.22 – Recovery of unchanged VPA and its metabolites as a percentage of the total dose in maternal urine after fetal steady-state drug infusion.

<table>
<thead>
<tr>
<th>Drug or Metabolite</th>
<th>Percent of Administered Dose in Maternal Urine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E4241</td>
</tr>
<tr>
<td>Unchanged VPA</td>
<td>18.7</td>
</tr>
<tr>
<td>VPA – Glucuronide</td>
<td>52.0</td>
</tr>
<tr>
<td>(E)-2-Ene VPA</td>
<td>0.015</td>
</tr>
<tr>
<td>(E)-3-Ene VPA</td>
<td>0.009</td>
</tr>
<tr>
<td>3-Keto VPA</td>
<td>1.96</td>
</tr>
<tr>
<td>4-Ene VPA</td>
<td>0.020</td>
</tr>
<tr>
<td>4-Keto VPA</td>
<td>0.45</td>
</tr>
<tr>
<td>3-OH VPA</td>
<td>0.25</td>
</tr>
<tr>
<td>4-OH VPA</td>
<td>2.05</td>
</tr>
<tr>
<td>5-OH VPA</td>
<td>0.51</td>
</tr>
<tr>
<td>2-PSA</td>
<td>0.09</td>
</tr>
<tr>
<td>2-PGA</td>
<td>0.36</td>
</tr>
<tr>
<td>Total</td>
<td>76.4</td>
</tr>
</tbody>
</table>
Table 5.23 – Recovery of unchanged VPA and its metabolites as a percentage of the total dose in newborn lamb urine after a 6 h drug infusion.

<table>
<thead>
<tr>
<th>Drug or Metabolite</th>
<th>Percent of Administered Dose in Newborn Urine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NL4241</td>
</tr>
<tr>
<td>Unchanged VPA</td>
<td>19.2</td>
</tr>
<tr>
<td>VPA – Glucuronide</td>
<td>12.4</td>
</tr>
<tr>
<td>(E)-2-Ene VPA</td>
<td>0.16</td>
</tr>
<tr>
<td>(E)-3-Ene VPA</td>
<td>0.016</td>
</tr>
<tr>
<td>3-Keto VPA</td>
<td>12.1</td>
</tr>
<tr>
<td>4-Ene VPA</td>
<td>0.024</td>
</tr>
<tr>
<td>4-Keto VPA</td>
<td>0.10</td>
</tr>
<tr>
<td>3-OH VPA</td>
<td>0.62</td>
</tr>
<tr>
<td>4-OH VPA</td>
<td>5.9</td>
</tr>
<tr>
<td>5-OH VPA</td>
<td>2.07</td>
</tr>
<tr>
<td>2-PSA</td>
<td>0.05</td>
</tr>
<tr>
<td>2-PGA</td>
<td>0.70</td>
</tr>
<tr>
<td>Total</td>
<td>53.3</td>
</tr>
</tbody>
</table>

* – significantly lower compared to the percentage of dose excreted as this metabolite in maternal urine during maternal infusion (p < 0.05); ** – significantly higher compared to the percentage of dose excreted as this metabolite in maternal urine during maternal infusion (p < 0.05).
5.3 Discussion

5.3.1 Maternal-Fetal VPA Plasma Concentrations, Plasma Protein Binding and Indices of Fetal Drug Exposure

The placental transfer of VPA was extremely rapid. Significant concentrations of VPA were detectable in fetal plasma after maternal administration and in maternal plasma after fetal administration at the first sampling time, i.e., 5 min (Figure 5.1). The steady-state maternal and fetal total plasma concentrations of VPA during maternal (106.5 ± 59.3 and 78.6 ± 55.0 µg/ml, respectively) as well as fetal (35.9 ± 18.9 and 58.7 ± 25.5 µg/ml, respectively) drug administration were near the suggested therapeutic range in humans (40-100 µg/ml, Davis et al., 1994). The steady-state fetal plasma concentrations of the total as well as unbound VPA after maternal drug administration were significantly lower compared to the corresponding maternal plasma concentrations. It has been suggested that lower fetal plasma concentrations of the unbound drug compared to the mother after maternal drug administration indicate irreversible drug elimination from the fetal compartment and/or by the placenta (Szeto et al., 1982a; Wang et al., 1986a). Similarly, after fetal drug administration, significantly lower total as well as unbound maternal plasma VPA concentrations were observed as compared to the fetus. This provides evidence of irreversible VPA elimination from the maternal compartment and/or by the placenta. The fetal-to-maternal steady-state plasma concentration ratios based on total and unbound drug were 0.70 ± 0.10 and 0.81 ± 0.09, respectively, indicating a high degree of fetal VPA exposure after maternal drug administration. The VPA $C_f/C_m$ ratio based on unbound drug concentrations appears to be higher compared to that of a number of other drugs studied in pregnant
sheep at this stage of gestation. These include DPHM (0.50 ± 0.30, Chapter 4, Section 4.2.5), morphine (0.14 ± 0.02, Szeto et al., 1982b), methadone (0.42 ± 0.19, Szeto et al., 1982b), and metoclopramide (0.67 ± 0.18, Riggs et al., 1990). However, the VPA C_f/C_m ratio is similar to that observed for acetaminophen (0.77 ± 0.03, Wang et al., 1986a). It has been shown that at steady-state, the fetal-to-maternal drug concentration ratio during maternal drug administration is determined by maternal placental clearance divided by the sum of fetal placental and non-placental clearances, \( i.e., \frac{C_f}{C_m} = \frac{CL_{mf}}{CL_{mf} + CL_{fo}} \) (Szeto et al., 1982a). In this regard, it is interesting to notice that VPA and acetaminophen also have the lowest values for \( CL_{fo} \) among all these drugs (see below).

Plasma protein binding of VPA, in both the mother as well as the fetus, was saturable (nonlinear). Maternal and fetal plasma unbound fractions ranged from 0.06 to 0.56 and from 0.10 to 0.78, respectively, during the course of experiments. Plasma protein binding parameters were estimated separately for the mother and the fetus using pooled maternal and fetal data from all experiments because enough data points were not available in individual animals to characterize binding at a wide range of concentrations. Fits of the bound vs. unbound plasma concentration data to a 2-site binding model with one high affinity saturable and a low affinity non-saturable binding site were very good and all the parameters were estimated with a high degree of confidence (low CV's) (Table 5.5). These analyses revealed similar maximal binding capacities of maternal and fetal plasma for VPA at the saturable binding site \( (B_{max}: 62.8 \text{ vs. } 65.0 \, \mu g/ml, \text{ respectively}) \). However, the overall VPA binding affinity at this saturable binding site appears to be considerably greater in maternal plasma as compared to fetal
plasma ($K_d$: 7.6 vs. 13.0 µg/ml for maternal and fetal plasma, respectively). Similar to the above data in maternal and fetal sheep, two classes of VPA binding sites (a high affinity saturable and a low affinity non-saturable) have also been demonstrated in the plasma of rats (Semmes and Shen, 1990; Haberer and Pollack, 1994), guinea-pigs (Yu and Shen, 1992), and non-pregnant and pregnant humans (Riva et al., 1984; Scheyer et al., 1990). Also, the binding parameters calculated in sheep are in reasonable agreement with those of the other species. Overall similarity of VPA plasma protein binding characteristics in different species is also supported by the fact that plasma protein binding of VPA is saturable in all these species at the therapeutic concentration range. The steady-state unbound fraction in maternal plasma during maternal drug administration (0.35 ± 0.13; Table 5.4) appears to be somewhat higher than the range (0.05 - 0.15) observed in epileptic non-pregnant humans (Gugler and Mueller, 1978; Cramer et al., 1986; Scheyer et al., 1990). However, the observed pregnant sheep plasma unbound fraction is in reasonable agreement with that measured in serum obtained from pregnant mothers at birth (0.27 ± 0.06) (Nau et al., 1984). It has been previously demonstrated that unbound fractions of VPA are up to 50% higher in pregnant women during the last few weeks of gestation compared to early pregnancy and controls (Riva et al., 1984; Nau and Krauer, 1986). This pregnancy-related increase in VPA unbound fraction appears to be due to lower plasma albumin concentrations and also possibly due to increased free fatty acid plasma concentrations during pregnancy (Nau et al., 1984; Riva et al., 1984; Nau and Krauer, 1986). Albumin is the major binding protein for VPA in plasma (Kober et al., 1980), and free fatty acids, due to their similar structure, may displace VPA from its binding sites (Nau et al., 1984; Nau and Krauer, 1986). Free fatty acid levels further increase during labor and at birth, and this may lead to an additional decrease in maternal plasma protein binding of the
drug to values lower than those in the fetus (Nau et al., 1984). Thus, in humans, binding of VPA in umbilical cord serum exceeds that in the maternal serum at birth, leading to fetal accumulation of the drug at birth (Nau et al., 1984; Nau and Krauer, 1986; Takeda et al., 1992). Approximately equal maximal VPA binding capacity of maternal and fetal sheep plasma a few days prior to delivery appears to be in line with the above data in humans.

5.3.2 Placental and Non-Placental Clearances of VPA in the Mother and the Fetus

As discussed in earlier chapters, a 2-compartment model of the maternal-fetal unit is most commonly employed to study maternal-fetal drug pharmacokinetics via the estimation of placental and non-placental clearances of the mother and the fetus (Szeto et al., 1982a; Szeto et al., 1982b; Wang et al., 1986a; Riggs et al., 1990; Yoo et al., 1993; Kumar et al., 1997). The estimates of maternal and fetal placental clearances provide a useful means to evaluate the placental permeability of the drug in question, whereas non-placental clearance values can be used to compare maternal and fetal non-placental drug elimination capacity (e.g., hepatic and renal elimination). The existence of the phenomenon of saturable plasma protein binding presented an interesting problem in the estimation of these clearance parameters for VPA. The above 2-compartment model is based on separate steady-state maternal and fetal drug administration coupled with the measurement of maternal and fetal plasma concentrations of the drug during both experiments. The model assumes linear pharmacokinetics and drug elimination from both fetal and maternal compartments. Further, it is assumed that the pharmacokinetics of the drug do not change significantly between the two experimental periods.
VPA is a low clearance drug with clearance values much lower than hepatic blood flow (Levy and Shen, 1995). Thus, systemic clearance of VPA can be approximated by the product of its plasma unbound fraction and unbound drug intrinsic clearance, i.e., \( CL = f_u \cdot CL_{int} \) (Wilkinson and Shand, 1975). Due to the phenomenon of saturable plasma protein binding, the lower maternal plasma concentrations of the drug during fetal VPA infusion resulted in a significantly lower maternal plasma unbound fraction compared to that during maternal infusion (0.18 ± 0.08 vs. 0.35 ± 0.13, respectively, Table 5.4). Thus, maternal clearance of the drug would actually be different during the 2 experimental periods, as predicted by the above relationship. This violates the basic assumption of the 2-compartment model and hence results in systematically negative estimates of \( CL_f \), if total maternal and fetal VPA steady-state concentrations are used to calculate these clearances (Table 5.6). To clearly understand this phenomenon, it is necessary to briefly consider the basic mathematical steps of the 2-compartment model as outlined below:

During maternal infusion, maternal total clearance is calculated as a function of maternal infusion rate and steady-state maternal plasma concentrations (\( C_m \)):

\[
CL_{mm} = f (k_0, C_m) \quad (11)
\]

Similarly, during fetal infusion, fetal total clearance is calculated as a function of fetal infusion rate and fetal steady-state plasma concentrations (\( C_f \)):

\[
CL_{ff} = f (k'_0, C_f) \quad (12)
\]
The rate of placental drug transfer to the mother during fetal drug administration ($R_{fm}$) is then calculated by the product of $CL_{mm}$ (estimated above during maternal experiments) and the maternal steady-state plasma concentration of the drug during fetal administration ($C_m^\prime$):

$$R_{fm} = CL_{mm} \times C_m^\prime \quad (13)$$

The rate of non-placental fetal drug elimination ($R_{fo}$) therefore equals the difference between fetal infusion rate and $R_{fm}$:

$$R_{fo} = k_0^\prime - R_{fm} \quad (14)$$

Since $CL_{mm}$ is higher during maternal experiments (because of a higher maternal plasma unbound fraction) compared to that during fetal experiments, the use of equation 13 actually overestimates the rate of fetal placental elimination. This eventually results in underestimation of the rate of fetal non-placental elimination using equation 14. Due to a large change in maternal plasma unbound fraction of VPA during maternal and fetal VPA infusions (~50%), the above $R_{fo}$ underestimation is so great that it results in negative estimates of $CL_{fo}$ (Table 5.6). The above mathematical description also clearly shows that like $CL_{fo}$, $CL_{fm}$ estimates presented in Table 5.6 are also inaccurate (equation 13). Similarly, it can be shown that if fetal plasma protein binding of the drug changes during maternal and fetal infusion experiments (as it did in our experiments, although not significantly, Table 5.4), inaccurate estimates of $CL_{mo}$ and $CL_{mf}$ will be obtained.
Due to the confounding effects of saturable plasma protein binding on total drug concentrations, it is essential that steady-state unbound plasma concentrations be used for the estimation of maternal and fetal placental and non-placental drug clearances. Table 5.7 presents the net, total, placental and non-placental clearances of the unbound drug in the mother and the fetus. The net maternal and fetal clearance estimates are lower compared to the corresponding 2-compartment estimates of total maternal or fetal clearance. This is because the net clearances are related to the corresponding 2-compartment total clearances as:

$$CL_{m(\text{net})} C_m = CL_{mm} C_m - CL_{fm} C_f$$ \hspace{1cm} (15)

and,

$$CL_{f(\text{net})} C_f' = CL_{ff} C_f' - CL_{mf} C_m'$$ \hspace{1cm} (16)

The placental clearance of the unbound VPA in the two directions is similar (mother to fetus: $69.0 \pm 20.2$ vs. fetus to mother: $61.9 \pm 24.2 \text{ ml/min/kg estimated fetal weight}$). With the exception of acetaminophen, this is in contrast to all other drugs studied in pregnant sheep where $CL_{fm}$ estimates are significantly higher than the corresponding $CL_{mf}$ estimates. Previously, we have demonstrated that for DPHM, a significant fraction of the drug (~44% on average) injected into the umbilical vein is taken up by the fetal liver before it reaches the fetal systemic circulation (Chapter 3). This fetal hepatic first-pass drug uptake also occurs with DPHM transferred from the maternal circulation across the placenta and into the umbilical vein, and eventually leads to a proportional underestimation of the $CL_{mf}$ clearance parameter for DPHM and possibly for a number
of other drugs (see Chapter 3). Since VPA is a low clearance drug, a significant fetal hepatic first-pass extraction of the drug would not be expected and hence accurate estimates of $CL_{mf}$ clearance are likely obtained. Also, the equal magnitude of placental clearance in both directions across the placenta indicates a passive diffusion mechanism for placental transfer of VPA in sheep.

