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The use of microdialysis for the study of drug kinetics: CNS pharmacokinetics of diphenhydramine in fetal, newborn and adult sheep

Sam CS Au Yeung, K Wayne Riggs, Nancy Gruber, and Dan W Rurak

PK/PD, Clinical Pharmacology, Quintiles (SCSAY), Faculty of Pharmaceutical Sciences (KWR) and Department of Obstetrics and Gynecology, Child & Family Research Institute (NC, DWR), University of British Columbia.

A) CNS Pharmacokinetics of Diphenhydramine in Fetal, Newborn and Adult Sheep

B) Corresponding Author: Dr. Dan W Rurak

Child & Family Research Institute

950 W.28th Ave

Vancouver, BC, V5Z 4H4, Canada

Tel: (604)875-2430

Fax: (604)875-2496

E-mail: drurak@cw.bc.ca

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D) List of Abbreviations:

$AUC_{0\rightarrow\infty}$ – Total area under the plasma concentration versus time curve

$AUC_{CSF0\rightarrow\infty}$ - Total area under the CSF concentration versus time curve

$AUC_{ECF0\rightarrow\infty}$ - Total area under the ECF concentration versus time curve

$AUMC_{0\rightarrow\infty}$ – Area under the first moment curve

BBB – Blood brain barrier

Cl_T – Total body clearance

C_{BRAIN} – Combined CSF and ECF DPHM concentrations in the brain

C_{Pt} – Total plasma DPHM concentration

C_{Pu} – Free plasma DPHM concentration

C_{CSF} – CSF DPHM concentration

C_{ECF} – ECF DPHM concentration

CNS – Central Nervous System

CSF – Cerebrospinal Fluid

DPHM – Diphenhydramine

$[DPHM]_{dialysate}$ – Diphenhydramine concentration in the output dialysate

ECF – Extracellular Fluid

f_{CSF} – the ratio of $AUC_{CSF0\rightarrow\infty}/AUC_{0\rightarrow\infty}$

f_{ECF} – the ratio of $AUC_{ECF0\rightarrow\infty}/AUC_{0\rightarrow\infty}$

$[^2H_{10}]$-DPHM – Deuterium-labeled diphenhydramine

MD – Microdialysis

PRN – Propranolol

SEM – Standard error of mean

$t_{1/2\alpha}$ - Distribution half-life

$t_{1/2\beta}$ - Elimination half-life

Vd_{ss} – Steady-state volume of distribution

E) Section assignment: Absorption, distribution, metabolism and excretion

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Abstract

The CNS pharmacokinetics of the H_1-receptor antagonist, diphenhydramine (DPHM), were studied in 100 and 120 d fetuses, 10 and 30 d newborn lambs, and adult sheep using *in vivo* microdialysis. DPHM was administered i.v. at 5 infusion rates, with each step lasting 7 h. In all ages, CSF and ECF concentrations were very similar to each other, which suggest that DPHM transfer between these two compartments is by passive diffusion. Also, the brain-to-plasma concentration ratios were ≥ 3 in all age groups suggesting the existence of a transport process for DPHM into the brain. Both brain and plasma DPHM concentrations increased in a linear fashion over the dose range studied. However, the ECF/unbound plasma and CSF/unbound plasma DPHM concentration ratios were significantly higher in the fetus and lambs (~5-6) than in the adult (~3). The factors f_{CSF} and f_{ECF}, the ratios of DPHM AUCs in CSF and ECF to the plasma DPHM AUC, decreased with age, indicating that DPHM is more efficiently removed from the brain with increasing age. The extent of plasma protein binding of the drug increased with age. This study provides evidence for a transporter-mediated mechanism for the influx of DPHM into the brain and also for an efflux transporter for the drug, whose activity increases with age. Moreover, the higher brain DPHM levels in the fetus and lamb compared to the adult may explain the greater CNS effects of the drug at these ages.

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Introduction

The perinatal period of development is a time of rapid physiological and anatomical changes that can profoundly affect drug disposition. Therefore, caution should be exercised for drug use during pre- and postnatal development due to a poor understanding of age-related changes in drug response and pharmacokinetics (Piper et al., 1987). Of all the issues related to xenobiotics use in the developing fetus and newborn, the effect of drugs on CNS development probably deserves the most attention since exposure to exogenous substances during this dynamic period of neurological growth can potentially cause deleterious effects on the developing brain (Rurak, 1992). Considering the fact that most drugs are lipophilic in nature, these compounds have the ability to cross biological membranes including the blood-brain barrier (BBB). Diphenhydramine, [2-(diphenylmethoxy)-N,N-dimethylethylamine (DPHM)], is a potent histamine H_1-receptor antagonist (Douglas, 1980) widely used for its anti-allergic properties, as well as for its anti-emetic, sedative, local anaesthetic and hypnotic effects (Runge et al., 1992; Ernst et al., 1993). Like other "first-generation" antihistamines, DPHM occupies central H_1-receptors to result in drowsiness, sedation, incoordination and with higher doses, convulsions and death (Douglas, 1980; Nicholson, 1983; Gengo et al., 1989).

In pregnancy, DPHM is used for conditions such as nausea and vomiting, insomnia in the first trimester (Magee et al., 2002), a pregnancy-related urticaria in the third trimester, cough and colds, and allergy (Piper et al., 1987). These findings suggest that a significant number of human fetuses may be exposed to this drug at some time during their gestation. Also, secretion into breast milk represents a potential route of neonatal exposure to DPHM (Schatz, 2002). The drug readily crosses the placenta, and

alters fetal behavioural state and may also do this in the newborn (Rurak et al., 1988). The DPHM-elicited behavioural effects occur at plasma concentrations lower than those required in adults (Rurak et al., 1988). While the mechanisms underlying this phenomenon are unknown, it may involve a greater exposure of the fetal and neonatal brain to the drugs. Because of ethical and technical constraints, study of this issue in humans is not possible. However, the fetal and newborn lamb are a useful model for the human fetus and infant, because of the similarities in the ontogenesis of both the BBB and behavioural functions in the 2 species (Mollgard and Saunders, 1986; Rurak, 1992). In addition, use of this species can overcome limitations in the available sampling volume of biological fluids associated with smaller animal models, and thus allows for more detailed studies. Therefore, studies in sheep can provide useful information on drug exposure in the fetal, newborn and adult brain. The purpose of this study is to assess blood-brain ECF and blood-CSF drug concentration relationships as a function of pre- and postnatal age, and in relation to variations in drug dose and hence plasma drug levels. By applying *in vivo* microdialysis (MD) in chronically-instrumented fetal and newborn lambs, and adult ewes, we were able to collect serial samples from the lateral ventricle and cerebral cortex and therefore elucidate DPHM pharmacokinetics in the CNS from these animals at different ages.

