Stereoselective Pharmacokinetics and Placental Transfer of Fluoxetine and Norfluoxetine in Pregnant Sheep Under Steady-State Conditions

by

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Abstract

Fluoxetine (FX) is one of the most popular SSRIs used for the treatment of depression. Its use in treating depressed pregnant woman has increased substantially in recent years. A considerable amount of attention in the literature regarding the use of FX in pregnancy has been devoted to the assessment of birth outcomes following *in utero* FX exposures while studies that focus on pharmacokinetics during pregnancy are relatively sparse. Previous FX experiments in pregnant sheep using single dose intravenous (*i.v.*) bolus administration have focused primarily on the acute exposure and effects of the drug on both the mother and fetus. In the present studies, experiments were conducted to investigate the stereoselective pharmacokinetics of FX and norfluoxetine (NFX) under steady-state conditions in nonpregnant sheep and pregnant sheep at late gestation via continuous 8-day *i.v.* infusion of FX to the ewe. Drug infusions were conducted at two dose levels (i.e. low dose and high dose) in nonpregnant sheep that allowed the examination of nonlinear pharmacokinetics of FX in sheep. The pregnant sheep infusion experiment was to determine the impacts of pregnancy on the stereoselective pharmacokinetics of FX and NFX, and to assess the exposure of the fetus to FX and NFX enantiomers at steady-state. Moreover, a continuous 4-day direct fetal *i.v.* FX infusion experiment was conducted to study the pharmacokinetics of FX and NFX in fetal lambs in attempt to explore intrinsic drug elimination capability by the fetus.

In nonpregnant sheep, FX and NFX enantiomers exhibited significant pharmacokinetic differences under steady-state conditions, confirming the observations made in previous *i.v.* bolus experiments. For FX, the R-enantiomer had a lower steady-state concentration (C_{ss})

level but a longer elimination half-life ($t_{1/2\beta}$) than the S-enantiomer while for NFX, both the R- and S-enantiomers had similar C_{ss} levels but the $t_{1/2\beta}$ was slightly longer for R-NFX compared to S-NFX. FX and NFX also displayed stereospecific protein binding, with free fractions of the R-enantiomers that were about 2 to 3-fold higher than those of the S-enantiomers. In comparison to humans, quantitative differences exist but qualitatively the overall stereoselective disposition of FX and NFX are similar between sheep and humans. Using the literature reported value for hepatic blood flow in sheep, the intrinsic clearances (CL_{int}) for the FX enantiomers were determined. CL_{int} of R-FX was estimated to be ~50% higher than that of S-FX, indicating that, in addition to stereoselective protein binding, stereoselective metabolism also played a pivotal role in the overall stereoselective disposition of FX. Moreover, FX displayed a disproportional change in pharmacokinetics in sheep upon long-term administration, characterized by a decrease in total body clearance (CL_{TB}) and increase in dose normalized area under the curve (AUC) with higher dose. This nonlinearity may be due to inhibition of a CYP2D6-like enzyme in the sheep liver, as this is the major drug metabolizing enzyme for FX in humans.

In pregnant sheep, the stereoselective pharmacokinetics of FX and NFX under steady-state conditions were similar to those observed in nonpregnant animals. No remarkable changes in the S/R ratio of FX and NFX were observed. Similarly, no change in FX clearance was observed as opposed to the previous finding of a higher clearance in pregnant sheep following single *i.v.* bolus administration. Protein binding did not appear to differ between pregnant and nonpregnant sheep for either FX and NFX. FX and NFX exhibited moderate degrees of placental transfer upon chronic administration, with a fetal-to-maternal (F/M) AUC ratios of about 0.40 and 0.33, respectively. When concentrations were corrected for the

difference in free fraction between the maternal and fetal compartments, the F/M ratio still remained less than unity, suggesting the presence of other nonplacental routes of elimination for FX in the fetus. Analyses of amniotic and fetal tracheal fluids showed that excretion of FX and NFX into these fluids resulted in levels not exceeding those in fetal plasma and significant accumulations were therefore unlikely. Comparisons of F/M ratios between the R- and S-enantiomers revealed no difference between R-FX and S-FX, while S-NFX was slightly higher (~25%) compared to R-NFX, indicating that NFX distributed between the mother and the fetus in a stereoselective manner.