The pregnant sheep placental clearance of unbound VPA is lower compared to that of DPHM (Yoo et al., 1993; Kumar et al., 1997; see Chapters 3 and 4), methadone (Szeto et al., 1982b), metoclopramide (Riggs et al., 1990), and compounds with blood flow limited placental diffusion (e.g., antipyrine and ethanol, ~200 ml/min/kg) (Wilkening et al., 1982); however, it is greater compared to morphine (Szeto et al., 1982b), labetalol (Yeleswaram et al., 1993) and acetaminophen (Wang et al., 1986a). This indicates an intermediate placental permeability for VPA in this species. Molecular size (weight) of VPA is smaller relative to all of the above compounds, except ethanol, and hence its intermediate placental clearance is likely related to its polarity (octanol/water partition coefficient 398) and high degree of ionization at the physiological pH (pKa 4.8).

The clearance data in Table 5.7 also indicate that fetal total and non-placental clearances are larger compared to the corresponding maternal clearances. The 2-compartment total fetal clearances of all compounds studied in pregnant sheep (DPHM, metoclopramide, labetalol, acetaminophen, morphine, methadone) are larger compared to maternal total clearances. Also, fetal non-placental clearance of DPHM and methadone is greater compared to the corresponding maternal value. The $CL_{fo}$ of unbound VPA, although consistent with significantly lower fetal plasma unbound concentrations compared to the mother during maternal drug administration (Table 5.2),
appears to be remarkably high. This is especially conspicuous considering the fact that total body clearance of unbound VPA in 1-day old newborn lambs was much lower compared to the mother (Table 5.12).

One limitation of the 2-compartment model is that the uptake/metabolism of the drug by the placenta, if present, is calculated as part of the CL_{fo} (and CL_{mo}) estimates (Wang et al., 1986a). VPA, due to its branched-chain fatty acid structure, is known to enter fatty-acid β-oxidation pathways (Baillie and Sheffels, 1995). A number of anabolic and catabolic intermediary lipid metabolism pathways are known to exist in the placenta of sheep and many other species (Coleman, 1989). These include triglyceride synthesis, fatty acid chain elongation, desaturation and partial as well as complete oxidation to CO_{2} (Diamant et al., 1980; Thomas et al., 1985; Coleman, 1989). Moreover it has been demonstrated that keto-acid compounds (e.g., acetoacetate and β-hydroxybutyrate), that are structurally similar to VPA, are in fact utilized by the sheep placenta, albeit at very slow rates (~30 μmol/min for β-hydroxybutyrate and ~6.0 μmol/min for acetoacetate) (Carver and Hay Jr., 1995). Utilization of VPA by the placenta at a rate of 6.0 μmol/min during both maternal and fetal infusion experiments, would introduce an error of ~15.5 ml/min/kg (~150%) in CL_{fo}, and of ~0.27 ml/min/kg (~7%) in CL_{mo}. This is because the contribution of this placental VPA uptake relative to ‘true’ non-placental clearance will be much greater in the fetus in comparison to the mother. Although we do not have direct evidence for placental utilization of VPA in our experiments, this appears to be a plausible explanation for the high magnitude of CL_{fo} observed in our studies. This is true especially in light of the fact that VPA clearance in newborn lambs immediately after birth is much lower compared to the estimated CL_{fo} (Table 5.12).
In addition to saturable plasma protein binding, another factor that could confound clearance estimations using the 2-compartment model is the nonlinear (saturable) Michaelis-Menten clearance of the drug. This phenomenon, if present, will also result in differences in unbound as well as total drug clearances during maternal and fetal infusion experiments due to different plasma drug concentrations. However, we have obtained evidence that due to a high $V_{\text{max}}$ and $K_m$ of VPA metabolism in maternal sheep, the extent of this nonlinearity will be minimal (on average < 10% change in maternal clearance during maternal and fetal infusions) at the plasma concentrations encountered during the course of our experiments (see Section 5.3.6).

The effective maternal and fetal placental and non-placental clearances of the total drug were also calculated after correcting for the alterations in maternal and fetal plasma protein binding during maternal and fetal infusions (Table 5.8). All maternal clearances are essentially the product of maternal plasma unbound fraction during maternal drug infusion and the corresponding clearance of the unbound drug (e.g., $\text{CL}_{\text{mo}} = \text{Maternal plasma unbound fraction during maternal infusion} \times \text{CL}_{\text{umo}}$). Similarly, fetal clearances are obtained as a product of fetal plasma unbound fraction during fetal infusion and the corresponding fetal clearance of the unbound drug. It should be emphasized that since maternal and fetal unbound fractions change with changes in plasma concentrations of the drug, these total drug clearances are plasma concentration and hence dose-dependent. In contrast, the unbound drug clearances would be unaltered with changes in dose or plasma drug concentrations if the clearance of the drug is linear (non-saturable or non-Michaelis-Menten).
Regardless of the presence or absence of placental VPA uptake/metabolism, the non-placental clearance accounts for a larger proportion of the net clearance in the mother as compared to the fetus (79.6 ± 13.2 vs. 20.4 ± 13.2%). This is similar to all other drugs studied in pregnant sheep (Szeto et al., 1982b; Wang et al., 1986a; Riggs et al., 1990; Yoo et al., 1993; Kumar et al., 1997; also see Chapter 3) and further emphasizes the importance of the placenta in fetal drug elimination.

5.3.3 Amniotic and Fetal Tracheal Fluid Concentrations of VPA during Maternal and Fetal VPA Infusions

In all animals, VPA was detectable in amniotic and fetal tracheal fluids at the first sampling point, i.e., 5 min. During the 6-24 h period when maternal and fetal plasma VPA concentrations were at steady-state, the drug concentrations in amniotic and fetal tracheal fluids also appeared to be at an apparent steady-state. In general, tracheal fluid concentrations exhibited somewhat larger fluctuations compared to amniotic fluid. One feature of amniotic and tracheal fluid disposition of VPA is a lack of its significant accumulation in these fluids relative to maternal and fetal plasma (Table 5.9). This is in contrast to a number of amine drugs such as, metoclopramide (Riggs et al., 1987), DPHM (Riggs et al., 1987), labetalol (Yeleswaram et al., 1992), and ritodrine (Wright et al., 1991), where fetal tracheal fluid drug concentrations reach values ~4-15 times higher compared to fetal plasma. Also, for most of these amine drugs, significant amounts of the drug accumulate in amniotic fluid; the amniotic fluid concentrations then decline relatively slowly and persist for long periods after the drug can be detected in either maternal or fetal plasma. However, VPA concentrations in amniotic fluid declined relatively rapidly and in parallel with maternal and fetal plasma during the post-infusion period. During the last third of gestation, fetal urine is a major source of amniotic fluid.
Hence, it has been suggested that amniotic fluid accumulation of drugs may occur via their excretion in fetal urine (Szeto et al., 1979). The appearance of drugs in amniotic fluid via renal excretion by the fetal lamb has been observed for several compounds including meperidine (Szeto et al., 1979), lidocaine (Morishima et al., 1979) and ethanol (Clarke et al., 1987). However, the accumulation of meperidine (Szeto et al., 1979), ethanol (Clarke et al., 1987) and possibly morphine-3-β-D-glucuronide (Olsen et al., 1988) in amniotic fluid occurs in spite of the complete diversion of fetal urine. Thus, it has been argued that other routes, such as passage of the drug from the fetal and/or maternal systemic circulation across the chorioallantoic membranes, may also play an important role in drug transfer into and out of amniotic and allantoic fluids (Szeto et al., 1979; Rurak et al., 1991). For several reasons, this intramembranous transport appears to be the major source of VPA present in amniotic fluid. Firstly, in contrast to a number of amine compounds such as DPHM (Kumar et al., 1997), meperidine (Szeto et al., 1979), cimetidine (Mihaly et al., 1983; Czuba et al., 1990), and ranitidine (Czuba et al., 1990), fetal renal excretion of VPA is negligible (Section 5.2.8). Secondly, VPA was detectable in amniotic fluid in all animals as early as 5 min after maternal or fetal drug administration. This would be unlikely if fetal urine was the only source of this drug, especially considering the very small amounts of VPA that could be excreted in fetal urine in 5 min. Thirdly, a rapid decline of amniotic fluid VPA concentrations during the post-infusion phase, almost in parallel with maternal and fetal plasma VPA concentrations, appears to indicate a relatively rapid equilibration of amniotic fluid drug concentrations with fetal and/or maternal plasma. This could be only possible via the intramembranous route.
5.3.4 Overall Total Body Clearance, Terminal Elimination Half-Life, Mean Residence Time, and Steady-State Volume of Distribution of VPA in the Mother and the Fetus

The CL\textsuperscript{u}_{tb} (calculated as total dose/AUC\textsubscript{0-\infty}\textsuperscript{u}, Table 5.10) of VPA in the mother is slightly (<4%) but significantly greater than the steady-state net clearance of the unbound drug (CL\textsuperscript{u}_{m(net)}, Tables 5.7 and 5.10). This likely occurs because of a slight Michaelis-Menten nonlinearity in VPA pharmacokinetics. During the post-infusion phase, VPA concentrations fall, leading to a slight increase in its clearance. Thus, CL\textsuperscript{u}_{tb}, which is the overall clearance over the range of the entire plasma profile, is somewhat greater compared to CL\textsuperscript{u}_{m(net)} calculated only from steady-state concentrations (which are higher relative to the post-infusion phase). In contrast to CL\textsuperscript{u}_{tb}, CL\textsubscript{tb} (Table 5.10) is significantly lower compared to the corresponding steady-state parameter (CL\textsuperscript{m(net)}, Table 5.8). This can be explained by the phenomenon of saturable plasma protein binding. During the post-infusion phase, the lower plasma VPA concentrations lead to a decrease in unbound fraction of the drug. Thus, clearance based on total drug concentrations also falls during this phase (because for VPA, CL\textsubscript{tb} = f\textsubscript{u} \times CL\textsuperscript{u}_{int}) and hence the resulting overall clearance (CL\textsubscript{tb}) is lower compared to that at steady-state. In contrast to the mother, the fetal CL\textsuperscript{u}_{tb} (Table 5.11) was not significantly different from CL\textsuperscript{u}_{f(net)} (Table 5.7). This could be due to the fact that the majority of fetal VPA clearance is comprised of placental elimination, which may not exhibit Michaelis-Menten type nonlinearity. Similar to the mother, however, fetal CL\textsubscript{tb} appears to be lower than CL\textsubscript{f(net)} (but not significantly, p = 0.05, Tables 5.8 and 5.11) possibly due to the phenomenon of saturable plasma protein binding.
The clearances of the unbound as well as total VPA in maternal sheep appear to be higher compared to adult humans. In adult humans on mono- or polytherapy, unbound and total clearance estimates range from 1.0 – 3.0, and 0.1 – 0.3 ml/min/kg, respectively (Perucca et al., 1978b; Gugler et al., 1977; Bowdle et al., 1980; Bialer et al., 1985; Schapel et al., 1980; Hoffman et al., 1981), with clearance being generally higher in epileptic patients on polytherapy. The observation of a higher VPA clearance in pregnant sheep compared to the human is similar to many other drugs, such as DPHM (Yoo et al., 1993), metoclopramide (Riggs et al., 1988) and acetaminophen (Wang et al., 1986a).

The apparent terminal elimination half-life of VPA ($t_{1/2p}$) in sheep based on unbound or total drug concentrations is much shorter compared to that observed in non-pregnant and pregnant humans (9-18 h) (Nau et al., 1982; Zaccara et al., 1988; Davis et al., 1994; Perucca et al., 1978a and b; Gugler et al., 1977; Bowdle et al., 1980; Bialer et al., 1985; Schapel et al., 1980; Hoffman et al., 1981). This may also be related to higher VPA clearance (but similar Vd, see below) in sheep compared to humans, as discussed above. The apparent fetal $t_{1/2p}$ based on unbound drug concentrations was shorter compared to the mother but that based on total drug concentrations was not significantly different. This is in contrast to metoclopramide (Riggs et al., 1988) and labetalol (Yeleswaram et al., 1992) where fetal $t_{1/2p}$ is longer compared to the mother in pregnant sheep. Maternal apparent $t_{1/2p}$'s of the unbound and total drug appear to be similar (Table 5.10), whereas the fetal apparent $t_{1/2p}$ of the unbound drug is significantly shorter compared to that of the total drug (Table 5.11). A number of theoretical analyses have shown that clearance, volume of distribution and half-life relationships for
drugs with saturable plasma protein binding are very complex (McNamara et al., 1979a; McNamara et al., 1979b; Øie et al., 1980). VPA appears to fit into the model of a drug with saturable binding to plasma and extracellular proteins because it has a very limited tissue distribution (see below). It has been shown that for drugs with such characteristics, apparent volume of distribution (Vd) of the total drug continuously decreases and that of the unbound drug increases with a fall in plasma drug concentrations (Øie et al., 1980). Also, these changes in Vd are proportional to the initial values for this parameter, being high for drugs with a high Vd and small for drugs with a small Vd. The result of these Vd alterations is that the total plasma concentration vs. time profile shows a subtle convexity, whereas the unbound concentration vs. time profile shows a concavity (Øie et al., 1980). These different shapes of total and unbound concentration vs. time profiles may lead to different estimates for apparent elimination half-life of the total and unbound drug. In our experiments, the above phenomenon appeared to be more pronounced in the fetus, likely due to a larger fetal Vd of VPA compared to the mother (Vd_\text{ss}' = 0.24 ± 0.10 vs. 1.78 ± 0.61 L/kg for the mother and fetus, respectively; Tables 5.10 and 5.11, also see below). This phenomenon is likely responsible for different estimates of fetal t_{1/2\beta} of the unbound and total drug in our studies.