Materials and Methods

Animals and Surgical Preparation

All studies were approved by the University of British Columbia Animal Care Committee, and the procedures performed on the sheep conformed to the guidelines of the Canadian Council on Animal Care. **Fetuses:** Time dated pregnant Dorset Suffolk cross-bred ewes (term, ~145 days) were operated on between 95 and 105 days for the 100 d group (n=3) and between 115 and 125 days of gestation for the 120 d group (n=7). The mean maternal weight was 81.5 ± 9.3 kg. Food was withheld for ~18 h prior to surgery, but the animals were allowed free access to water. Approximately 30 min before surgery, a 6 mg *i.v*. dose of atropine (Glaxo Laboratories, Montreal, Canada) was administered *via* the jugular vein to control salivation. Surgery was performed asceptically under isoflurane (1-2%) and nitrous oxide (60%) anesthesia (balance O_2), after induction with *i.v*. sodium pentothal (1 g) and intubation of the ewe. Silicone rubber catheters (Dow Corning Corp., Midland, MI) were implanted in a fetal femoral artery and vein, fetal trachea, amniotic cavity, and a maternal femoral artery and vein. In addition, through 1.5 mm holes drilled through the skull, flexible MD probes (CMA 20, Stockholm, Sweden) were implanted in the lateral ventricle and ipsilateral parietal cortex for collection of CSF and ECF respectively. The probes were secured in place with tissue glue and dental cement. The MD probe input and output tubing was extended with FEP catheters (CMA, Stockholm, Sweden) for access outside the animal. The catheters were tunneled underneath the neck skin of the fetus and exited through a small incision on the dorsal neck. All catheters were exteriorized *via* a small incision on the flank of the ewe; they were stored in a Ziploc® bag, protected with tensor bandages around the ewe's abdomen

when not in use. All vascular catheters were flushed daily with approximately 2 mL of sterile 0.9% sodium chloride solution containing 12 U heparin/mL to maintain catheter patency. The MD probes were flushed with sterile, degassed lactated ringer solution daily to avoid air bubble formation along the tubing. Antibiotics, including Trivetrin® (Schering Canada Inc., Pointe Claire, QC) and ampicillin, were administered to the ewe on the day of the surgery and for 3 days postoperatively. After surgery, animals were kept in holding pens with other sheep and were allowed free access to food and water. The ewes were allowed to recover for at least 3 days before experimentation.

Post-natal Lambs: A total of 9 Dorset Suffolk cross-bred newborn lambs were used. The lambs were divided into a 10 day old group (n=5) and a 30 day old group (n=4). Surgery was performed aseptically under isoflurane (1-2%) and nitrous oxide (60%) anesthesia (balance O_2) and intubation of the lamb. Silicone rubber catheters (Dow Corning Corp., Midland, MI) were implanted in a carotid artery and a jugular vein. In addition, as described above for the fetus, flexible MD probes (CMA 20, Stockholm, Sweden) were implanted in the lateral ventricle and ipsilateral parietal cortex for collection of CSF and ECF respectively. The MD probe input and output catheters were extended with FEP catheters (CMA, Stockholm, Sweden) and tunneled *s.c.* and exteriorized *via* a small incision on the back of the neck for access. Post-surgical treatments were the same as described in the above section. After surgery, animals were kept in holding pens with their mothers and allowed to recover for at least 3 days before experimentation. **Adult Sheep:** Eight non-pregnant Dorset Suffolk cross-bred ewes were employed. The same preoperative and anesthetic procedures used with the pregnant ewes (see above) were employed with the non-pregnant sheep. Polyvinyl or silicone

rubber catheters (Dow Corning Corp., Midland, MI) were implanted in both the carotid artery and jugular vein. In addition, flexible MD probes (CMA 20, Stockholm, Sweden) were implanted in the lateral ventricle and ipsilateral parietal cortex for collection of CSF and ECF respectively. The MD probe input and output catheters were extended with FEP catheters (CMA, Stockholm, Sweden) and tunneled *s.c.* and exteriorized *via* a small incision on the back of the neck for access. Post-surgical treatments of the ewes are the same as described above for fetuses and lambs. After surgery, animals were kept in holding pens with other sheep and were allowed free access to food and water. The ewes were allowed to recover for at least 3 days before experimentation