Following fetal *i.v.* infusion of FX, alterations in fetal blood gas status, characterized by decreases in P_{O_2} and O_2 saturation, and increases in P_{CO_2} and pH, were noted. Due to variability between animals, the majority of these effects did not reach statistical significance. The stereoselective pharmacokinetics of FX and NFX in fetal lambs were similar to those observed in adult sheep, but the extent of stereoselectivity in terms of the S/R ratio was less. The differential steady-state concentrations of the FX enantiomers observed in the fetal circulation were likely due to stereoselective protein binding in fetal plasma and differential maternal-to-fetal placental transfer of the enantiomers preceded by maternal stereoselective metabolism. Based on the F/M ratios for NFX (~0.62) following fetal FX infusion, it appears that the fetus has limited metabolic capability, if any, to biotransform FX to NFX. It is not fully known, however, whether other metabolic pathways such as the formation of trifluoromethylphenol via *O*-dealkylation are functional in the fetus. Renal excretion of FX and NFX did not contribute substantially to their overall clearance in the fetus (<1% of CL_{TB}). In comparison to maternal and nonpregnant adult sheep, weight normalized CL_{TB}, CL_{renal}, and Vd_{ss} were significantly higher in the fetus. Unbound fractions of FX and NFX

were also higher in the fetus owning to the lower concentration of plasma protein in fetal plasma. The intrinsic capability of the fetus to eliminate FX was assessed by calculating the maternal and fetal placental and nonplacental clearances based on the 2-compartment model for the maternal-fetal unit. The results indicated that both placental and nonplacental routes were equally important in fetal elimination of FX. It is concluded that other elimination routes are likely present in the fetus but these remain to be elucidated.

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List of Abbreviations

5-HT	5-Hydroxytryptamine or serotonin
μ	Micron
°C	Degree Celsius
μg	Microgram
μl	Microliter
μm	Micrometer
μM	Micromolar
~	Approximately
AAG	α_1-acid glycoprotein
ADME	Absorption, distribution, metabolism, elimination
AIC	Akaike's Information Criterion
AMN	Amniotic
ANOVA	Analysis of variance
AUC	Area under the plasma concentration *vs.* time curve
AUC^{0-t}	Area under the curve from time zero to the last time point sampled
$AUC^{0-\infty}$	Area under the curve from time zero to infinity
AUC_m	Area under the curve of metabolite
$AUMC^{0-\infty}$	Area under the first moment curve from time zero to infinity
β	Terminal elimination rate constant
BW	Body weight
C_b	Drug concentration in buffer
C_f	Fetal drug concentration
C_m	Concentration of metabolite or maternal drug concentration
C_t	Last time point sampled
CL_H	Hepatic clearance
CL_{ff}	Fetal total clearance

CL_{fm}	Placental clearance from the fetus to the mother
CL_{fo}	Fetal nonplacental clearance
CL_{int}	Intrinsic clearance
CL_{m}	Elimination clearance of metabolite
CL_{mm}	Maternal total clearance
CL_{mo}	Maternal nonplacental clearance
CL_{mf}	Placental clearance from the mother to the fetus
$CL_{non\text{-}renal}$	Non-renal clearance
CL_{renal}	Renal clearance of the total drug
CL_{TB}	Total body clearance based on total drug concentrations
C_{max}	Maximal plasma concentration
C_{p}	Plasma concentration of the total drug
C_{ss}	Steady-state concentration
$C_{ss(m)}$	Steady-state plasma concentration of metabolite
C_{u}	Plasma concentration of the unbound drug
CYP	Cytochrome P-450 enzyme
d	Day
Da	Dalton
DM	Dextromethorphan
DPHM	Diphenhydramine
DPMA	Diphenylmethoxyacetic acid
DX	Dextrorphan
E	Extraction ratio
EDTA	Ethylenediaminetetraacetic acid
EI	Electron impact
EM	Extensive metabolizer
eV	Electron volt