By definition, the steady-state volume of distribution (Vd_\text{ss}) is a proportionality constant relating the amount of drug in the body at steady-state to its steady-state plasma concentration (Gibaldi and Perrier, 1982). For drugs with linear plasma protein binding, Vd_\text{ss} is a constant drug-specific parameter that describes the steady-state distribution of the drug. The computation of a meaningful Vd_\text{ss} parameter for drugs with saturable plasma protein binding is, however, much more complex; in these situations, Vd_\text{ss} is a
concentration-, and hence, time- and dose-dependent parameter. McNamara et al. (1983) discussed the properties of various Vd\(_{ss}\) terms in describing the distribution of drugs with saturable plasma protein binding. By way of computer simulations, it was demonstrated that Vd\(_{ss}^u\) (Vd\(_{ss}\) of the unbound drug), calculated using equation (6), is a valid pharmacokinetic parameter for describing the distribution of this group of drugs. However, unlike the clearance of the unbound drug, this parameter is unbound fraction- (and hence concentration-) dependent. The value of the calculated Vd\(_{ss}^u\) is a constant only for a particular area weighted unbound fraction of the drug (f\(_p\), calculated using equation 5). The computed value of Vd\(_{ss}^u\) at a particular f\(_p\) 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). Unlike drugs with linear plasma protein binding, the Vd\(_{ss}\) of the total drug (Vd\(_{ss}\)), calculated using equation 7 (section 5.1.8), overestimates the “true” Vd\(_{ss}\) and should not be used. Instead, Vd\(_{ss}^*\), calculated using equation (8), provides a more reliable estimate of “true” Vd\(_{ss}\). As with Vd\(_{ss}^u\) above, this Vd\(_{ss}^*\) parameter is also constant only for a particular f\(_p\) or a steady-state plasma unbound fraction equivalent to f\(_p\). Thus, all these Vd\(_{ss}\) terms must be expressed along with their corresponding f\(_p\) estimates. The f\(_p\) parameter accounts for the influence of dose and time on the fraction unbound in plasma, and thus transforms apparently dose-, time- and concentration-dependent pharmacokinetic parameters into functions of binding changes (McNamara et al., 1983).

The corrected Vd\(_{ss}^*\) of VPA in maternal sheep (0.24 ± 0.10 L/kg) appears to be similar to that estimated for humans in different studies (0.13 – 0.20 L/kg; Davis et al., 1994; Perucca et al., 1978a and b; Gugler et al., 1977; Bowdle et al., 1980; Bialer et al., 1985;
Schapel et al., 1980; Hoffman et al., 1981). This suggests that VPA is primarily confined to the systemic circulation and extracellular fluids in both humans and sheep. All the calculated fetal $V_d$ terms were much higher compared to the mother; a finding similar to DPHM (Chapter 3) and labetalol (Yeleswaram et al., 1992). Apart from the possible differences in maternal and fetal body composition, the higher fetal $V_d$ may in part be due to the fact that a significant proportion of the drug administered to the fetus rapidly distributes to the ewe via placental transfer. Hence, the calculated fetal $V_d$ terms may include a maternal component, resulting in a higher apparent fetal $V_d$. The fetal $V_d$ for VPA is, however, smaller compared to that of amine drugs such as DPHM (Chapter 3), labetalol (Yeleswaram et al., 1993) and ritodrine (Wright, 1992), indicating its less extensive distribution in fetal tissues.

5.3.5 Pharmacokinetics and Plasma Protein Binding of VPA in Newborn Lambs

In contrast to maternal and fetal experiments, the drug was infused to newborn lambs only for a period of 6 h. Long-term (24 h as in the mother and the fetus) infusions could not be conducted in lambs for a number of reasons. Firstly, VPA caused marked sedation in lambs so much so that it interfered with their feeding. Secondly, long-term infusion necessitated separation of the lambs from their mothers for at least the duration of infusion, and hence restriction of their free movement and nursing from the mother. Due to the potential confounding effects of these factors on newborn drug disposition, the infusion duration was restricted to 6 h. The pharmacokinetic behavior of VPA appears to be very different in newborn lambs compared to the mother and the fetus. In contrast to the mother and the fetus, a continuous accumulation of the drug was observed throughout the infusion duration (Figure 5.4). Also, during the post-infusion
phase, a typical convexity was evident in the plasma concentration vs. time profile of the unbound as well as total drug. The convexity in unbound plasma concentration vs. time profile was observed notwithstanding the expected concavity due to the phenomenon of saturable plasma protein binding (see above). This convexity is clearly indicative of a pronounced Michaelis-Menten drug elimination in the newborn lamb (Gibaldi and Perrier, 1982; Wagner 1993). This is in contrast to the mother where apparently linear kinetics are observed (Figure 5.1; also see Section 5.3.6).

Estimated clearances of the unbound as well as total drug in the newborn lamb are much lower compared to the mother indicating impaired VPA elimination in lambs (Tables 5.10 and 5.12). Also, newborn VPA clearances are much lower compared to the fetal total body as well as non-placental clearances (Tables 5.7, 5.8, 5.11 and 5.12). The lower newborn clearance compared to the fetal total body clearance is possibly due to the loss of the placenta at birth. As discussed above, the higher fetal non-placental clearance compared to the newborn lamb total body clearance may be due to placental utilization of "fatty acid-like" VPA. The volume of distribution terms for the newborn lamb are not significantly different compared to those of the mother (Tables 5.10 and 5.12), indicating similarities in their drug distribution characteristics. The Vd of VPA in newborn lambs is, however, much smaller compared to the fetus (Tables 5.11 and 5.12). As discussed before, the large fetal Vd for VPA and a number of other drugs is possibly due to the distribution of a significant proportion of the fetal dose into the maternal circulation.

It is interesting to note that the estimated total clearances in newborn lambs are very similar to the 0.15 – 0.48 ml/min/kg range observed in different studies in human
neonates less than a month of age (Brachet-Liermain and Demarquez, 1977; Irvine-Meek et al., 1982; Gal et al., 1988). In one study (Gal et al., 1988), clearances of the unbound drug were also measured in four human neonates and were 0.7, 1.1, 1.2 and 4.2 ml/min/kg. The newborn lamb clearances of the unbound drug also agree reasonably well with these values, in spite of the fact that different doses were administered and the kinetics appear to be of the Michaelis-Menten type. Similar to clearance, the volume of distribution of VPA in newborn lambs based on total drug concentrations (Table 5.12) is similar to that of the human neonates in the above studies (0.28 – 0.46 L/kg).

The apparent elimination half-life of the unbound as well as total drug in lambs is much longer compared to the mother or the fetus. It must be emphasized that the drugs obeying Michaelis-Menten kinetics exhibit a continuously changing apparent elimination half-life which is difficult to estimate due to the convexity in their log plasma concentration vs. time profile. Thus, t₁/₂ values presented in Table 5.12 are only approximations of the average apparent t₁/₂ observed during the entire post-infusion phase. Similar to the fetus, the t₁/₂ of the unbound drug in newborn lambs was shorter compared to that of the total drug, again likely due to the previously discussed phenomenon of saturable plasma protein binding. A number of studies have reported longer apparent elimination half-lives of VPA in human neonates compared to adults. This includes studies where neonates were exposed to the drug via direct administration for control of seizures or via persistence of the drug during the newborn period after in utero placental transfer (Nau et al., 1981; Nau et al., 1984; Gal et al., 1988; Irvine-Meek et al., 1982; Dickinson et al., 1979b; Ishizaki et al., 1981). In these studies, apparent elimination half-lives ranging from 15.1 – 80 h have been observed in
human newborns less than 1 month of age, and these appear to be much longer compared to the 9 – 18 h range observed in epileptic adults (Davis et al., 1994; Levy and Shen, 1995). Similar to the sheep and human, longer elimination half-lives of the drug have also been observed in the newborns of other species such as the rat and guinea pig (Yu et al., 1985; Yu et al., 1987; Haberer and Pollack, 1994).

The overall plasma protein binding characteristics of VPA are different in the newborn lambs compared to the mother or the fetus (Figures 5.2 and Figure 5.5). The expected relationships between the plasma concentrations of the bound and the unbound drug do not appear to exist in the newborn lamb. The mechanism of this difference between the VPA binding properties of newborn vs. maternal or fetal plasma becomes somewhat apparent when the bound and unbound plasma concentration data from newborn lambs are partitioned into those obtained on day 1 (0-24 h, Figures 5.6A-C) and those obtained after day 1 (24-96 h, Figures 5.6D-F) of the experiments. In the Rosenthal plot of the data from day 1, the bound/unbound concentration ratio is positively related to the bound concentration (Figure 5.6A). This is in contrast to the maternal and fetal situation where these variables are inversely related (Figures 5.2A and 5.2D). This means that in the newborn plasma, with an increase in unbound drug concentration, there is a more pronounced increase in bound drug concentration, thus resulting in an increase in bound/unbound concentration ratio. In other words, with an increase in total plasma concentration of VPA in the newborn lamb, the bound concentration increases more than the unbound concentration. This is in contrast to maternal and fetal plasma where the opposite situation exists, i.e., unbound concentration increases more than the bound concentration, leading to a fall in bound/unbound concentration ratio. This phenomenon could be possible if significant concentrations of some competitive VPA plasma protein
binding displacer (inhibitor) were present in the newborn lamb plasma. In this situation, an increase in VPA plasma concentration will lead to competitive displacement of the displacer from the binding sites and hence to an increased binding of VPA. This would then result in an increase in bound/unbound concentration ratio with increasing VPA concentration until all of the displacer has been displaced from the binding sites.

The plasma unbound fractions of VPA have been found to correlate inversely to some extent with plasma concentrations of free fatty acids (Nau et al., 1984; Riva et al., 1984). Hence, increased plasma free fatty acids during pregnancy and the newborn period have been suggested as potential competitive inhibitors of VPA binding to plasma proteins (Nau et al., 1984; Riva et al., 1984). It has been found in a number of studies that the newborn lamb plasma concentrations of free fatty acids rise dramatically soon after birth, and within 6 h increase almost 10 fold compared to the concentrations immediately after birth (Noble, 1980). This increase mainly results from a rapid mobilization of fatty acids from the adipose tissue stores in order to meet the energy needs of the newborn (Noble, 1980). A similar increase in plasma free fatty acid concentrations also occurs in the human newborn (Warshaw; 1979; Nau et al., 1984). Thus, this increase in plasma free fatty acids at birth may be responsible for the anomalous VPA plasma protein binding characteristics observed in our newborn lamb studies. Also, the plasma concentrations of free fatty acids begin to decline after the initial 2-3 days of life both in the lamb and the human newborn (Noble, 1980; Nau et al., 1984). In parallel with this, the VPA binding characteristics of newborn lamb plasma after day 1 of our experiments (after day 2 of life) appear to approach those of maternal and fetal plasma, with the estimates of binding parameters being similar to those of the fetus (Table 5.5).
In the current section, striking differences in the pharmacokinetics of VPA in newborn lambs as compared to the mother have been outlined. These include a significantly lower clearance, a longer apparent elimination half-life and a pronounced Michaelis-Menten type nonlinear pharmacokinetic profile. Interestingly, these differences also appear to exist in human newborns exposed to VPA either via direct neonatal administration for control of seizures or via in utero placental transfer and persistence of the drug in the neonatal circulation after birth. It was thus of interest to compare the overall VPA elimination capacity in the newborn lamb relative to the mother. Also, we felt that it would be worthwhile elucidating the underlying mechanisms of these differences. This includes possible differences in the renal elimination of VPA and functional capacity of various VPA metabolism pathways in the newborn lamb compared to maternal sheep.

5.3.6 Overall VPA Elimination Capacity in the Newborn Lamb and Maternal Sheep

For drugs undergoing nonlinear Michaelis-Menten elimination, clearance does not provide the most suitable comparison of drug elimination capacity among different populations. This is because for such drugs, clearance is plasma concentration, and hence time- and dose-dependent. The in vivo Michaelis-Menten pharmacokinetic parameters, \( V_{\text{max}} \) and \( K_m \), are the most suitable parameters for comparing the overall drug elimination capacity under such circumstances (Gibaldi and Perrier, 1982; Wagner, 1993). Since, in our studies in newborn lambs, we observed clear evidence of Michaelis-Menten elimination, we decided to compare the in vivo \( V_{\text{max}} \) and \( K_m \) parameters in the newborn and maternal sheep. However, the reliable estimation of in vivo \( V_{\text{max}} \) and \( K_m \) requires that randomized experiments be conducted in each animal at
3-4 dose levels, at least some of which exhibit pronounced saturation kinetics (Gibaldi and Perrier, 1982; Metzler and Tong, 1981). The data from all doses are then simultaneously fit to the Michaelis-Menten pharmacokinetic equations in order to estimate $V_{\text{max}}$ and $K_m$ (Metzler and Tong, 1981; Wagner, 1993). This was, however, not possible in our studies in newborn lambs. The 3-4 dose-ranging experiments, plus the appropriate washout periods in between experiments, would take a considerable amount of time (at least 20-30 days) due to the long apparent elimination half-life of VPA in the newborn lamb. In our other studies, we have observed that significant changes in VPA pharmacokinetics and metabolism begin to occur even within the initial 10 day post-natal period in lambs (Harvey Wong, Sanjeev Kumar, K. Wayne Riggs and Dan W. Rurak, unpublished data). Since our primary interest was to compare the elimination capacity in the immediate newborn period relative to the mother, we felt that these dose-ranging studies would not yield useful data for our purposes. Hence, we decided to conduct newborn studies with an infusion protocol. We felt that the pharmacokinetic profile after infusion administration would inherently contain more information, i.e., accumulation kinetics of the drug during the infusion period and convex Michaelis-Menten decline during the post-infusion phase. Thus, it may be possible to estimate the Michaelis-Menten pharmacokinetic parameters in a single experiment with a reasonable degree of confidence.