Experimental Protocols

The protocol involved a bolus *i.v.* loading dose of the drug (to hasten the achievement of steady-state), followed by *i.v.* infusion of the drug at 5 different rates, with each infusion rate lasting 7 h. All DPHM (Diphenhydramine hydrochloride, Sigma Chemical Co., St. Louis, MO) doses were prepared in 0.9% sodium chloride solution and were sterilized by filtering through a 0.22 μm nylon syringe filter (MSI, Westboro, MA) into a capped empty sterile injection vial. For the 100 d fetus the DPHM loading dose was 0.5 mg/kg and the infusion rates were 17, 76.5, 136, 195.5 and 255 μg/kg/min. For the 120 d fetus the DPHM loading dose was 0.5 mg/kg and the infusion rates were 13.6, 61.2, 108.8, 156.4 and 204 μg/kg/min. For the 10 d lamb the DPHM loading dose was 0.7 mg/kg and the infusion rates were 5.25, 19.25, 33.25, 47.25 and 61.25 μg/kg/min. For the 30 d lamb the DPHM loading dose was 0.7 mg/kg and the infusion rates were 6, 22, 38, 54 and 70 μg/kg/min. In adult ewes, the DPHM loading dose was 0.15 mg/kg and the

infusion rates were 1.5, 5.5, 9.5, 13.5 and 17.5 μg/kg/min. The various dosages were determined based on 2 sources: 1) results obtained from previous pharmacokinetic studies performed in newborn lambs, pregnant and adult sheep (Yoo et al., 1993; Kumar et al., 2000; Wong et al., 2000), which indicated that the systemic clearance of DPHM was high in the fetus and newborn and decreased in adult sheep, and 2) results obtained from preliminary studies. The doses were adjusted so that they would target a plasma concentration range of ~ 35 – 450 ng/mL. This particular plasma concentration range was used because it was associated with behavioral changes in fetal lambs in a study performed previously (Rurak et al., 1988).

During the infusions arterial blood samples (3 mL adult, 0.5 mL fetus and lamb) were collected at -5, 5, 15, 30 min, and 1, 2, 3, 4, 5, 6, 7, 7.083, 7.25, 7.5, 8, 9, 10, 11, 12, 13, 14, 14.083, 14.25, 14.5, 15, 16, 17, 18, 19, 20, 21, 21.083, 21.25, 21.5, 22, 23, 24, 25, 26, 27, 28, 28.083, 28.25, 28.5, 29, 30, 31, 32, 33, 34, 35, 35.083, 35.25, 35.5, 36, 36.5, 38, 40, 43, 46, 49, and 53 h. Samples (0.5 mL) were also collected from the fetus at intervals for assessment of blood gas and metabolic status. Due to the relatively small total blood volume in the fetus [~125 – 300 mL, (Kwan et al., 1995)], maternal blood (8 mL) was infused into the fetus every 7 hours for replacement. Microdialysis sampling began at the onset of the infusion.

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

polytetrafluoroethylene-lined caps. MD dialysate samples were collected directly into clean borosilicate test tubes. Plasma and MD samples were stored frozen at -20^{o}C until the time of analysis.

Retrodialysis

Microdialysis sampling began at the onset of the infusion. The microdialysis pump (Harvard Apparatus Inc., Holliston, MA) infusion rate was 2 µL/min and 60-min cumulative samples of CSF and ECF were collected throughout the duration of the experiment. MD probe recovery was determined using the retrodialysis technique (De Lange et al., 1998). The MD dialysate (degassed, sterile lactated ringer solution) contained a calibrator ([$^{2}H_{10}$]-DPHM) at a concentration of 400 ng/mL. The probe recovery rate can be determined by comparing the input and output concentrations of the calibrator as follows:

$$\text{Recovery} = \frac{[\text{Calibrator}_{\text{input}}] - [\text{Calibrator}_{\text{output}}]}{[\text{Calibrator}_{\text{input}}]} \qquad \text{(Equation 1)}$$

Free fraction drug concentration (C_{CSF} or C_{ECF}) at the MD sampling site = $[DPHM]_{dialysate}$/Recovery Rate.

Plasma Protein Binding of DPHM

Determination of plasma protein binding/unbound fraction (C_{Pu}) of DPHM was achieved using the equilibrium dialysis procedure described by Kumar et al. (Kumar et al., 2000) in steady-state plasma samples from each infusion step of the 5-step infusion studies.

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Drug Analysis

The concentrations of DPHM (C_{Pt}, C_{Pu}, C_{CSF} and C_{ECF}) in all samples were measured using a gas chromatographic-mass spectrometric assay capable of simultaneously measuring DPHM and [$^2H_{10}$]-DPHM with a limit of quantitation of 2.0 ng/mL (Tonn et al., 1993).

Data Analysis

All pharmacokinetics modeling was performed using WinNonlin®, version 1.1 (Scientific Consulting Inc., Apex, NC). Volume of distribution (Vd_{ss}) and total body clearance (Cl_T) were calculated using the following respective equations (Gibaldi and Perrier, 1982):

Vd_{ss} = Rate of DPHM infusion/($C_{Ptss} \bullet \beta$) (Equation 2)

Cl_T = Rate of DPHM infusion/C_{Ptss} (Equation 3)

where C_{Ptss} is the plasma total steady-state DPHM concentration, and β is the terminal elimination constant.

The extent of DPHM transfer into the brain in this study were calculated by relating the CSF and ECF $AUC_{0\rightarrow\infty}$ values to the plasma $AUC_{0\rightarrow\infty}$ value to yield the f_{CSF} and f_{ECF} ratios. Specifically, using f_{CSF} as an example, the CSF $AUC_{0\rightarrow\infty}$ was divided by the plasma $AUC_{0\rightarrow\infty}$ as follows:

$f_{CSF} = AUC_{CSF\ 0\rightarrow\infty}/AUC_{0\rightarrow\infty}$ (Equation 4)

The f_{ECF} value was calculated in the same manner using $AUC_{ECF\ 0\rightarrow\infty}$. This method of characterizing drug transfer across the BBB has been used in numerous other MD studies for many different drugs (Wang et al., 1993; Wong et al., 1993; de Lange et al., 1994; Potschka et al., 2002).

Statistical Analysis

Data were plotted using SigmaPlot® 9.0 (SPSS Inc., Chicago, IL). All data are reported as mean ± SEM. Statistical analyses were performed using NCSS 2000 (NCSS, Kaysville, UT). Pharmacokinetic parameters and concentrations were compared using one-way ANOVA followed by a Duncan's multiple comparison test. For statistical analysis of the difference in ECF and CSF DPHM concentrations, the paired t test was used. In occasions where normal distribution of data was not observed, a Kruskal-Wallis test was used instead of ANOVA. The significance level was $p < 0.05$ in all cases.