F	Systemic bioavailability
F/M	Fetal-to-maternal
FA	Fetal femoral arterial plasma
f_a	Total fraction of the administered dose absorbed
f_m	Fraction of drug converted to metabolite
f_u	Unbound fraction
FX	Fluoxetine
g	Gram
GC	Gas chromatography
GC-MS	Gas Chromatography Mass Spectrometry
GFR	Glomerular filtration rate
GI	Gastrointestinal
h	Hour
HD	High dose
HPLC	High performance liquid chromatography
i.d.	Internal diameter
i.e.	*id est;* that is
i.v.	Intravenous
k	Overall first order elimination rate constant
k_a	First order absorption rate constant
k_f	Formation rate constant of metabolite from parent drug
kg	Kilogram
K_i	Inhibitor affinity for an enzyme, the inhibitory constant
k_m	Elimination rate constant of metabolite
K_m	Substrate concentration at which the reaction velocity is at half -maximal, a Michaelis-Menten parameter
k_o	Infusion rate
l	Liter

LC	Liquid chromatograph
LD	Low dose
log P	Logarithm of partition coefficient (octanol-to-water)
LOQ	Limit of quantitation of the assay
M	Molar (moles/litre)
MA	Maternal arterial plasma
mEq	Milliequivalent
mg	Milligram
min	Minute
ml	Milliliter
mm	Millimeter
mM	Millimolar
mm Hg	Millimeter of mercury
MRT	Mean residence time
MS	Mass spectrometry
MW	Molecular weight
n	Number of subjects or animals
NFX	Norfluoxetine
ng	Nanogram
nmol	Nanomole
o.d.	Outer diameter
O_2 Sat	Oxygen Saturation
PBS	Phosphate-buffered saline
P_{CO_2}	Partial gas pressure of carbon dioxide
PD	Pharmacodynamic
P-gp	P-glycoprotein
pH	Negative logarithm of hydrogen ion concentration

PK	Pharmacokinetic
pKa	Negative logarithm of acid association constant
PM	Poor metabolizer
pmol	picomole
P_{O_2}	Partial gas pressure of oxygen
psi	Pound per square inch
Q1	Bottom quartile
Q3	Top quartile
QC	Quality control
Q_H	Hepatic blood flow
r^2	Coefficient of determination
r_s	Spearman rank correlation
R-FX	R-fluoxetine
R-NFX	R-norfluoxetine
RSD	Relative standard deviation
SC	Schwarz Criterion
S/R	S-to-R ratio
S.D.	Standard deviation
S.E.	Standard error of mean
S-FX	S-fluoxetine
SIM	Single ion monitoring
S-NFX	S-norfluoxetine
SSRI	Selective serotonin reuptake inhibitor
τ	Dosing interval
$t_{1/2\beta}$	Apparent terminal elimination half-life
TCAs	Tricyclic antidepressants
TEA	Triethylamine

TFAP	(S)-Trifluoroacetylprolyl chloride
TFMP	Trifluoromethylphenol
t_{max}	Time of occurrence of maximal plasma concentration
TR	Fetal tracheal
UGT	UDP-glucuronosyltransferase
Vd	Volume of distribution
Vd_m	Volume of distribution of metabolite
Vd_{ss}	Apparent steady-state volume of distribution
V_{max}	Maximal velocity of an enzymatic reaction, a Michaelis-Menten parameter
X_u	Cumulative amount of drug or metabolite collected in urine

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A final tribute is given to my Lord Jesus Christ for His countless blessings throughout every path of my life. The completion of this Ph.D. work is not possible without Him.