Similar to the newborn lambs, it was also not possible to conduct dose-ranging experiments in maternal sheep due to the limited time window available during which experiments can be conducted in the late-gestational pregnant sheep preparation before labor and delivery (maximum 2 weeks). However, in contrast to the lambs, the unbound as well as total VPA plasma concentrations appeared to exhibit the
characteristics of a linear pharmacokinetic profile in the mother, i.e., rapid achievement of steady-state with no apparent drug accumulation, and log-linear post-infusion decline in plasma concentrations. The independent and reliable estimation of $V_{\text{max}}$ and $K_m$ from such a pharmacokinetic profile is not possible. Hence, we followed a different experimental approach for the estimation of maternal Michaelis-Menten pharmacokinetic parameters. We have found that VPA glucuronidation is the major pathway of VPA elimination in maternal sheep and accounts for 77.0 ± 7.8% (range 69-87%; Table 5.21) of the total VPA dose. In recent years, considerable evidence has been gathered suggesting that the \textit{in vitro} microsomal estimates of $V_{\text{max}}$ and $K_m$ may be representative of their \textit{in vivo} values and can be successfully used to predict the \textit{in vivo} pharmacokinetics of a number of drugs (Houston 1994; Iwatsubo \textit{et al}., 1996; Iwatsubo \textit{et al}., 1997; Lave \textit{et al}., 1997; Obach \textit{et al}., 1997). Thus, we reasoned that the \textit{in vitro} estimate of the $K_m$ of VPA glucuronidation would likely approximate the $K_m$ of \textit{in vivo} VPA elimination. Also, this estimate should be similar in different sheep because the same enzyme is likely involved in VPA glucuronidation in different sheep and this enzyme is present in a similar biological milieu. However, the \textit{in vivo} $V_{\text{max}}$ is more likely to differ among different sheep because of a host of genetic and environmental factors. Thus, we decided to use the \textit{in vitro} estimate of $K_m$ in Michaelis-Menten pharmacokinetic modeling of the unbound VPA pharmacokinetic data in maternal sheep. The $K_m$ value was fixed at the \textit{in vitro} estimate of 363.4 µg/ml, and the $V_{\text{max}}$ parameter in different sheep was estimated by model fitting.

The \textit{in vitro} pooled microsomal estimate of $V_{\text{max}}$ (15.2 nmol/min/mg microsomal protein or 2.19 µg/min/mg microsomal protein) indicates that glucuronidation is a high capacity pathway in the mother and supports its major role in VPA clearance. Similarly, a high
$K_m$ of VPA glucuronidation (2.52 mM or 363.4 µg/ml) relative to maternal unbound VPA concentration range encountered in our experiments (average < 50 µg/ml, Table 5.2) indicates that this pathway will not be easily saturable at these plasma concentrations. This may explain the apparently linear pharmacokinetic profiles of VPA in the mother.

The one-compartment Michaelis-Menten model fits to the observed data were adequate in the newborn lamb as well as the mother (Figures 5.8A and 5.8B). In the lamb, the accumulation of the drug during the infusion phase, and apparent convexity in the pharmacokinetic profile during the post-infusion phase was well predicted by the proposed Michaelis-Menten model (Figure 5.8A).

Table 5.13 presents the estimated *in vivo* values of $V_{\text{max}}$ and $K_m$ in maternal sheep and newborn lambs. All parameters are estimated with a reasonably good degree of confidence (relatively low CV's) indicating the validity of our *in vivo* Michaelis-Menten analyses. Also, if the *in vitro* $V_{\text{max}}$ of VPA glucuronidation (2.19 µg/min/mg microsomal protein) is scaled up to the whole body level using the most widely accepted values of the scaling factors, *i.e.*, 45 mg microsomal protein/g of liver (Houston, 1994; Obach *et al.*, 1997) and an adult sheep liver weight equivalent to 1.65% of total body weight (Boxenbaum, 1980), a value of 1626.1 µg/min/kg body weight is obtained. This scaled-up *in vitro* $V_{\text{max}}$ value is in reasonable agreement with the average *in vivo* value estimated in five maternal sheep (*i.e.*, 2207.1 ± 1075.3 µg/min/kg body weight; Table 5.13), especially considering the fact that the latter value includes ~20% contribution from maternal renal elimination of the unchanged drug (see below). This again verifies the validity of the use of the *in vitro* $K_m$ mean value in the estimation of *in vivo* $V_{\text{max}}$. 
The weight-normalized estimates of $V_{\text{max}}$ in all four newborn lambs are much lower compared to the mother, with the average being ~18 fold lower than the mother. These data are in agreement with the lower clearances and apparent Michaelis-Menten saturable pharmacokinetic profile of VPA observed in newborn lambs compared to the higher clearances and apparent linear pharmacokinetic profiles in adult maternal sheep. The estimated $K_m$ values in the lambs range from 66.9 – 304.3 µg/ml and appear to be lower than the *in vitro* estimate in the mother (363.4 µg/ml). We have found that the metabolic profile of VPA is quantitatively different in newborn lambs compared to the mother. In the lamb, the β-oxidation and hydroxylation pathways account for a larger proportion of VPA clearance as compared to the mother (see below). In contrast, VPA glucuronidation appears to be impaired in newborn lambs and accounts for a lesser percentage of VPA clearance compared to the mother (see below). Since, the *in vivo* $K_m$ is a hybrid parameter of all elimination pathways, the differences in newborn lamb and maternal sheep $K_m$ estimates may be due to the quantitative differences in their metabolic routes. In this regard, it is interesting to note that in the lamb NL2243(1), the $K_m$ estimate is higher and more closer to adult values compared to NL4241 and NL0123z; in NL2243(1), a larger percentage of the VPA dose is also excreted as its glucuronide (38.9%) compared to NL4241 (12.4%) and NL0123z (25.9%) (Table 5.23).

### 5.3.7 Pharmacokinetics of the Renal Excretion of Unchanged VPA in the Mother, Fetus and the Newborn Lamb

The renal clearances of the unbound as well as total VPA are much lower in the fetus as compared to adult sheep. This is in agreement with the negligible renal excretion of a number of acidic compounds such as indomethacin (Krishna *et al.*, 1995), diphenylmethoxyacetic acid (DPHM metabolite; Kumar *et al.*, 1997; also see Chapter 3)
and para-aminobipirinate (Elbourne et al., 1990) in the fetal lamb, as discussed earlier (Chapter 3). This limited fetal VPA renal excretion ability compared to adult sheep also appears to exist in the immediate newborn period, with the average weight-normalized unbound and total newborn lamb renal clearances being ~10 fold lower compared to the mother (Tables 5.14 and 5.15). Although the neonatal renal clearances of VPA appear to be somewhat higher compared to the fetus, a meaningful statistical comparison cannot be performed due to a low n value, high inter-animal variability, and relatively small differences in these parameters.

In the four animals, where maternal renal clearance data were available during both maternal and fetal administration, the $\text{CL}_u$ was similar during maternal and fetal experiments, in spite of the fact that the maternal plasma concentrations of the unbound drug differed ~5-8 fold during these experiments. This indicates an apparent linearity of the renal excretion pathway within this range of VPA concentrations in maternal sheep. In these four animals, the $\text{CL}_r$ estimate, however, was significantly lower during fetal infusion experiments compared to that during maternal administration. This is presumably related to a lower maternal plasma unbound fraction of the drug during fetal infusion due to lower total plasma concentrations. In the three animals, where fetal renal clearance data were available during both maternal as well as fetal drug administration, the fetal renal clearances of the total or unbound drug appear to be similar; statistical analysis of these data, however, are not possible due to a low n value. Renal excretion of the unchanged VPA accounts for ~20% (range 12.6 – 27.3%) of the total VPA dose in the mother (during maternal administration) and ~11% (range 5.6 – 19.2%) in newborn lambs (Tables 5.14 and 5.15). This is somewhat higher compared to humans, where in one study, on average 1.8% and 3.2% of the total VPA dose was
excreted unchanged in urine during single dose and steady-state administration, respectively (Gugler et al., 1977). Also, the renal clearance of the unbound drug in humans is much lower compared to sheep (0.03 – 0.06 ml/min/kg; Levy and Shen, 1995). Thus, the differences between maternal sheep and human VPA renal clearance may be related either to a reduced tubular reabsorption or increased tubular secretion of the drug in sheep. In the four animals, where data were available during both maternal and fetal experiments, the percentage of the total VPA dose excreted unchanged in maternal urine during fetal drug administration was significantly lower compared to that during maternal experiments (13.3 ± 5.2 vs. 19.9 ± 6.5%; Table 5.14). Although the exact reasons for this remain unclear, especially considering the fact that maternal CL\textsuperscript{u}, was similar during the two experimental periods, fetal and/or placental metabolism/utilization of a portion of the fetal dose is a likely possibility.

### 5.3.8 Maternal, Fetal and Newborn Metabolism of VPA

Our overall objective was to compare the \textit{in vivo} functional capacity of different VPA metabolism pathways and their quantitative roles in the \textit{in vivo} clearance of VPA in maternal, fetal and newborn sheep. Hence, we studied the plasma and urine pharmacokinetics of different VPA metabolites in the mother, fetus and newborn.

#### 5.3.8.1 Maternal and Fetal Plasma Concentrations of VPA Metabolites

Identification of the fetal ability to metabolize drugs \textit{in utero} is a difficult task. This is because many of the metabolites formed in the maternal circulation can easily transfer across the placenta and hence resolution of the fetal metabolite formation becomes
difficult. However, relative maternal and fetal plasma concentrations of drug metabolites after maternal and fetal drug administration can sometimes be used to detect *in utero* fetal drug metabolism. Using this approach, we have identified the ability of the fetal lamb to metabolize DPHM to DPMA and DPHM-N-oxide *in utero* (see Chapter 3). However, this approach would be useful only in situations where linear pharmacokinetics are existent, and the fetal ability to form the metabolite is significant, being somewhat comparable to that of the mother. Also, ideally, maternal and fetal plasma unbound concentrations of the metabolites should be compared because total plasma concentrations may be highly dependent on the relative extent of protein binding of the metabolite in maternal and fetal plasma (Nau *et al.*, 1984). Since, VPA kinetics are very complex, with possible nonlinearities due to the phenomena of saturable plasma protein binding and nonlinear Michaelis-Menten clearance, this approach is less likely to provide reliable detection of fetal drug metabolism. However, since some VPA metabolites have been implicated in rare VPA-induced hepatotoxicity (*e.g.*, 4-ene VPA) and its pharmacological effects (*e.g.*, 2-ene VPA), we felt it was relevant to measure the extent of fetal exposure to different VPA metabolites during steady-state drug administration. Hence, we measured the plasma concentrations of various VPA metabolites during both maternal and fetal drug infusion experiments.

Similar to the human (Baillie and Sheffels, 1995), a number of metabolites of VPA were detectable in maternal and fetal plasma during these experiments. These include the products of mitochondrial fatty acid β-oxidation ((E)-2-ene, (E)-3-ene, 3-keto and possibly 3-OH VPA) as well as of microsomal cytochrome P-450 mediated pathways (4-ene, 4-keto, 3-OH, 4-OH, and 5-OH VPA, and 2-PSA and 2-PGA). All of the detected metabolites, except (E)-2-ene and 3-keto VPA, appeared to be at an apparent steady-
state in maternal and fetal sheep plasma during the 20-24 h period of VPA infusion (Figures 5.10 - 5.13). The steady-state maternal plasma concentrations of a number of these metabolites during maternal drug administration were within the range encountered in human adult epileptics. These include, 4-ene VPA (0.36 ± 0.34 vs. trace - 0.64 µg/ml; sheep vs. human), 4-keto VPA (0.22 ± 0.10 vs. trace - 4.50 µg/ml), 4-OH VPA (3.3 ± 2.6 vs. trace – 2.97 µg/ml), 5-OH VPA (0.73 ± 0.67 vs. trace – 1.06 µg/ml), and 2-PGA (0.14 ± 0.08 vs. trace – 0.22 µg/ml) (Rettenmeier et al., 1989; Kassahun et al., 1990).

After maternal drug infusion, the maternal sheep plasma concentration of the (E)-2-ene VPA metabolite (0.54 ± 0.07 µg/ml during the 20-24 h period of infusion) was near the low end of the range encountered in human adults (0.55 – 4.66 µg/ml; Rettenmeier et al., 1989; Kassahun et al., 1990). Also, the maternal plasma concentrations of the other two metabolites formed via the β-oxidation pathway, i.e., (E)-3-ene and 3-keto VPA, appear to be lower compared to the range observed in human patients on VPA therapy (0.11 ± 0.03 vs. 0.41-1.68 µg/ml for (E)-3-ene VPA; 0.38 ± 0.21 vs. 2.26-14.7 µg/ml for 3-keto VPA) (Rettenmeier et al., 1989; Kassahun et al., 1990). It must be emphasized that these lower plasma concentrations of the β-oxidation metabolites in sheep are observed at VPA plasma concentrations similar to those in human epileptic patients (Rettenmeier et al., 1989; Kassahun et al., 1990; Baillie and Sheffles, 1995). These lower plasma concentrations of the β-oxidation VPA metabolites in sheep, compared to the human, may be related to inter-species differences in the formation or elimination kinetics of these metabolites or to the differences in VPA metabolism during chronic oral dosing in humans vs. a relatively short-term infusion protocol in sheep. However, it has
also been suggested that pregnancy is associated with a significantly reduced fatty acid β-oxidation capacity, especially during the later part of gestation, possibly due to the effects of elevated estrogenic and progestative steroids (Grimbert et al., 1993; Grimbert et al., 1995). Thus, these pregnancy-related alterations in fatty acid β-oxidation capacity may be responsible for lower plasma concentrations of the β-oxidation VPA metabolites observed in maternal sheep. In agreement with this, higher plasma concentrations of these β-oxidation VPA metabolites have been observed during preliminary studies in non-pregnant sheep in our lab (Harvey Wong, Sanjeev Kumar, K. Wayne Riggs and Dan W. Rurak, unpublished data). Also, at least one report in humans showed lower maternal serum concentrations of (E)-2-ene, (E)-3-ene and 3-OH VPA during the early second trimester compared to the late first-trimester of pregnancy, providing evidence of reduced valproate β-oxidation with advancing gestation (Omtzigt et al., 1992). The pregnancy-related reductions in VPA β-oxidation may be related either to a reduced β-oxidation capacity or to a competition for β-oxidation pathways between VPA and increased maternal plasma free fatty acids during pregnancy (Omtzigt et al., 1992). In another study, the maternal serum concentrations of (E)-2-ene and 3-keto VPA metabolites in 6 pregnant women at birth were 0.99 ± 0.59 µg/ml (range 0.43-1.95) and 4.2 ± 3.6 µg/ml (range 1.2 - 11.2) (Nau et al., 1981). Unfortunately, more detailed data on VPA metabolism during the last part of gestation in humans are not available.