Results

The average age of the fetuses on the day of their experiments was 103.5 ± 1.2 days for the 100 d fetus group and 124.1 ± 0.5 days for the 120 d fetus group. For the lambs, the average age on the day of their experiments was 11.5 ± 0.7 days and 33.8 ± 0.5 days for the 10 d and 30 d lamb groups, respectively. For the adults, the average age on the day of their experiments was 5.2 ± 1.1 years old. Estimated mean fetal body weight for the 100 d fetal group was 1.1 ± 0.1 kg, and for the 120 d fetal group was 2.4 ± 0.1 kg. Fetal weights *in utero* were estimated from the weight at birth and the time between the experiment and the birth as described by Gresham *et al.* (Gresham et al., 1972). Mean lamb body weight for the 10 d lamb group was 7.1 ± 0.7 kg and for the 30 d

lamb group was 12.2 ± 0.5 kg, and mean adult ewe body weight was 74.6 ± 8.3 kg. Fetal blood gas and pH values during the experiment were within the normal range and did not change significantly (data not shown).

MD probe recovery rates ranged between 39.2 ± 4.2 to 48.0 ± 1.1 % across the different age groups (Table 1). However, the failure rate was high (~ 1/3 of the probes failed to work shortly after surgeries) and only animals that had functional probes are listed in Table 1. Most animals had one CSF and one ECF probe; however, 3 of the 30 d lambs had both probes implanted in the brain tissue (*i.e.* ECF) due to difficulties in locating the lateral ventricles for CSF probe insertion. Figure 1 presents semilogarithmic plots of mean DPHM CSF, ECF, and plasma concentrations *vs*. time for all age groups, while the mean concentrations for each infusion step are given in Table 2. From the profiles, it can be seen that CSF, ECF, and plasma concentrations increased in proportion to increases in infusion rate. The mean difference (±SEM) in DPHM concentration between CSF and ECF was 38.2 ± 14.2 ng/mL in all 5 infusion steps across all age groups, and this difference was not significantly different from zero; thus the concentrations in the 2 fluid compartments were identical. Steady-state concentrations were reached at the 4th hour of each infusion step, as no significant differences were observed among the concentrations in all three fluids beyond this point (ANOVA, $p>0.05$).

Upon examination of the overall C_{CSF}/C_{Pt} and C_{ECF}/C_{Pt} ratios (Tables 2 and 3), a trend existed where the brain concentrations started higher than plasma concentrations in the fetal groups, then became roughly equal to each other in the newborn lamb groups, and eventually dropped to levels lower than the plasma concentrations in the adult group.

The ratios for total drug started at about 2-3 in the fetal groups dropped to between 1 and 2 in the postnatal lambs and were below 1 in the adult group. Equilibrium dialysis was performed on the steady-state plasma samples to determine the extent of plasma protein binding (Table 4) and therefore yielding the plasma concentrations of unbound DPHM. Since plasma protein binding increased with advancing age and it is the free drug that crosses the BBB, the steady-state CSF (C_{CSF}) and ECF (C_{ECF}) concentrations were compared with the unbound plasma levels (C_{Pu}) to further examine the brain/blood concentration relationships at each infusion step (Table 3). When the ratios were calculated using the unbound plasma DPHM concentrations, the values at all ages were significantly greater than one (Table 3). There was a significant increase in the extent of protein binding between the 120 d fetus and 10 d lamb groups (Table 4). After birth, a significant increase in the level of plasma protein binding also occurred from 30 d lambs to adults . In contrast to the decreasing trend in the overall C_{CSF}/C_{Pt} and C_{ECF}/C_{Pt} ratios, the overall C_{CSF}/C_{Pu} and C_{ECF}/C_{Pu} ratios increased after birth (*i.e.* relative to the 120 d fetus values before dropping to adult values (Table 3).

As shown in Figure 1, DPHM CSF, ECF, and plasma elimination profiles appeared to follow two-compartmental pharmacokinetics following discontinuation of the drug infusion. Table 5 provides a summary of pharmacokinetics parameters. Total body (Cl_T) clearance increased with age until 30 d post-natal followed by a marked decrease in the adult. f_{CSF} and f_{ECF} values decreased significantly after birth, with both values at their lowest in the adult. This is more evident upon examination of the f_{CSF} and f_{ECF} values in Table 5, which shows a decreasing trend for f_{CSF} and f_{ECF} with respect to increasing age. A significant decrease in Vd_{ss} was observed with increasing age until 30 d postnatal, with

a significant, 5 fold increase in the adult. There was a significant increase in ECF elimination half-life with age, and a similar trend also existed for CSF and plasma half-lives.

Discussion

CSF, ECF, and Plasma Relationships in Relation to Age

CSF and ECF concentrations were not significantly different from each other in all age groups throughout the dose ranges studied (Figure 1, Table 2). This rapid equilibrium in concentrations suggests that transfer of DPHM between the 2 compartments involves a passive diffusion process. In fact, no transporters have been identified to date on the neuroependyma, which is the cell layer separating the ECF and CSF compartments (Davson and Segal, 1996). Also, this homogeneous distribution of the drug over the brain suggests lack of local metabolism (Kerr et al., 1984). As shown in Table 3, the C_{CSF}/C_{Pu} and C_{ECF}/C_{Pu} ratios from the different age groups were higher than 1, suggesting that DPHM is transported into the CNS in sheep against a concentration gradient. Many transporters are present at the BBB to deliver a range of substances into the CNS (Oldendorf, 1973), including sodium independent organic cation transporters (OCT) (Koepsell, 1998). A diverse group of organic cations, including endogenous bioactive amines (*i.e.* acetylcholine, choline, dopamine, epinephrine, norepinephrine, guanidine, thiamine), and therapeutic drugs (*i.e.* cimetidine, amiloride, morphine, quinidine, verapamil) are actively transported by the OCT system (Koepsell, 1998; Wu et al., 1998; Zhang et al., 1998; Lee et al., 2001). There is also evidence for saturable transporter mechanisms in the BBB for a number of lipophilic amine drugs

including propranolol, lidocaine, amphetamine, rimantadine. amantidine, pentazocine, and the histamine H_1-antagonist, mepyramine (Pardridge and Connor, 1973; Pardridge et al., 1984; Spector, 1988; Yamazaki et al., 1994; Suzuki et al., 2002a & b). Moreover, in rat studies involving the carotid injection technique (Suzuki et al., 2002a) and the *in situ* brain perfusion method (Suzuki et al, 2002b), the transport of pentazocine into the brain was inhibited by several compounds including DPHM, mepyramine and propranolol. This led the authors to suggest that these compounds (including DPHM) utilize a common cationic carrier-mediated influx system; however its precise identity is not yet known.