"*Unless the Lord builds the house, its builders labor in vain. Unless the Lord watches over the city, the watchmen stand guard in vain.*"

Psalm 127:1

CHAPTER 1

INTRODUCTION

Preface

Although it has been long known that stereoisomers of a chiral drug often exhibit pronounced differences in their pharmacokinetic and pharmacodynamic properties both in quantitative and qualitative terms, more than 500 drugs are currently marketed as racemic mixtures without relevant pharmacokinetic and pharmacodynamic information for each individual stereoisomer (Aboul-Enein and Wainer, 1997). This neglect of stereochemistry in pharmaceutical research was wide spread until Ariens' famous critical review of sophisticated scientific nonsense was published (Ariens, 1984). It was Ariens' review that finally incited drug researchers to consider the importance of stereoselective differences. The advent of efficient methods for large-scale stereoselective organic synthesis and the introduction of chromatographic systems for stereoselective analysis now make it much easier to study enantiomer drugs and their metabolites in biological fluids. As a consequence our knowledge of differences in individual enantiomer pharmacodynamic and/or pharmacokinetic properties as a result of stereoselective interaction with optically active biological macromolecules is mounting (Campbell, 1990; Wright and Jamali, 1993; Srinivas et al., 2001). The study of stereoselective pharmacokinetics in the context of pregnancy, however, remains very limited.

During pregnancy, a number of physiological functions undergo substantial changes which will significantly alter the pharmacokinetics/pharmacodynamics of drugs as well as the subsequent degree of exposure to the developing fetus (Loebstein et al., 1997). The naive

assumption that for therapeutic purposes a pregnant woman is no more than a nonpregnant woman with a fetus attached is seriously flawed. Although it is not totally clear whether pregnancy will affect the stereoselective properties of drug disposition, physicians should be aware of these changes since this knowledge will define the principles that guide the therapeutic use of racemic drugs in pregnant women.

Unfortunately, information on stereoselective pharmacokinetics and fetal drug exposure in human pregnancy is fragmentary and generally unsatisfactory. There is not a single drug with which a reasonably complete picture can be presented. In an experimental setting, drugs can only be given ethically for a clinical need so that standardized conditions can seldom be achieved. Well-designed experiments using appropriate animal models are therefore necessary to provide detailed pharmacokinetic and fetal drug exposure data that cannot be obtained ethically and practically in human subjects.

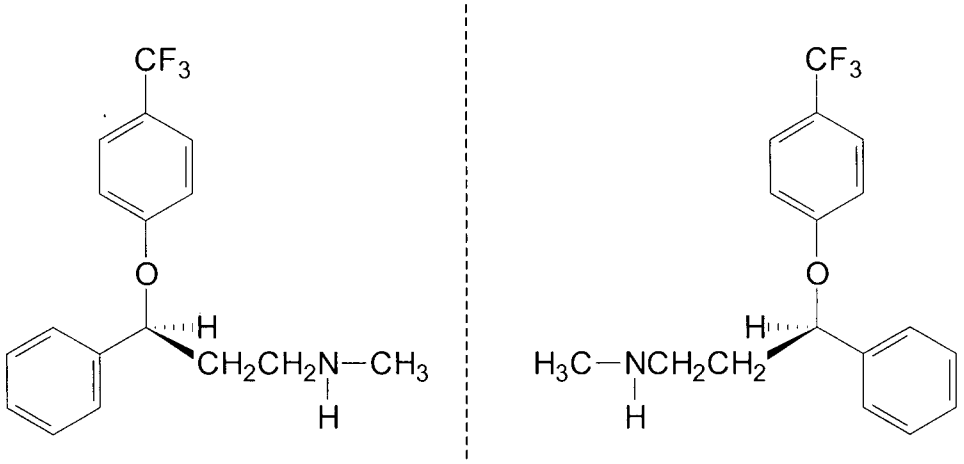
This thesis is an investigation of the stereoselective pharmacokinetics and placental transfer of the selective serotonin reuptake inhibitor fluoxetine (FX) and its metabolite, norfluoxetine (NFX) in chronically catheterized pregnant sheep. Fluoxetine was chosen as the study compound based on its 1) extensive use in the pregnant population, 2) unique stereoselective pharmacokinetic and pharmacodynamic properties, and 3) nonlinear characteristics upon multiple dosing. Our previous studies involving a single *i.v.* bolus dose of racemic fluoxetine revealed significant stereoselective disposition and considerable fetal exposure of fluoxetine in sheep (Kim et al., 2004). The present study was undertaken to further examine the stereoselective pharmacokinetics and placental transfer of fluoxetine in the mother and fetus under steady-state conditions, a more clinically relevant situation in human therapeutics. It is hoped that the information obtained from our studies will add to the