In contrast to the human, the di-unsaturated VPA metabolites (e.g., (E,E)-2,3-diene VPA, and (E)-2,4, diene VPA) were either absent or present only in trace amounts in maternal and fetal plasma. Also, the 3-OH VPA metabolite was below the limit of
quantitation of our assay (0.1 μg/ml) in contrast to the human, where significant plasma concentrations of this metabolite are observed (Rettenmeier et al., 1989; Kassahun et al., 1990). Similar to the human, the 2-PSA metabolite was present only in trace amounts in maternal plasma and was below the limit of quantitation (< 0.0125 μg/ml) in all but two animals where steady-state concentrations were 0.05 (E4241) and 0.02 (E5108) μg/ml.

All of the above metabolites were also detected in maternal and fetal plasma during fetal drug infusion (Table 5.17, Figures 5.10 – 5.13). The maternal plasma concentrations of the β-oxidation products during the 20-24 h period of fetal infusion were similar to those measured during maternal drug infusion; (E)-2-ene VPA (0.54 ± 0.07 vs. 0.53 ± 0.11 μg/ml during maternal and fetal infusions, respectively), (E)-3-ene VPA (0.11 ± 0.03 vs. 0.08 ± 0.04 μg/ml) and 3-keto VPA (0.38 ± 0.21 vs. 0.24 ± 0.14 μg/ml). This occurred in spite of ~5-7 fold lower maternal plasma unbound VPA concentrations during fetal infusion experiments and may indicate saturation of the β-oxidation pathway at these concentrations, as has been suggested earlier in humans (Granneman et al., 1984a). However, the maternal plasma concentrations of VPA metabolites formed via the cytochrome P-450 pathways (4-ene, 4-keto, 4-OH, and 5-OH VPA, and 2-PGA) were proportionately lower during fetal infusion experiments due to the lower unbound VPA maternal plasma concentrations, indicating an overall linearity of these pathways. This observation also appears to be similar to humans (Granneman et al., 1984a).
During the post-infusion phase, maternal plasma concentrations of all VPA metabolites, except (E)-2-ene and 3-keto VPA, declined relatively rapidly and were below the LOQ of the assay within 12-24 h after the end of infusion in most animals. However, maternal plasma concentrations of the 3-keto and particularly the (E)-2-ene VPA metabolite still appeared to be increasing at the end of the infusion. The plasma concentrations of 3-keto VPA began declining within 1-6 h after the end of the infusion. However, the plasma concentrations of (E)-2-ene VPA stayed at an approximately constant level or increased slightly for ~12 h after the end of infusion and then started declining. These 2 metabolites were detectable in maternal plasma for 36-72 h after the end of VPA infusion. The prolonged persistence of these two metabolites does not appear to be related to their slow elimination because their plasma concentrations declined relatively rapidly after the beginning of the decline phase (Figure 5.10). It may, however, be related to the apparent saturation of the β-oxidation pathway such that this pathway continues to operate at its maximal (saturation) capacity for a prolonged period of time until VPA plasma concentrations have fallen to very low levels.

Fetal exposure to VPA metabolites appears to be high, with a number of metabolites detected at relatively equivalent ((E)-2-ene, (E)-3-ene and 4-OH VPA) or higher (3-keto and 5-OH VPA) concentrations in fetal plasma relative to the mother during the final 4 h period of VPA infusions (Tables 5.16 and 5.17). However, the fetal plasma concentrations of 4-ene VPA, 4-keto VPA and 2-PGA appear to be lower compared to the mother (Tables 5.16 and 5.17). Fetal plasma concentrations of the 3-keto and 5-OH VPA metabolites increased steadily during the infusion and were higher compared to maternal plasma concentrations during the majority of the experimental period (Figures 5.10 and 5.13). Also, in contrast to the mother, the fetal plasma concentrations of 3-
keto VPA increased continuously and significantly for ~12 h after the end of infusion. Similar to the mother, however, the fetal plasma (E)-2-ene concentrations stayed at a constant level or increased slightly for ~12 h after the end of infusion. As discussed above, these prolonged high plasma concentrations of β-oxidation products may be related to the saturation of this pathway at the experimental VPA concentrations, thus leading to the operation of this pathway at or near its maximal capacity until VPA concentrations have fallen to a very low level. The longer residence and much higher concentrations of 3-keto VPA in fetal plasma compared to the mother may be related to its increased formation or reduced elimination in the fetus.

In relation to the above data, it is interesting to note that higher concentrations of these β-oxidation VPA metabolites compared to maternal serum are also found in cord serum samples obtained at birth from epileptic mothers (Nau et al., 1981; Nau et al., 1984; Kondo et al., 1987). A number of mechanisms, based on the fetus acting as a deep compartment, active transport of metabolites across the placenta, and maternal and fetal differences in plasma protein binding of these compounds, have been proposed in order to explain the apparent fetal accumulation of these metabolites (Nau et al., 1981; Nau et al., 1984). However, our data provide strong evidence that the apparent fetal accumulation of these metabolites may result from increased fetal metabolism of VPA via these pathways (see below).

As discussed before, the relative maternal and fetal plasma concentrations of drug metabolites are dependent on comparative maternal and fetal metabolite formation capacity and plasma protein binding of the metabolite. In order to circumvent the effects of plasma protein binding on the maternal-fetal total plasma concentrations of
these metabolites, we did attempt to measure their unbound plasma concentrations. However, in many cases the unbound concentrations were below or near the LOQ of the assay and could not be reliably measured, and hence detailed data on plasma protein binding of VPA metabolites are not presented. The maternal and fetal plasma protein binding of the 3-keto and 5-OH VPA metabolites was low (unbound fractions >50-90%), and similar to the total concentrations, the fetal unbound plasma concentrations of these two metabolites were higher compared to the mother. This provides strong evidence of fetal formation of these metabolites. Similarly, the fetal unbound plasma concentrations of the 4-OH VPA metabolite also appeared to be higher compared to the mother during the post-infusion phase, again indicating that the fetus may be capable of forming this metabolite in utero. The unbound plasma concentrations of the other prominent \(\beta\)-oxidation metabolite, (E)-2-ene VPA in maternal and fetal plasma could not be measured due to its very high plasma protein binding (>95%). The unbound plasma concentrations of other VPA metabolites (4-ene, 4-keto, and (E)-3-ene VPA, and 2-PGA) appeared to be higher in maternal plasma compared to the fetus during maternal as well as fetal drug administration, indicating that the fetus may or may not be able to form these metabolites.

### 5.3.8.2 Plasma Concentrations of VPA Metabolites in Newborn Lambs, and Fetal and Newborn Metabolite Exposure Relative to the Mother

As discussed above, clear evidence of in utero fetal formation of a number of VPA metabolites could not be obtained due to their low unbound plasma concentrations and possible rapid placental transfer of the metabolites formed in the mother. Also, if the fetal ability to form a particular metabolite is relatively low compared to the mother, the above approach may not yield clear identification of in utero fetal drug metabolism.
Hence, we measured the concentrations of VPA metabolites in plasma obtained from newborn lambs. Most of the metabolites detected in maternal plasma during pregnant sheep experiments were also detectable in significant concentrations in newborn lamb plasma, indicating that these pathways are likely functional in the late-gestational fetus as well. Although, VPA did not reach steady-state in newborn lambs during the experimental period, the observed plasma concentrations of VPA were either higher than or within the range observed in the mother and the fetus during pregnant sheep experiments (Tables 5.16 – 5.20). Thus, valid comparisons of metabolite concentrations can be made between the mother, fetus and the newborn in order to assess the relative maternal, fetal and newborn exposure to these metabolites. In addition, the newborn ability to form these metabolites can be clearly identified and compared to that of the mother.

Similar to the mother, the plasma concentrations of the 2-PSA and 3-OH VPA metabolites were below the LOQ of the assay in newborn lamb plasma. The newborn lamb plasma concentrations of the 2-PGA metabolite were also below the LOQ. This is in contrast to the mother where measurable plasma concentrations of this metabolite were present (during the maternal infusion experiments), and may indicate a lower newborn capacity to form this metabolite. None of the metabolites detected in measurable concentrations in newborn plasma reached steady-state during the 6 h infusion period. Instead plasma concentrations of the majority of these metabolites increased continuously for prolonged periods of time after the end of infusion, with \( C_{\text{max}} \)'s occurring 0.5 to 54 h after the end of infusion (Table 5.20). Low concentrations of many VPA metabolites ((E)-3-ene, 4-ene, 4-keto and 4-OH VPA) were detectable in the newborn plasma until 54-66 h after the end of infusion (Figures 5.11 and 5.12). This
is in contrast to pregnant sheep where maternal and fetal plasma concentrations of these metabolites declined to below LOQ levels within 12-24 h after the end of infusion period in most animals. Also, (E)-2-ene, 3-keto and 5-OH VPA metabolites were still detectable in newborn plasma in significant concentrations (12.7 ± 7.9%, 24.4 ± 16.0% and 7.0 ± 7.3 % of the respective C\text{max} for (E)-2-ene, 3-keto and 5-OH VPA) at the end of the 96 h experimental protocol. This may be related to slower elimination of these metabolites in the newborn lamb via secondary metabolism or renal excretion, and indicates a much more prolonged newborn exposure to these compounds compared to the mother as well as the fetus. Fetal exposure to a number of these metabolites appears to be lower compared to the newborn, presumably because of relatively efficient removal of these compounds from fetal circulation via the placenta.

Perhaps the most significant observation with respect to newborn VPA metabolism was the detection of higher plasma concentrations of many VPA metabolites (i.e., (E)-2-ene, 3-ene, 3-keto, 5-OH and possibly 4-OH VPA) in the newborn compared to the fetus, and in some cases even compared to the mother. As discussed above, lower fetal C\text{max}'s of many of these metabolites compared to the newborn as well as the mother could be related to relatively rapid placental elimination of these low molecular weight compounds from the fetus. However, the average plasma C\text{max}'s of some metabolites in newborn lambs are from approximately two ((3)-ene VPA) to more than 10 fold (3-keto VPA) higher compared to those in the mother. This could be related to increased metabolism of VPA via these metabolic pathways compared to the adult and/or a slower elimination of these metabolites via secondary metabolism or renal elimination in the newborn. VPA is not very frequently used in human neonates and hence detailed information on VPA metabolism in this population is not available. A few isolated
reports exist describing VPA metabolite concentrations in cord serum samples and the similarities of these data to our fetal data have been discussed above (see above, Section 5.3.8.1). However, VPA is commonly used to control seizures in epileptic children and considerable data are available on the plasma or serum profiles of various VPA metabolites in this population (Nau et al., 1991; Fischer et al., 1992, Kondo et al., 1992; Siemes et al., 1993). Nau et al., (1991) presented a compilation of VPA metabolite profiles in 195 epileptic children divided into groups of <2 years and >2 years of age. The comparison of these data with our newborn lamb data reveals some striking similarities. It has been demonstrated that the plasma concentrations of (E)-2-ene, (E)-3-ene, 3-keto, and 5-OH VPA metabolites are generally higher in children < 2 years of age compared to those in >2 years of age. In contrast, the plasma concentrations of 4-ene and 4-keto VPA and 2-PGA metabolites are generally lower in the younger age group, whereas those of 4-OH VPA are similar between the 2 age groups (Nau et al., 1991). Similar observations have been reported by other investigators (Fischer et al., 1992; Kondo et al., 1992; Siemes et al., 1993).

The higher plasma concentrations of the β-oxidation VPA metabolites at younger ages in children and also in newborn lambs may be explained by the time course of developmental alterations in the fatty acid β-oxidation pathway. In all species studied (e.g., rat, rabbit, guinea pig, and human), it has been demonstrated that hepatic fatty acid β-oxidation activity is low in utero and at birth (Krahling et al., 1979; Duee et al., 1985; Shipp et al., 1982; De Vivo et al., 1991). However, these activities increase dramatically during the first few hours after birth and may reach values higher than the adult within 1 day; subsequently the β-oxidation activities decline steadily to adult levels until weaning (Stanley et al., 1979; Krahling et al., 1979; Duee et al., 1985; Shipp et al.,
This increase in β-oxidation capacity at birth may be responsible for the higher plasma concentrations of β-oxidation VPA metabolites in the newborn lamb. However, as discussed above, our and others' data also provide strong evidence for significant fetal formation of the 3-keto metabolite in the sheep as well as the human (Nau et al., 1981). This may indicate that this pathway is at least partly functional in utero.

In contrast to (E)-2-ene, (E)-3-ene and 3-keto VPA, the 5-OH VPA metabolite is produced via microsomal ω-oxidation and the specific isozymes involved in its formation are not known. The identification of these isozymes and the time course of their ontogenetic development may explain the higher plasma concentrations of this metabolite observed in newborn lambs as well as younger children (< 2 years) as compared to adult sheep and adult humans, respectively. The detection of other VPA metabolites (3-OH and 4-OH VPA) in newborn lamb plasma and urine (see below) also appears to be in agreement with the human data from one report where significant amounts of these metabolites were detected in tissue homogenates (liver, lung, adrenal, brain) from aborted human fetuses as early as 55-77 d gestation (Rettie et al., 1986).