Both CNS and plasma DPHM concentrations showed corresponding increases with dose. Furthermore, a significant trend existed where CNS concentrations started higher than plasma concentrations in the fetal groups, approximated each other in the lamb groups, and finally became lower than plasma concentrations in the adult group (Figure 1, Table 2). Examination of C_{CSF}/C_{Pt} and C_{ECF}/C_{Pt} ratios reveals a similar trend (Tables 2, 3). Since only the unbound drug in plasma can cross the BBB, the relationships between brain and free plasma concentrations were also examined. Although the C_{CSF}/C_{Pu} and C_{ECF}/C_{Pu} ratios increased in the 10 and 30 day lambs compared to the fetal values (Table 3), this was primarily caused by the increase in protein binding that occurred after birth (Table 4). This resulted in a decrease in free DPHM concentration. Therefore, the increase seen in [Brain]/[Free Plasma] ratios for the postnatal lambs was due to division by smaller values (*i.e.* lower free DPHM concentrations), rather than an increase in transfer of DPHM into the CNS. A significant

increase in protein binding was also observed in the adults. However, the [Brain]/[Free Plasma] ratios in the adult are lower than that in the 120 d fetal group because of much lower brain concentrations of DPHM in the adult compared to the fetus. For example, step 5 C_{CSF} and C_{ECF} in the adult were 29.9% and 29.4% of the corresponding 120 d fetal values. Overall, trends from these ratios indicate that brain clearance of DPHM increased with age, which will be discussed further later with comparison of pharmacokinetic parameters.

Goldberg *et al*. have examined transport of DPHM in the CNS using a brain perfusion technique in rats (Goldberg et al., 1987). The concentration of DPHM in the CSF at steady state was approximately twice the unbound plasma concentration, which is similar to our results from the adult sheep (Table 3). Co-administration of choline with DPHM did not significantly inhibit DPHM transport through the BBB, indicating that a documented BBB transporter for weak bases, including choline, (Cornford et al., 1978) is not involved in DPHM transport into the CSF.

DPHM Pharmacokinetics in CSF, ECF, and Plasma

The total body clearance (Cl_T) of DPHM was higher in the postnatal lambs compared to the other age groups studied (Table 5), which is consistent with previous findings in our laboratory (Wong et al., 2000). In contrast to Cl_T, both f_{CSF} and f_{ECF} decrease following birth indicating that CNS drug elimination became more efficient with increase in age (Table 5). This is most likely due to development and maturation of efflux mechanisms with age (Saunders and Dzielgielewska, 1998).

In terms of distributional parameters, protein binding increased with age (Table 4) and this finding is consistent with results from previous studies in sheep (Kumar et al., 2000). This increase in protein binding is largely responsible for the lower total body clearance value in the adult compared to the fetus and lamb (Table 5), consistent with our previous study (Kumar et al., 2000). On the other hand, Vd_{ss} dropped postnatally compared to fetal values (Table 5). A decrease in Vd_{ss} following birth is expected since drug administered post-natally cannot distribute to the maternal compartment that is available to the fetus. Wong *et al*. also observed similar decreases in Vd_{ss} in their study in post-natal lambs (Wong et al., 2000). Vd_{ss} in the adult is significantly higher than the fetal and lamb values, likely due to the buildup of body fat (Smith et al., 1987; Owens et al., 1993). At the same time, Cl_T is significantly lower in adults. Since $t_{1/2}$ varies as Vd/Cl_T, a higher Vd but lower Cl_T value in the adult, relative to the fetal and lamb groups, should lead to a significantly longer elimination half life in the adult, which is what we observed (Table 5).

The relatively short $t_{1/2\beta}$ values in CSF and ECF indicated that DPHM was rapidly eliminated from these compartments. Goldberg *et al*. also observed rapid rate of efflux of DPHM from rabbit choroid plexus determined using a brain homogenate method (Goldberg et al., 1987). Because DPHM is, to a great extent, ionized at physiologic pH, simple diffusion of the drug back to cerebral circulation alone does not seem adequate to explain this observation. Besides diffusion, substances can leave the CNS via two other mechanisms. One comprises efflux (transporter-mediated or not) *via* brain or choroidal

blood. The second route involves efflux *via* bulk flow of CSF draining into either the lymphatic system or venous blood through the arachnoid villi in the superior sagittal sinus (Saunders and Dzielgielewska, 1998). The latter phenomenon is responsible for what is termed the sink effect. While CSF flow is a normal physiological process that happens regardless of the substance, transporter-mediated efflux is a substrate specific process. As noted previously, there is evidence for a cationic carrier-mediated influx system for pentazocine, DPHM and other cationic substances (Suzuki et al., 2002 a & b). Moriki et al (2004) have provided evidence that P-glycoprotein acts as BBB efflux transporter for this compound. However, DPHM is not a P-glycoprotein substrate (Chen et al., 2003), although we have previously shown that propranolol, which inhibits P-glycoprotein (Bachmakov et al., 2006), significantly increases the brain/plasma ratios of DPHM in adult sheep (Au-Yeung et al., 2006). The identity of CNS efflux transporter for DPHM remains to be determined.