knowledge currently available in the literature and allow for a more rational approach to drug therapy in pregnancy.

1.1 BACKGROUND

1.1.1 Physicochemical Properties

Fluoxetine (FX), (±)-*N*-methyl-3-phenyl-3-[(α,α,α-trifluoro-*p*-tolyl)-oxy] propylamine contains one asymmetric center which gives rise to the R- and S-enantiomers (Figure 1.1). The commercially available drug is a racemic mixture of 2 optical isomers. It has a low molecular weight of 309.33 Daltons (Da). It is a weakly basic amine of high lipophilicity, with pK_a and octanol/water partition coefficient (Log P) of 9.97 and 4.05, respectively (Florey et al., 1990; Hansch et al., 1990).



S-fluoxetine R-fluoxetine

FIGURE 1.1 Chemical structure of fluoxetine enantiomers.

1.1.2 Pharmacology and Therapeutic Uses

FX belongs to a class of therapeutic agents called the ***S***elective ***S***erotonin ***R***euptake ***I***nhibitors (SSRI). It acts by inhibiting the serotonin reuptake pump thereby increasing the amount of serotonin (5-HT) available at the cerebral receptor sites, which is related to antidepressant action. The 1988 introduction of fluoxetine revolutionized the treatment of depression. Due to its low affinity for norepinephrine and dopamine reuptake sites and the lack of effects on the muscarinic, cholinergic, histaminic, and $alpha_1$-adrenergic receptors at usual therapeutic dosages, FX is devoid of life-threatening side effects of traditional tricyclic antidepressants (TCAs), such as cardiotoxicity and CNS toxicity, as well other intolerable side effects that include dry mouth, abnormal vision, memory impairment, constipation, and urinary retention (Gram, 1994). The favorable safety profile of the SSRIs makes FX widely used in the treatment of depression as well as other psychological disorders, including anxiety, obsessive-compulsive disorders, bulimia nervosa and premenstrual dysphoric disorders. FX is used at dosages of 20 to 80 mg/day (PDR, 2002). The recommended starting dose of 20 mg has been shown to be the most frequently used dose in the majority of patients and also the best tolerated (Beasley et al., 2000).

1.1.3 Pharmacokinetics

In humans, FX is well absorbed with a bioavailability of ~72-90% after oral administration. Following a single oral 40 mg dose, peak plasma concentrations of FX ranging from 15 to 55 ng/ml were observed after 6 to 8 hours (PDR, 2002). It is highly protein bound with 95% of the drug bound to plasma proteins (Hiemke and Hartter, 2000). Alpha 1-acid- glycoprotein (AAG) is believed to be the major plasma protein responsible for