5.3.8.3 Excretion of Unchanged VPA and its Metabolites in Maternal and Fetal Urine

During maternal drug infusion, almost the entire administered dose of VPA was eventually recovered in maternal urine as unchanged VPA (~20%) and in the form of a number of VPA metabolites (~80%) (Table 5.21). The major components of the VPA urinary metabolite profile were unchanged VPA and VPA-glucuronide (19.3 ± 5.8% and
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77.0 ± 7.8% of the total dose, respectively), with all other metabolites collectively accounting for ~5% of the dose. The 3-keto VPA, 4-keto VPA, 4-OH VPA, 5-OH VPA and 2-PGA accounted for ~0.4 – 2.3% of the total administered dose, whereas (E)-2-ene VPA, (E)-3-ene VPA, 4-ene VPA, 3-OH VPA and 2-PSA accounted for <0.4%. Small amounts of conjugated (E)-2-ene, (E)-3-ene, 4-ene, 3-OH, 4-OH and 5-OH VPA metabolites were also detected and accounted for <0.5% of the total dose. In contrast, conjugated counterparts of 3-keto and 4-keto VPA metabolites were not detectable. In our other studies in sheep, we have observed negligible biliary excretion of VPA and its glucuronide (Harvey Wong, K. Wayne Riggs and Dan W. Rurak, unpublished data). This is in contrast to the rat where significant amounts of VPA-glucuronide are excreted in bile, resulting in pronounced entero-hepatic cycling of the drug (Dickinson et al., 1979a). Similar to the human, we did not observe any entero-hepatic cycling of VPA in sheep. However, there appear to be a number of differences in the sheep VPA urinary metabolite profile compared to the human. Firstly, the renal excretion of the unchanged drug accounts for a much larger percentage of the total dose in sheep as compared to the human where on average only 1-2% (range 0.1-9%) of the total VPA dose is recovered unchanged in urine (Gugler et al., 1977; Dickinson et al., 1989). This may be related to a higher renal clearance of VPA in maternal sheep compared to the human, as discussed earlier (Section 5.3.7). The second significant difference between the sheep and the human lies in the fraction of the total dose recovered as VPA-glucuronide. In general, a larger percentage of the dose was recovered as VPA-glucuronide in sheep (77.0 ± 7.8%) compared to the human, where averages of 20.7 ± 21.9% (Gugler et al., 1977), 32.6 ± 14.4% (Dickinson et al., 1989), and 39.2 ± 18.3% (Levy et al., 1990) have been reported in different studies. However, the range of percent dose recovered as VPA-glucuronide in humans is rather wide (10-70%) and
may increase with increasing dose due to the saturation of the $\beta$-oxidation pathway (Granneman et al., 1984a; Pollack et al., 1986; Dickinson et al., 1989). The third major difference between maternal sheep and humans appears to be the much lower contribution of the $\beta$-oxidation pathway to total VPA elimination. In humans, in addition to VPA-glucuronide, 3-keto VPA is a major urinary metabolite and may account for 10-60% of the total VPA dose (Dickinson et al., 1989; Levy et al., 1990; Sugimoto et al., 1996). At higher doses, however, the 3-keto pathway could be saturated and its contribution may decrease (Granneman et al., 1984a; Sugimoto et al., 1996). The other $\beta$-oxidation metabolites, (E)-2-ene and 3-OH VPA, may each account for an additional 1-3% of the VPA dose in humans (Pollack et al., 1986; Dickinson et al., 1989; Levy et al., 1990; Sugimoto et al., 1996). It should be noted, however, that in addition to $\beta$-oxidation, the 3-OH VPA metabolite may also be formed via microsomal cytochrome P-450 mediated pathways (Rettenmeier et al., 1987). The contribution of all these $\beta$-oxidation metabolites to total VPA metabolism in maternal sheep appears to be much smaller compared to the human. As discussed earlier (Section 5.3.8.1), the lower contribution of the $\beta$-oxidation pathway in overall VPA metabolism in maternal sheep may be related to pregnancy-related reductions in $\beta$-oxidation capacity (Grimbert et al., 1993) or to the competition between VPA and elevated plasma free fatty acids for this pathway (Omtzigt et al., 1992). In agreement with this, we have observed a greater percentage (10-20%) of the total VPA dose excreted as the 3-keto VPA metabolite in urine during our studies in non-pregnant sheep (Harvey Wong, Sanjeev Kumar, K. Wayne Riggs and Dan W. Rurak, unpublished data). The contribution of most other measured VPA metabolites to total elimination of VPA appears to be within the range encountered in humans (Dickinson et al., 1989; Levy et al., 1990). Also, as in plasma,
the di-unsaturated VPA metabolites ((E)-2,4-diene and (E,E)-2,3'-diene VPA) were either absent or detectable only in trace amounts in maternal urine. This appears to be a species difference compared to the human where a significant fraction of the VPA dose (~0.1-0.2% for (E)-2,4-diene VPA and ~2-3% for (E,E)-2,3'-diene VPA) may be excreted in the form of these metabolites.

In three out of four animals (E4241, E5108, E105x) where maternal urine was collected during both maternal and fetal infusion experiments, the percentage of the total VPA dose recovered in maternal urine following fetal drug infusion appears to significantly less than 100%. The reasons for this remain unclear; however, fetal and/or placental metabolism/utilization of a portion of the fetal VPA dose may be responsible for this phenomenon.

Small amounts of VPA, VPA-glucuronide and other VPA metabolites were also detected in fetal urine. However, since cumulative fetal urine collection was not performed, it was not possible to estimate the total amounts of various metabolites excreted in fetal urine. Also, the presence of many of these metabolites in fetal urine could be the result of their formation in the maternal circulation and subsequent placental transfer. However, the fetal renal clearance of the unchanged drug is on average ~0.03% of the fetal total body clearance indicating that approximately this percentage of the total fetal VPA dose will be excreted in fetal urine. The contribution of fetal renal clearance to fetal non-placental clearance will, however, be ~0.15% because the latter is ~20% of total fetal clearance (Table 5.7). The presence of VPA-glucuronide in fetal urine likely indicates the fetal origin of this conjugate. This is because the permeability of the epiteliocorial sheep placenta to hydrophilic glucuronide conjugates is very low (Wang et al., 1985) and it is
unlikely that VPA-glucuronide formed in the mother could transfer across the placenta and subsequently be excreted in fetal urine. The amounts of VPA detected as VPA-glucuronide in fetal urine were equal to or less than those of the unchanged VPA (Figure 5.16), indicating that this metabolite will also likely account for a very small fraction of fetal VPA clearance (~0.03% of total fetal clearance, and ~0.15% of fetal non-placental clearance). This low fetal ability to form VPA-glucuronide in utero does not appear to be related to limiting concentrations of the substrate, UDP-glucuronic acid, because extremely low rates of VPA glucuronidation were also observed during our in vitro studies in fetal liver microsomes where large concentrations of this cofactor were added exogenously (Section 5.1.6).

Similar to VPA, very small amounts of the glucuronide conjugate have also been found in fetal lamb urine for another carboxylic acid drug, indomethacin, in our lab (Krishna, 1995). In contrast to these two carboxylic acid drugs, the ability to glucuronidate alcoholic/phenolic moieties appears to be much more developed in the late-gestation fetal lamb. It has been demonstrated that roughly 63%, 22% and 40% of the total fetal dose of morphine, ritodrine and labetalol, respectively, is glucuronidated by the fetal lamb at this stage of gestation (Olsen et al., 1988; Wright et al., 1991; Yeleswaram et al., 1993). Significant amounts of acetaminophen and para-nitrophenol glucuronides are also formed by the fetal lamb in utero and by the isolated perfused fetal lamb liver in vitro, respectively (Wang et al., 1986a; Ring et al., 1996). However, the acetaminophen glucuronide formation capacity in the fetal lamb is much less compared to adult sheep (Wang et al., 1986a; Wang et al., 1986b). These data may indicate the involvement of different isoforms of UDP-glucuronosyl transferases in the glucuronidation of acidic and alcoholic/phenolic compounds and their differential ontogenetic development in the fetal
lamb. Similar differential development of glucuronidation of a number of substrates has also been observed in the rat and the human fetus. In the rat, significant glucuronidation activity towards many substrates (e.g., 4-nitrophenol, 4-aminophenol, 5-hydroxytryptamine and 1-naphthol) also appears to develop in utero, whereas that towards others (e.g., bilirubin, morphine, testosterone and phenolphthalein) remains relatively low until after birth (Wishart, 1978). The human fetal liver appears to be able to glucuronidate 5-hydroxytryptamine effectively, but activities towards morphine, 1-naphthol, 4-nitrophenol, bilirubin and 2-aminophenol are much reduced (Dvorchik et al., 1986; Leakey et al., 1987; Burchell et al., 1989). Unfortunately, there appear to be no data on the ability of the human fetus or newborn to metabolize VPA via glucuronidation. In general, the fetal ability for glucuronidation is much less developed compared to the adult in all species examined. The acetaminophen (Wang et al., 1986a and 1986b), morphine (Dvorchik et al. 1986), indomethacin (Krishna, 1995) and VPA data (this work) in the fetal lamb appear to be consistent with this proposition.

5.3.8.4 Excretion of Unchanged VPA and its Metabolites in the Newborn Lamb Urine

The urinary metabolite profile of VPA in newborn lambs appears to be quantitatively different compared to maternal sheep. In particular, the percentage of the total dose recovered as the unchanged drug and VPA-glucuronide was smaller compared to the mother (Tables 5.21, 5.22 and 5.23). The former appears to be related to a lower renal clearance of the drug in the newborn lamb. As discussed before, it has been demonstrated in a number of studies that renal excretion of acid or organic anion compounds is negligible in the late-gestation fetal lamb. This lower fetal renal excretion capacity for VPA also appears to exist in the immediate newborn period. The lower
amount of VPA-glucuronide in newborn lamb urine is due to the reduced formation of this metabolite. Although, the percentage of dose recovered as VPA-glucuronide in the newborn lamb is only ~2-3 fold lower compared to the mother, the maternal-neonatal kinetic differences in this pathway are much more pronounced. In the mother, the majority of the glucuronide was recovered in urine within 12 h after the end of VPA infusion (Figure 5.9A). In contrast, in the lamb, significant amounts were still detectable in urine at the end of the 96 h experimental protocol (i.e., 90 h after the end of infusion) (Figure 5.9B). Thus, in the lamb, this pathway was likely saturated during a significant portion of the total duration of the experiment due to its much lower maximal capacity, as predicted from our in vivo Michaelis-Menten pharmacokinetic analysis (Section 5.3.6).

In contrast to the mother, the metabolites formed via the β-oxidation and cytochrome P-450 pathways appear to account for a much larger portion of the VPA dose in newborn lambs. These include (E)-2-ene, (E)-3-ene, 3-keto, 3-OH, 4-OH and 5-OH VPA. Interestingly, however, larger amounts of the potentially hepatotoxic cytochrome P-450 metabolite, 4-ene VPA, were not excreted in newborn lambs. Also, the fractions of dose recovered as 2-PSA and 2-PGA metabolites were similar in the newborn lambs and the mother. As discussed above, the fatty-acid β-oxidation capacity of the newborn lamb may exceed that of the mother, and this may result in a larger fraction of the newborn VPA dose being metabolized via this pathway. However, this may not be true for cytochrome P-450 mediated pathways, because the concentrations of total cytochrome P-450 in hepatic microsomes obtained from the late-gestation fetal lamb and from the newborn lamb during first few days of life are very low (~1/10 compared to the mother) (Dvorchik et al., 1986; Sanjeev Kumar, K. Wayne Riggs and Dan W. Rurak,
unpublished data). However, it is possible that significant quantities of specific isozymes involved in the formation of the above VPA metabolites are present at this stage of development. Regardless, it appears that due to a low capacity and possible saturation of VPA-glucuronidation, as well as reduced renal VPA elimination, a larger fraction of the newborn VPA dose is channeled to the cytochrome P-450 and β-oxidation pathways of VPA metabolism. Although a significantly larger fraction of the dose is metabolized via these routes in the newborn lamb compared to the mother, kinetically these pathways do not appear to be much more efficient in VPA elimination than glucuronidation. Hence, apparently nonlinear kinetics of VPA are observed in newborn lambs with a significantly reduced clearance. The time course of the ontogenetic development of VPA glucuronidation and renal excretion in lambs is not known at this time and is the subject of current investigations in our lab. In contrast to the mother, a significant portion of the total VPA dose (~30-50%) could not be accounted for in the newborn lamb. Hence, it is possible that additional routes of VPA metabolism are present during the newborn period, but this remains to be investigated.

Glycine conjugates and carnitine and coenzyme A esters of VPA have been identified in humans as well as other species; albeit the amounts present are small (Baillie and Sheffels, 1995). In addition, incorporation of VPA into endogenous lipids, such as triglycerides, phospholipids and lipoproteins, during the metabolically very active newborn period is also a possibility (Dr. Thomas A. Baillie, personal communication).

In summary, we have investigated detailed maternal, fetal and newborn pharmacokinetics and metabolism of VPA in sheep. Placental transfer of the drug is rapid and extensive, and results in a high degree of fetal drug exposure. Placental clearance data demonstrate an intermediate placental permeability for VPA in sheep,
possibly due to its polarity and high degree of ionization at physiological pH. Plasma protein binding of the drug is saturable in both the mother and the fetus and confounds maternal and fetal clearance estimations using the 2-compartment model. Corrected clearance estimates provide evidence of irreversible fetal drug elimination; however, the estimated fetal non-placental clearance is remarkably high, and may indicate possible placental utilization of the “fatty-acid like” VPA. The pharmacokinetics of VPA in the newborn lamb are strikingly different compared to the mother as well as the fetus but are generally very similar to those seen in the human newborn. The newborn clearance of the drug is much lower compared to both the mother and the fetus, whereas the apparent elimination half-life is much longer. In addition, VPA appears to exhibit a pronounced saturable (nonlinear) Michaelis-Menten type kinetics in the newborn lamb compared to an apparently linear pharmacokinetic profile in the mother and the fetus.

VPA appears to be extensively displaced from the binding sites in newborn lamb plasma during the initial 2 days of life, possibly as a result of elevated plasma free fatty acids. The maximal VPA binding capacities of the maternal, fetal and newborn plasma (after the initial 2-days of life) are similar, whereas, the binding affinity of maternal plasma appears to be about twice that of fetal and newborn plasma.