CNS Effects of DPHM in Fetus

In a previous study of DPHM in pregnant sheep, DPHM administration decreased the incidence of fetal low voltage electrocorticographic (ECoG) pattern, rapid eye movements, and fetal breathing (Rurak et al., 1988). These sedative-like effects occurred at fetal plasma drug concentrations (~ 36 ng/mL) lower than those (> 50 ng/mL) which result in discernible CNS effects in adults (Carruthers et al., 1978). The fetal plasma concentration of 36 ng/mL is slightly higher than the concentration of 26.9 ng/mL achieved during the step 1 infusion in the 120 d fetal group in the current study (Table 2). The brain ECF concentration at step 1 was 51 ng/mL, and in adult ewes this ECF concentration was not reached until infusion step 2, when the plasma DPHM level was

169.3 ng/mL. This is considerably higher than the plasma concentration of the drug associated with CNS effects in adult humans. Thus, greater exposure of the fetal brain to DPHM appears to explain its greater CNS effects.

In terms of the relevance of these observations to human, fetal exposure to DPHM following maternal bolus administration is extensive (*i.e.* AUC fetal/AUC maternal = 0.85) (Yoo et al., 1986). In humans, peak plasma concentrations following a 50 mg oral dose are between 40 - 80 ng/mL (Carruthers et al., 1978). Assuming that placental transfer of DPHM in human is similar to sheep, a 50 mg oral dose to a pregnant woman would result in peak plasma concentrations of 34 – 68 ng/mL in fetal plasma. This would result in fetal brain concentrations of ~ 70 – 140 ng/mL. Thus, alterations in the behavior of human fetus are likely to occur with maternal administration of normal oral doses of the drug.

In summary, use of in vivo microdialysis probes has allowed us to study the disposition of DPHM in the CNS of sheep as a function of post-conceptual age. Brain ECF and CSF concentrations of the drug were higher in the fetal and postnatal lambs than in the adult and this may be due to immaturity of a CNS efflux transporter for the drug. Finally, the higher levels of DPHM in the fetal CNS appear to explain our previous observations of greater CNS effects of the drug at this age.

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Footnotes

A) Financial support for this work was provided by the Canadian Institute of Health Research (CIHR). Sam CS Au-Yeung is a recipient of a CIHR Doctoral Fellowship. The current project is a part of Sam CS Au-Yeung's Ph.D thesis at UBC. Part of this work was previously presented as a poster in the 2002 ISSX meeting held in Orlando, FL. Below is the citation of the abstract: Sam CS Au Yeung, Nancy Gruber, K Wayne Riggs, and Dan Rurak (2002) Investigation of the CNS pharmacokinetics of diphenhydramine (DPHM) in sheep using microdialysis. *Drug Metab Rev* 34(1): 134.

Figure 1. Average (±SEM) plasma, CSF, and ECF concentrations versus time plots in 100d fetuses (A, n=3); 120 day fetuses (B, n=7), 10 day lambs (C, n=5), 30 day lambs (D, n= 4) and adult ewes (E, n=8) during 5 step i.v. infusions of diphenhydramine. The duration of each infusion step was 7 h.

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Table 1. Summary of MD probe recovery rates in the 5-step infusion experiment

		Recovery (%)[a]				
Age	**Probe**	**Step 1**	**Step 2**	**Step 3**	**Step 4**	**Step 5**
Fetus (100 d)	**CSF** (n=2)	39.9	41.7	40.5	39.2	40.1
	ECF (n=2)	42.6	43.6	42.4	42.8	41.9
Fetus (120 d)	**CSF** (n=4)	41.6 ± 2.3	41.6 ± 2.0	40.8 ± 1.5	39.9 ± 1.8	40.2 ± 2.1
	ECF (n=5)	42.8 ± 1.7	42.6 ± 1.9	42.3 ± 1.7	41.8 ± 1.4	40.5 ± 1.8
Lamb (10 d)	**CSF** (n=4)	44.0 ± 1.6	43.5 ± 2.1	44.9 ± 2.0	44.1 ± 2.2	43.5 ± 1.4
	ECF (n=5)	42.9 ± 1.3	44.0 ± 1.6	43.9 ± 1.4	42.8 ± 1.8	41.4 ± 1.3
Lamb (30 d)	**CSF** (n=1)	44.8	44.5	43.0	43.2	44.2
	ECF (n=6)	46.6 ± 0.9	44.8 ± 1.1	45.1 ± 1.2	44.3 ± 1.1	44.9 ± 1.1
Adult	**CSF** (n=6)	46.3 ± 1.6	48.0 ± 1.1	46.1 ± 1.6	45.8 ± 1.1	43.3 ± 1.4
	ECF (n=5)	47.9 ± 1.6	47.4 ± 1.1	46.2 ± 0.9	44.8 ± 1.5	45.4 ± 1.6

Data are shown as mean ± SEM

[a]Probe recovery rates were calculated using Equation 1.

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Table 2. Mean DPHM CSF, ECF, and Plasma Concentrations at Steady State for each infusion step.

Biological Fluid	Step	100d Fetus[a]	120d Fetus[b]	10d Lamb[c]	30d Lamb[d]	Adult[e]
C_{CSF} (ng/mL)	1	40.3	43.1±8.5	42.7±7.2	43.7	20.0±1.7
	2	76.8	168.7±40.0	109.7±22.5	147.3	54.4±6.5
	3	194.9	357.1±63.2	252.5±39.5	279.5	112.8±17.4
	4	303.4	520.9±101.0	575.2±111.0	539.9	158.0±23.7
	5	368.2	741.5±153.2	861.4±65.4	854.5	221.9±36.3
C_{ECF} (ng/mL)	1	34.3	51.0±7.5	60.3±12.5	53.1±5.5	20.2±2.0
	2	94.9	174.6±31.7	135.4±25.4	227.0±23.1	56.7±11.1
	3	209.0	345.5±56.7	291.2±47.8	413.8±33.2	119.6±28.1
	4	306.1	542.7±96.4	583.3±111.1	667.8±26.2	165.6±32.2
	5	386.2	727.2±129.5	897.4±120.2	1186.4±93.5	214.0±44.5
C_{Pt} (ng/mL)	1	13.2±3.8	26.9±5.7	43.5±20.0	47.4±4.1	62.9±7.1
	2	36.7±4.5	79.4±11.4	133.8±73.3	168.1±32.1	169.3±23.2
	3	73.3±11.8	138.3±18.5	340.9±240.0	311.8±54.6	306.6±41.1
	4	127.4±7.7	205.4±27.4	600.9±337.2	473.4±100.9	453.8±76.7
	5	161.5±9.0	297.6±42.1	1004.2±499.3	757.9±121.0	599.3±104.7