Overall disposition and metabolism of VPA in sheep appears to be similar to the human. We have obtained strong evidence of in utero fetal formation of a number of VPA metabolites, such as 3-keto, 5-OH, and 4-OH VPA and the VPA-glucuronide. Also, we have demonstrated that fetal exposure to most metabolites of VPA is comparable to that in the mother. Studies in newborn lambs clearly indicate that all major pathways of VPA metabolism are functional in the immediate newborn period. The newborn VPA metabolite exposure is relatively high and much prolonged compared to the mother, and
this is likely related to reduced newborn elimination of many of these metabolites. Newborn plasma concentrations of the $\beta$-oxidation VPA metabolites are much higher compared to the mother and this may be related to a high $\beta$-oxidation capacity at birth. This phenomenon is also apparent in VPA metabolite profiles in younger human children, especially <2 years of age. We have demonstrated that renal excretion of the unchanged VPA and formation of the VPA-glucuronide are the major determinants of VPA elimination in maternal sheep. Evidence has been obtained that both these routes of VPA clearance are significantly underdeveloped in the newborn lamb; this may underlie the profoundly different pharmacokinetic characteristics of VPA in the newborn lamb compared to the mother including a much longer apparent elimination half-life and reduced clearance. Since, a number of similarities have been observed with respect to the pharmacokinetics and metabolism of VPA in human newborns and newborn lambs, it is tempting to speculate that reduced VPA glucuronidation and/or renal clearance may also underlie the long half-life of VPA observed in human newborns. The quantitative urinary metabolite profile of VPA is different in newborn lambs compared to maternal sheep. Due to the reduced VPA-glucuronidation and renal clearance, a significantly larger percentage of the dose is metabolized via the $\beta$-oxidation and cytochrome P-450 mediated pathways.
Chapter 6

Global Summary and Conclusions

A series of studies were undertaken in chronically-catheterized pregnant sheep in order to assess the relative importance of various maternal, fetal and placental pharmacokinetic factors in determining fetal exposure to DPHM and VPA. In addition, attempts were made to examine the extent of development of fetal drug metabolism capacity for these two drugs in comparison to the mother. DPHM and VPA were studied as model compounds with several contrasting features in terms of their physicochemical properties (VPA carboxylic acid vs. DPHM basic amine) as well as pharmacokinetics (VPA low clearance vs. DPHM high clearance). Also, these two drugs are metabolized via a number of distinct metabolic pathways in many other species. Thus, the study of their metabolism in the fetus may provide information on the ontogenetic development of drug metabolizing enzymes in general.

Initial studies involved the identification of organs and pathways of DPHM elimination in the mother and the fetus. A high hepatic first-pass extraction of DPHM across the adult sheep liver indicates a major role of this organ in adult DPHM clearance. However, pharmacokinetic analysis of the parent drug and metabolite data after portal venous and i.v. administration indicated a significant role of the gut in systemic DPHM clearance in adult sheep as well. This gut uptake of DPHM in adult sheep was subsequently confirmed in direct studies where arterial and portal venous concentrations of the drug were measured. Although the gut extraction of the drug appears to be lower compared to the liver, the contribution of the gut to systemic clearance of DPHM is ≥50% in adult sheep. This is because the series arrangement of the gut with respect to the liver undermines the
role of the latter organ in systemic drug elimination in spite of its higher efficiency for drug clearance. This extensive gut uptake of the drug from the systemic circulation is a relatively rare finding and has broader implications in the study of the role of the gut in bioavailability and clearance of drugs in general. Overall, the gut and liver appear to be the major organs responsible for systemic DPHM clearance in adult sheep.

The study of the role of fetal liver in DPHM clearance (non-placental clearance) is much more complicated due to the complex geometry of the fetal circulation and multiple vascular inputs into the fetal liver. Maternally administered drugs cross the placenta and reach the fetal circulation via the umbilical vein. Also, the umbilical vein provides a major portion of fetal hepatic blood flow (~75%), and ~50% of the umbilical venous blood flow returning from the placenta passes via the fetal liver; the remaining 50% bypasses the liver via the ductus venosus. Thus, fetal hepatic first-pass uptake of the drug from the umbilical vein, if present, could likely be the most effective way of minimizing fetal exposure to drugs. An earlier study attempted to examine the extent of this fetal hepatic DPHM uptake from umbilical venous blood (Tonn et al., 1996). However, based on the measurement of parent drug fetal plasma concentrations after umbilical venous and inferior vena caval administration, fetal hepatic extraction from the umbilical vein was found to be negligible. In the current project, additional metabolite (DPMA) data were obtained from the fetal plasma samples collected during the previous study. These data clearly indicated fetal hepatic first-pass metabolism of the drug administered via the umbilical vein; the earlier conclusion of a lack of fetal hepatic drug uptake appears to be due to the unique blood flow patterns within the fetal circulation and high placental permeability of DPHM. A theoretical approach (Appendix I), based on the principles of metabolite kinetics and the well-stirred model of hepatic elimination, was developed to quantitate this fetal hepatic
first-pass extraction from the umbilical vein using the metabolite plasma concentration data in combination with the parent drug data. Using this approach, an average fetal hepatic first-pass extraction of 44% from umbilical venous blood was estimated. Since, ~50% of the umbilical venous blood bypasses the fetal liver, the total fetal hepatic extraction (assuming uniform DPHM uptake capacity in all parts of the fetal liver) would be ~88%. This indicates that the fetal liver during late gestation exhibits a significant ability for DPHM uptake/metabolism and is likely the major site of fetal non-placental clearance of the drug.

The role of the gut in fetal DPHM non-placental clearance remains to be investigated; however, it may be less significant compared to the adult because gut blood flow is only ~20% of the total hepatic blood flow in the fetus, as compared to ~80% in the adult.

Significant fetal hepatic first-pass uptake/metabolism of the drug was also evident in the paired infusion study where DPHM and [2H10]-DPHM were infused simultaneously but separately to the mother and the fetus, respectively (Chapter 3, Study A). In this case, DPHM, which reaches the fetal circulation via the placenta and the umbilical vein, undergoes fetal first-pass hepatic uptake and was found to be metabolized to a greater extent as compared to [2H10]-DPHM, which was infused i.v. to the fetus. This fetal first-pass hepatic uptake/metabolism of the drug reaching the fetal circulation via placental transfer and the umbilical vein also appears, in part, responsible for an underestimation of maternal-to-fetal DPHM placental clearance. This may also be the case for other drugs studied in pregnant sheep using the 2-compartment open model that demonstrate higher values of CLfm compared to CLmf even after the maternal and fetal plasma protein binding differences are taken into account. Conversely, the difference between fetal and maternal placental clearance for these drugs may provide evidence for their fetal hepatic uptake/metabolism.
The studies of the identification of organs of DPHM clearance were followed by the elucidation of specific metabolic pathways for this drug in the mother and the fetus. Two major metabolic pathways of DPHM clearance in many other species, DPMA and DPHM-N-oxide formation, were examined in this study. Using an approach based on the simultaneous administration of differently labeled parent drug and metabolite to the mother and the fetus, the contribution of DPMA formation to DPHM non-placental elimination in the mother and the fetus was estimated to be ~0.5-1%. Hence, this is a minor pathway in overall DPHM elimination in this species. However, the in vivo functional capacity of this metabolic pathway appears to be similar in maternal sheep and the late-gestation fetal lamb. Almost the entire maternal as well as fetal dose of the DPMA metabolite was eventually recovered in maternal urine, indicating that DPMA is not secondarily metabolized in fetal or adult sheep. Furthermore, DPMA is eliminated solely via the renal pathway in the mother and via the placenta (and eventually in maternal urine) in the fetus. The impaired fetal renal excretion and slow placental transfer of the DPMA metabolite is responsible for its long half-life in the fetus. In terms of the N-oxide pathway, the renal elimination of the intact DPHM-N-oxide also accounts for <1% of the total DPHM elimination in the mother as well as the fetus. However, the nature and extent of secondary metabolism of DPHM-N-oxide in the mother or the fetus or any other species remains to be investigated. The minor contribution of the DPMA pathway and DPHM-N-oxide renal elimination to sheep DPHM clearance is in contrast to other species (dog, rhesus monkey, man) where these routes account for ~40-60% and ~10-20% of the total DPHM dose, respectively. Thus, the exact pathways of DPHM metabolism are likely different in sheep compared to other species. Moreover, the mechanism and fate of extensive gut uptake of the drug from the systemic circulation in
adult sheep is not known. Overall, the exact routes of a large portion of maternal and fetal sheep DPHM elimination still remain unidentified. However, the pathways that were examined in this study appear to be almost equally functional in utero in the fetal lamb during late gestation in comparison to maternal sheep.

Since DPHM maternal and fetal placental and non-placental clearance and plasma protein binding data were available for a total of 18 pregnant sheep from all our studies, an extensive correlational analysis was performed to identify the factors determining overall DPHM disposition within the maternal-fetal unit. Our data demonstrate that DPHM fetal total, placental and possibly non-placental clearances decrease during the last 2 weeks of gestation in sheep. An increase in fetal plasma protein binding of the drug and a decrease in weight-normalized umbilical blood flow with advancing gestation appear responsible for these clearance changes. A similar phenomenon is apparent in the literature data of acetaminophen, a drug with much lower placental clearance compared to DPHM, indicating that it is not limited only to high placental clearance drugs such as DPHM. Plasma protein binding also appears to be an important determinant of maternal and fetal non-placental DPHM clearances. Fetal placental clearance of the drug is highly dependent on the extent of plasma protein binding in the fetus but not in the mother. An analogous relationship between maternal placental clearance and maternal plasma protein binding could not be demonstrated, probably because of errors in the estimation of this placental clearance due to fetal hepatic first-pass uptake of the drug from umbilical venous blood. The major determinant of plasma drug concentrations in the mother after maternal as well as fetal administration is maternal plasma protein binding and maternal non-placental clearance. In contrast, the major determinant of fetal plasma concentrations after maternal drug administration is the extent of fetal first-pass hepatic drug uptake from
the umbilical vein. DPHM provides a good example of a drug which results in only a limited fetal drug exposure after maternal administration (~20% compared to the mother) in spite of its high placental permeability. This reduced degree of fetal exposure to DPHM is, however, achieved at the expense of a much greater and prolonged fetal exposure to some of its metabolites (e.g., DPMA). Thus, for DPHM at least, fetal hepatic first-pass uptake of the drug from the umbilical vein overrides the effects of placental permeability, and maternal and fetal plasma protein binding and systemic clearance in determining the extent of fetal drug exposure after maternal administration. However, after fetal drug administration, fetal plasma concentrations are related to the extent of fetal plasma protein binding, and fetal placental and non-placental clearances. The index of fetal-to-maternal placental drug transfer (steady-state \( C_m/C_f \) ratio) was related to steady-state fetal plasma unbound fraction, and fetal placental and non-placental clearance; its magnitude decreased significantly with advancing gestation due to a fall in all these variables. However, this index was not related to the magnitude of the factors operating on the other side of the placenta such as maternal plasma protein binding and maternal non-placental clearance, as is generally postulated. These variables thus do not seem to provide any significant driving force for placental transfer of the drug and have a minimal effect on the kinetics of placental transport. This might indicate a lack of complete equilibration of the unbound drug concentrations on the two sides of the placenta, leading to departures from ideal placental transport characteristics. Similar types of relationships, however, could not be described for maternal-to-fetal placental transfer and the fetal drug exposure index after maternal drug administration (steady-state \( C_f/C_m \) ratio) because of variable underestimation of \( C_f \) due to fetal hepatic first-pass uptake of the drug from the umbilical venous blood.
As with DPHM, detailed maternal and fetal pharmacokinetics and metabolism of VPA were also investigated in pregnant sheep during the last part of gestation. In addition, VPA disposition was also studied in the immediate newborn period because fetal metabolic capacity for the drug could not be accurately determined. The placental transfer of the drug was rapid and extensive, resulting in a high degree of fetal exposure. Fetal VPA exposure after maternal drug administration, based on the steady-state fetal-to-maternal plasma concentration ratio, was $0.70 \pm 0.10$, as compared to $0.20 \pm 0.14$ for DPHM. On the other hand, placental clearance of VPA was much lower compared to DPHM, indicating a lower placental permeability for this drug, possibly due to its greater polarity and high degree of ionization at physiological pH. Also, in contrast to DPHM, placental clearances of the unbound VPA in the two directions (CL$_{mf}$ and CL$_{fm}$) were similar. This indicates a lack of any significant fetal hepatic uptake of VPA from the umbilical vein, which is to be expected from a low clearance drug. Thus, the higher degree of fetal exposure to VPA after maternal administration is primarily related to a low fetal non-placental elimination capacity in comparison to DPHM and also to a relatively similar degree of maternal and fetal plasma protein binding. Similar to VPA, fetal non-placental clearance for most low clearance compounds would be low, and at best would be in the range of the adult values. Hence, if placental permeability is not limiting, fetal exposure to this class of compounds in general would be greater as compared to high clearance compounds. Fetal non-placental elimination and hepatic first-pass drug uptake from the umbilical vein, if at all present, would be sufficiently high only for high clearance compounds so as to be significant in minimizing fetal drug exposure. An exception to this would be the drugs which are metabolized to a large extent by the placenta.
The plasma protein binding of VPA is saturable in both the mother and the fetus, and confounds the maternal-fetal clearance estimations using the 2-compartment model. Corrected clearance estimates provide evidence of irreversible fetal VPA elimination. However, the estimated fetal non-placental clearance is remarkably high, indicating the possibility of placental utilization of "fatty-acid like" VPA. The pharmacokinetics of VPA in the newborn lamb are strikingly different compared to the mother as well as the fetus, but are very similar to those seen in the human newborn. Clearance of the drug in the newborn is much lower compared to both the mother and the fetus, whereas the apparent elimination half-life is much longer. In addition, VPA appears to exhibit pronounced saturable (nonlinear) Michaelis-Menten type kinetics in the newborn lamb compared to an apparently linear pharmacokinetic profile in the mother and the fetus. VPA appears to be extensively displaced from binding sites in the newborn lamb plasma during the initial 2 days of life, possibly as a result of elevated plasma free fatty acids. The maximal VPA protein binding capacities of the maternal, fetal and newborn plasma (after initial 2-days of life) are similar, whereas, the binding affinity of the maternal plasma appears to be about twice that of the fetal and newborn plasma.