Data are presented as Mean ± SEM

Asterisks (*) depict C_{BRAIN} values that are significantly different from the respective C_{Pt} values as determined by one way ANOVA ($p<0.05$).

[a] n=2 for C_{CSF}, n=2 for C_{ECF}, n=3 for C_{Pt}
[b] n=4 for C_{CSF}, n=5 for C_{ECF}, n=7 for C_{Pt}
[c] n=4 for C_{CSF}, n=5 for C_{ECF}, n=5 for C_{Pt}
[d] n=1 for C_{CSF}, n=6 for C_{ECF}, n=4 for C_{Pt}
[e] n=6 for C_{CSF}, n=5 for C_{ECF}, n=8 for C_{Pt}

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Table 3. Overall C_{CSF}/C_{Pt}, C_{ECF}/C_{Pt}, C_{CSF}/C_{Pu}, and C_{ECF}/C_{Pu} ratios for each infusion step.

	C_{CSF}/C_{Pt}	C_{ECF}/C_{Pt}	C_{CSF}/C_{Pu}	C_{ECF}/C_{Pu}
100 d Fetus	2.3^{a}	2.3^{a}	6.5^{a}	6.5^{a}
120 d Fetus	2.3 ± 0.5^{1b}	2.1 ± 0.4^{1d}	4.7 ± 1.2^{1b}	4.5 ± 0.7^{1d}
10 d Lamb	1.1 ± 0.1^{2b}	1.3 ± 0.3^{2d}	5.6 ± 0.5^{1b}	6.6 ± 0.4^{2d}
30 d Lamb	0.7^{e}	1.6 ± 0.3^{2c}	4.9^{e}	6.6 ± 0.8^{2c}
Adult	0.4 ± 0.1^{3c}	0.4 ± 0.0^{3d}	3.4 ± 0.1^{2c}	3.4 ± 0.3^{3d}

Data are shown as mean ± SEM

The overall ratios are averages of individual ratios from the 5 steps.

$^{a}n=2$; $^{b}n=4$; $^{c}n=6$; $^{d}n=5$; $^{e}n=1$

Values with different number superscripts (1-3) in each column are statistically different as determined by the Duncan's multiple comparison test ($p<0.05$).

Tables (4 of 5)

Table 4. Extent of protein binding in steady-state plasma samples for each infusion step.

Age Group	Step 1	Step 2	Step 3	Step 4	Step 5	Overall
100d Fetus n=3	0.60 ± 0.09	0.60 ± 0.10	0.61 ± 0.09	0.66 ± 0.14	0.65 ± 0.10	**0.63 ± 0.12[1]**
120d Fetus n=7	0.62 ± 0.08	0.51 ± 0.02	0.52 ± 0.02	0.54 ± 0.02	0.54 ± 0.03	**0.55 ± 0.02[1]**
10d Lamb n=5	0.76 ± 0.04	0.80 ± 0.05	0.79 ± 0.06	0.80 ± 0.05	0.80 ± 0.05	**0.79 ± 0.01[2]**
30d Lamb n=4	0.82 ± 0.05	0.79 ± 0.05	0.76 ± 0.04	0.73 ± 0.01	0.74 ± 0.04	**0.77 ± 0.02[2]**
Adult n=8	0.86 ± 0.02	0.89 ± 0.02	0.87 ± 0.02	0.86 ± 0.03	0.83 ± 0.03	**0.86 ± 0.01[3]**

Data are shown as mean ± SEM

Values with different numbers (1-3) are statistically different as determined by the Duncan's multiple comparison test ($p<0.05$).

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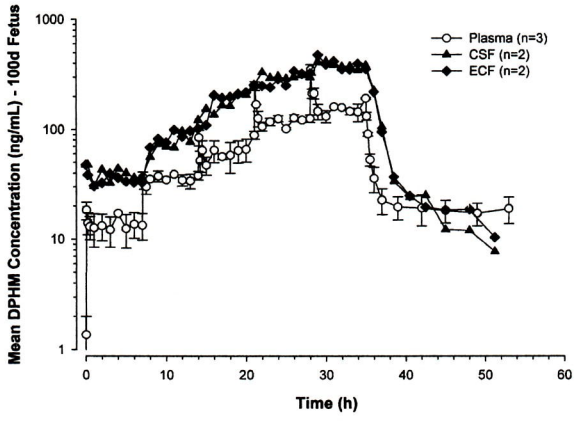
Table 5. Pharmacokinetic parameters for fetal (100 & 120d), lamb (10 & 30d), and adult sheep.

		AGE GROUP			
Sample	**Parameter**	**100d Fetus**	**120d Fetus**	**10d Lamb**	**30d Lam**
Plasma	Cl_T(mL/min/kg)	106.5 ± 19.6^{a1}	110.0 ± 4.6^{b1}	126.4 ± 3.6^{c2}	$145.4 \pm 5.$
	Vd_{ss}(L/kg)†	14.8 ± 1.7^{1}	13.9 ± 0.8^{1}	9.7 ± 0.6^{2}	9.1 ± 0.2
	$t_{1/2\beta}$(h) †	4.6 ± 1.1^{1}	3.6 ± 0.4^{1}	4.9 ± 0.9^{1}	4.5 ± 1.3
	$t_{1/2\alpha}$(h) †	0.3 ± 0.1^{1}	$0.4 \pm 0.0^{1,4}$	1.2 ± 0.4^{2}	0.7 ± 0.2
CSF	f_{CSF} †	1.9^{h}	2.5 ± 0.2^{g1}	1.1 ± 0.3^{g2}	0.6^{e}
	$t_{1/2\beta CSF}$(h)	1.2	1.6 ± 0.2^{1}	2.2 ± 0.8^{1}	2.2
	$t_{1/2\alpha CSF}$(h)	1.0	0.9 ± 0.1	1.8 ± 0.3^{2}	1.7
ECF	f_{ECF} †	2.0^{h}	2.2 ± 0.2^{c1}	0.9 ± 0.2^{c2}	1.4 ± 0.3
	$t_{1/2\beta ECF}$(h)	1.0	1.5 ± 0.2	2.5 ± 0.8^{2}	2.4 ± 1.1
	$t_{1/2\alpha ECF}$(h)	0.9	0.8 ± 0.2	1.5 ± 0.6^{2}	1.6 ± 0.6

Data are shown as mean±SEM
^{a}n=3; ^{b}n=7; ^{c}n=5; ^{d}n=8; ^{e}n=1; ^{f}n=6; ^{g}n=4; ^{h}n=2
Values with different number superscripts (1-4) in each row are statistically different as determined by the Duncan's multiple comparison test ($p<0.05$); Comparisons were made by ANOVA;
†Comparisons were made by Kruskal-Wallis Test followed by Duncan's multiple comparison test

A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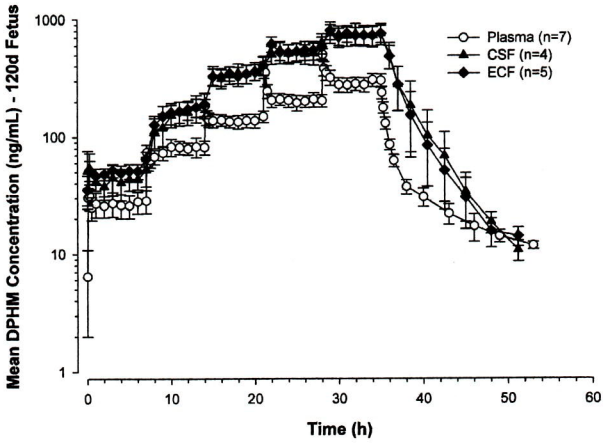
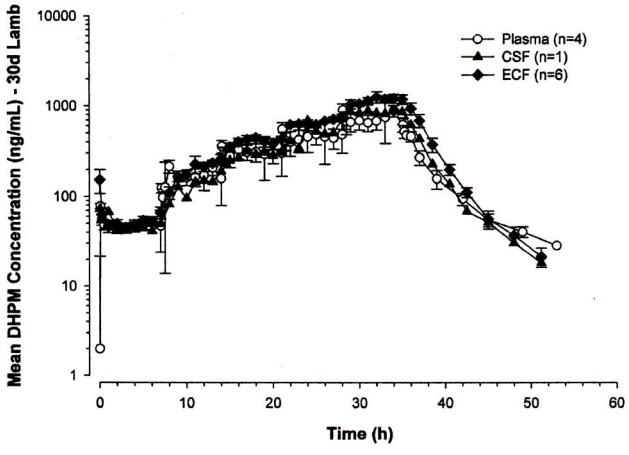
Plasma (n=3)
CSF (n=2)
ECF (n=2)
Time (h)

B


Mean DPHM Concentration (ng/mL) - 120d Fetus
Plasma (n=7)
CSF (n=4)
ECF (n=5)
Time (h)

C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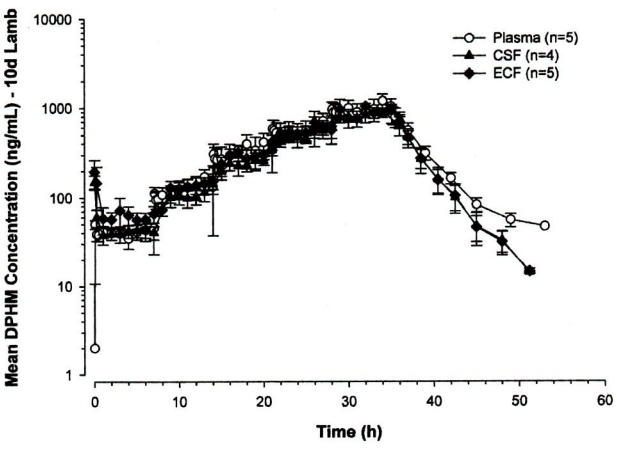
Plasma (n=4)
CSF (n=1)
ECF (n=6)
Time (h)

D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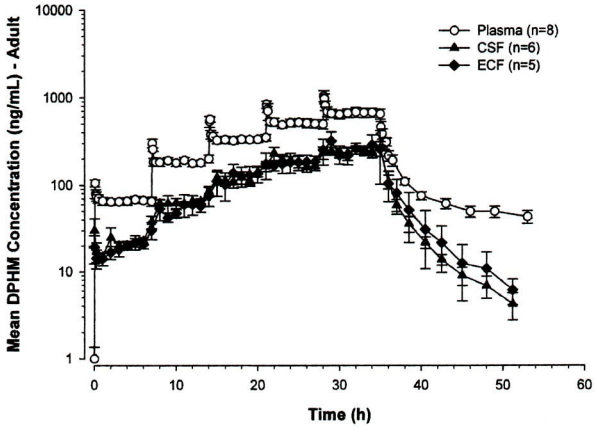
ECF (n=5)
Time (h)

E


Mean DPHM Concentration (ng/mL) - Adult
Plasma (n=8)
CSF (n=6)
ECF (n=5)
Time (h)