The overall disposition and metabolism of VPA in sheep appears to be similar to the human. We have obtained strong evidence of in utero fetal formation of a number of VPA metabolites, such as 3-keto, 5-OH, and 4-OH VPA and VPA-glucuronide. The higher fetal plasma 3-keto VPA concentrations as compared to the mother suggest that the increase in the capacity of the β-oxidation pathway seen at birth may actually begin in utero in sheep. Also, we have demonstrated that fetal exposure to most metabolites of VPA is comparable to that in the mother. Studies in newborn lambs clearly indicate that all major pathways of VPA metabolism are functional in the immediate newborn
period. Newborn VPA metabolite exposure is relatively high and very prolonged compared to the mother; this is likely related to reduced newborn elimination of many of these metabolites. Newborn plasma concentrations of VPA metabolites formed via β-oxidation are much higher compared to the mother and this may be related to a high β-oxidation capacity at birth. This phenomenon is also apparent in VPA metabolite profiles in younger human children, especially <2 years of age. We have demonstrated that renal excretion of the unchanged VPA and formation of VPA-glucuronide are the major determinants of VPA elimination in maternal sheep. Evidence has been obtained that both these routes of VPA clearance are significantly underdeveloped in the newborn lamb. This may underlie the profoundly different pharmacokinetic characteristics of VPA in the newborn lamb compared to the mother, including a much longer apparent elimination half-life and reduced clearance. Since, a number of similarities have been observed with respect to the pharmacokinetics and metabolism of VPA in human newborns and newborn lambs, it is tempting to speculate that this reduced VPA glucuronidation and/or renal clearance may also underlie the long half-life and low clearance of VPA in human newborns. The quantitative urinary metabolite profile of VPA is different in newborn lambs compared to maternal sheep. Due to reduced VPA glucuronidation and renal excretion capacity, a significantly larger percentage of the dose is metabolized via the β-oxidation and cytochrome P-450 mediated pathways.

The known differences in the renal handling of organic acid and base compounds in the late-gestation fetal lamb also appear to extend to DPHM, DPMA and VPA. Previous studies have indicated that renal tubular secretion of the basic amine compounds such as cimetidine (Mihaly et al., 1983), ranitidine (Czuba et al., 1990), meperidine (Szeto et al.,
1979), and tetraethylammonium (Elbourne et al., 1990), is functional in the fetal lamb during late gestation. Significant fetal renal clearance of DPHM relative to adult sheep, observed in the current as well as the previous study (Tonn, 1995), is in agreement with these findings with other compounds. In contrast, fetal (and also newborn in case of VPA) renal clearance of two carboxylic acid compounds, DPMA and VPA, is much lower compared to the mother. In addition, the long half-lives of many VPA metabolites, which also have a carboxylic acid structure, in the newborn may also be related partly to their reduced renal elimination. This appears to be similar to the limited fetal renal tubular secretion of other acid compounds such as para-aminohippurate (Elbourne et al., 1990), acetaminophen glucuronide and sulfate conjugates (Wang et al., 1986), indomethacin (Krishna et al., 1995) and morphine glucuronide (Olsen et al., 1988) in sheep. Overall, the data on DPHM, DPMA and VPA renal excretion in adult and fetal sheep, taken together with the studies of other compounds, suggest that while organic cation tubular secretion is developed in utero during late gestation, renal elimination pathways for organic anions must develop some time after birth.

Although considerable in vitro data are available on the expression and activity of human fetal drug metabolizing enzymes (Pacifici et al., 1982; Perucca, 1987; Burchell et al., 1989; Krauer and Dayer, 1991; Jacqz-Aigrain and Cresteil, 1992; Raucy and Carpenter, 1993; Hakkola et al., 1994; Cazeneuve et al., 1994; Shimada et al., 1996), the in utero role of these enzymes in human fetal drug metabolism can not be examined in detail due to obvious practical and ethical considerations. In the current studies, we have clearly demonstrated that many metabolic pathways involved in the metabolism of DPHM and VPA are at least partly functional in utero in the fetal lamb during late gestation, and in certain cases the fetal metabolic capacity is similar to that of the adult
(e.g., DPMA). Also, in the case of DPMA, we have demonstrated direct involvement of the fetal liver in metabolite formation. Many cytochrome P-450 mediated pathways of VPA metabolism (e.g., the formation of 4-OH and 5-OH VPA) are also noticeably functional in the immediate post-natal period and hence likely in utero as well. This is in spite of the very low total cytochrome P-450 concentrations generally found in the fetal or neonatal lamb liver microsomes at this stage, and indicates that certain specific isozymes involved in these reactions may be present in significant quantities at earlier stages of life. Also, significant fetal ability to form DPHM-N-oxide was observed in our studies. The elucidation of the possible role of flavin-containing-monoxygenases in this pathway may yield important new information on the fetal development of this group of enzymes. Certain other metabolic pathways (e.g., VPA glucuronidation) are, however, much less developed in utero and in the immediate post-natal period. Overall, these findings combined with previous data from other studies in different animal models suggest the involvement of many different enzymes in these metabolic reactions and their differential ontogenetic development. The individual isozymes involved in any of these metabolic pathways, however, have not been identified and hence detailed understanding of their ontogeny remains poorly understood.
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Appendix I

Equation (7) (Chapter 3) is derived from principles of the well-stirred model of hepatic drug elimination (Wilkinson and Shand, 1975; Pang and Rowland, 1977). In the current study, the fetal umbilical venous input of the drug (form of drug given to mother, i.e., DPHM) is considered equivalent to portal venous administration after birth because ~50% of the umbilical venous blood flow passes through the fetal liver before reaching the fetal circulation (Edelstone et al., 1978). The bypass of the remaining ~50% of umbilical blood flow from the fetal liver does not jeopardize the validity of this assumption because we were in fact interested in the fetal hepatic drug extraction ratio after umbilical input only. Tarsal venous administration (form of drug given directly to the fetus, i.e., $[^2H_{10}]$-DPHM) is equivalent to the usual i.v. input.

In the following section, the symbols 'D' and 'M' denote parent drug (DPHM or $[^2H_{10}]$-DPHM) and metabolite (DPMA or $[^2H_{10}]$-DPMA), respectively. The subscripts 'u' and 'l' represent unlabeled and labeled drug or metabolite, respectively.

The fraction of DPHM (or $[^2H_{10}]$-DPHM) metabolized to DPMA (or $[^2H_{10}]$-DPMA) ($F_m$) can be described in terms of intrinsic clearances of the liver as:

$$F_m = \frac{(CL_{f,\text{int}})_{M_u \text{ or } M_l}}{(CL_{\text{int}})_{D_u \text{ or } D_l}} = \frac{(CL_{f,\text{int}})_{M_u \text{ or } M_l}}{(CL_{\text{int}})_{D_u \text{ or } D_l} + (CL_{\text{int}})_\text{other}}$$  \hspace{1cm} (i)

where, $(CL_{f,\text{int}})_{M_u \text{ or } M_l}$ is the intrinsic formation clearance of DPMA or $[^2H_{10}]$-DPMA from DPHM or $[^2H_{10}]$-DPHM, respectively. The symbol $(CL_{\text{int}})_{D_u \text{ or } D_l}$ represents the total
intrinsic clearance of DPHM or \([^2\text{H}_10]\)-DPHM, and \((\text{CL}_{\text{int}})_{\text{other}}\) is the intrinsic clearance of DPHM or \([^2\text{H}_10]\)DPHM via pathways other than the formation of DPMA or \([^2\text{H}_10]\)-DPMA.

Also, according to the well-stirred model of hepatic drug elimination (Wilkinson and Shand, 1975; Pang and Rowland, 1977):

\[
(\text{CL})_{D_u \cup D_i} = \frac{Q_H \cdot (\text{CL}_{\text{int}})_{D_u \cup D_i}}{Q_H + (\text{CL}_{\text{int}})_{D_u \cup D_i}} = \frac{Q_H \cdot [(\text{CL}_{\text{f, int}})_{M_u \cup M_i} + (\text{CL}_{\text{int}})_{\text{other}}]}{Q_H + [(\text{CL}_{\text{f, int}})_{M_u \cup M_i} + (\text{CL}_{\text{int}})_{\text{other}}]} \tag{ii}
\]

where, \(Q_H\) is the total hepatic blood flow.

The formation clearance of DPMA (or \([^2\text{H}_10]\)-DPMA) can be expressed as:

\[
(\text{CL}_{\text{f}})_{M_u \cup M_i} = F_{\text{m}} \cdot (\text{CL})_{D_u \cup D_i} \quad \tag{iii}
\]

where, \((\text{CL})_{D_u \cup D_i}\) is the systemic clearance of DPHM or \([^2\text{H}_10]\)-DPHM.

Using equations (i) & (ii), equation (iii) becomes:

\[
(\text{CL}_{\text{f}})_{M_u \cup M_i} = \frac{Q_H \cdot (\text{CL}_{\text{f, int}})_{M_u \cup M_i}}{Q_H + [(\text{CL}_{\text{f, int}})_{M_u \cup M_i} + (\text{CL}_{\text{int}})_{\text{other}}]} \quad \tag{iv}
\]

As discussed above, \([^2\text{H}_10]\)-DPHM administration is equivalent to \(i.v\). input, and the total amount of labeled metabolite (\([^2\text{H}_10]\)-DPMA) formed in the fetus from \([^2\text{H}_10]\)-DPHM during
the experimental period is equal to the total amount of metabolite eliminated (all of the formed metabolite is ultimately eliminated). This can be described by the following relationship (assuming a one compartment fetus):

\[ k_f \cdot V_d \cdot AUC_D = k_m \cdot V_m \cdot AUC_M \]

(v)

where, \( k_f \) and \( k_m \) are the formation and elimination rate constants of the \([^2\text{H}_{10}]\)-DPMA metabolite. \( V_d \) and \( V_m \) are the volumes of distribution of \([^2\text{H}_{10}]\)-DPHM and \([^2\text{H}_{10}]\)-DPMA, respectively. \( AUC_D \) and \( AUC_M \) are the systemic arterial AUC’s of \([^2\text{H}_{10}]\)-DPHM and \([^2\text{H}_{10}]\)-DPMA, respectively.

On rearranging, equation (v) becomes:

\[
\frac{AUC_M}{AUC_D} = \frac{k_f \cdot V_d}{k_m \cdot V_m} \cdot \frac{(CL_f)_M}{(CL_e)_M}
\]

(vi)

where, \((CL_f)_M\) and \((CL_e)_M\) are the systemic formation and elimination clearances of the \([^2\text{H}_{10}]\)-DPMA metabolite.

Substituting the right hand side of equation (iv) for \((CL_f)_M\) in equation (vi):

\[
\frac{AUC_M}{AUC_D} = \frac{Q_h \cdot (CL_{f, \text{int}})_M}{(CL_e)_M \cdot [Q_h + (CL_{f, \text{int}})_M + (CL_{\text{int}})_{\text{other}}]}
\]

(vii)
After umbilical venous input (equivalent to portal or oral route in postnatal circulation), systemic formation clearance of the metabolite is in fact equivalent to its intrinsic formation clearance (Wilkinson and Shand, 1975; Pang and Rowland, 1977).

Hence, for umbilical input of the drug (unlabeled DPHM in the case of Study A in Chapter 3), an expression equivalent to equation (vi) can be written as:

\[
\left( \frac{\text{AUC}_{M_u}}{\text{AUC}_{D_u}} \right)_{\text{umbilical input}} = \frac{(\text{CL}_{f, \text{int}})_{M_u}}{(\text{CL}_e)_{M_u}}
\]

Using equation (viii), equation (vii) becomes:

\[
\left( \frac{\text{AUC}_{M_i}}{\text{AUC}_{D_i}} \right)_{\text{i.v. input}} = \frac{\text{AUC}_{M_u}}{\text{AUC}_{D_u}} \cdot \frac{Q_H}{[Q_H + (\text{CL}_{f, \text{int}})_{M_i} + (\text{CL}_{\text{int}})_{\text{other}}]}
\]

Again, according to the well-stirred model of hepatic drug elimination, the hepatic extraction ratio is given by (Wilkinson and Shand, 1975; Pang and Rowland, 1977):

\[
E_H = \frac{(\text{CL}_{\text{int}})_{D_i} \text{ or } D_l}{Q_H + (\text{CL}_{\text{int}})_{D_i} \text{ or } D_l} = \frac{(\text{CL}_{f, \text{int}})_{M_i} \text{ or } M_l + (\text{CL}_{\text{int}})_{\text{other}}}{Q_H + (\text{CL}_{f, \text{int}})_{D_i} \text{ or } D_l + (\text{CL}_{\text{int}})_{\text{other}}}
\]

And hence, systemic availability \((F = 1 - E_H)\) will be:

\[
F = 1 - E_H = \frac{Q_H}{Q_H + (\text{CL}_{f, \text{int}})_{M_i} \text{ or } M_l + (\text{CL}_{\text{int}})_{\text{other}}}
\]
Using equation (xi), equation (ix) then becomes:

\[
\frac{\text{AUC}_{M_i}}{\text{AUC}_{D_i}} \text{ i.v. input} = \frac{\text{AUC}_{M_i}}{\text{AUC}_{D_i}} \text{ umbilical input} \times (1 - E_H)
\]

or

\[
E_H = \frac{\frac{\text{AUC}_{M_i}}{\text{AUC}_{D_i}} \text{ umbilical input} - \frac{\text{AUC}_{M_i}}{\text{AUC}_{D_i}} \text{ i.v. input}}{\frac{\text{AUC}_{M_i}}{\text{AUC}_{D_i}} \text{ umbilical input}} \quad \text{(xii)}
\]

This is the same expression as described in equation (7).

Similar relationships have been employed by other workers to study parent drug and metabolite kinetics after i.v. and portal (oral) venous drug administration (Rollins et al., 1980; Lane and Levy, 1981).