FX binding (Holladay et al., 1998). FX is highly distributed into tissues and has a volume of distribution (Vd) of 14-100 l/kg. The lungs show the highest accumulation of FX, and lysosomal trapping is considered to play a role in the high Vd of FX (Daniel and Wojcikowski, 1997). The elimination half-life in humans is about 1-4 days, which is the longest among other agents in this class of drugs. Urinary excretion of the intact drug is low, with less than 11% of administered dose excreted unchanged; therefore metabolism accounts for the majority of the clearance of fluoxetine (Catterson and Preskorn, 1996). Fluoxetine exhibits nonlinear pharmacokinetics based on evidence of a prolonged half-life (to approximately 4-5 days) and a disproportionate increase in plasma concentration, which occurred after administration of multiple versus single doses of the drug (DeVane, 1994; Hiemke and Hartter, 2000).

1.1.4 Metabolism

Fluoxetine undergoes extensive biotransformation. One of its major metabolites is the N-demethylated product, norfluoxetine (NFX) (Figure 1.2).

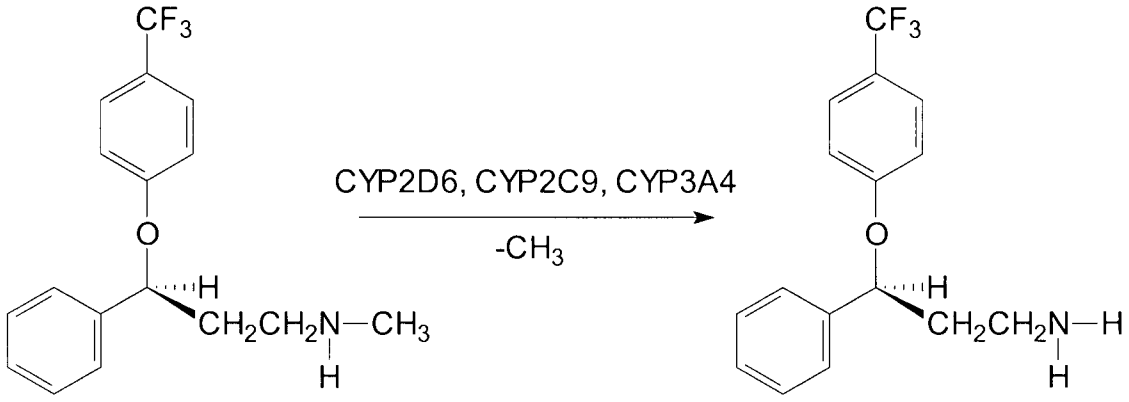


FIGURE 1.2 *N*-demethylation of fluoxetine to norfluoxetine.

This *N*-demethylation pathway is mediated by cytochrome P450 (CYP) enzymes. Data from clinical pharmacokinetic studies suggest a pivotal role for CYP2D6 in the clearance of FX because the area under the FX concentration versus time curve was higher in CYP2D6 poor metabolizers than extensive metabolizers (Hamelin et al., 1996; Fjordside et al., 1999), while *in vitro* studies show that multiple CYP enzymes are involved in the formation of NFX (von Moltke et al., 1997). Scaled intrinsic clearance values, used to estimate the contribution of individual CYP enzymes in human liver microsomes, suggest that CYP2D6, CYP2C9 and CYP3A4 contribute the greatest amount of FX *N*-demethylation (Margolis et al., 2000; Ring et al., 2001). Previous investigations from our laboratory using purified human cDNA-expressed CYP isozymes demonstrated that in addition to CYP2D6 and CYP2C9, CYP2C18 and CYP2C19 are also involved in the formation of NFX, however no activity was observed with CYP3A4 (Kim, 2000). Like the parent drug, NFX is also chiral in nature with both R- and S-NFX being formed in the body. The elimination half-life of NFX is about 7-15 days in humans, which is considerably longer than that of the parent drug. Another important feature of NFX is its preserved pharmacological activity in 5-HT reuptake inhibition (Brosen and Skjelbo, 1991; Wong et al., 1993).

While much is known about the conversion of FX to NFX, limited study has been conducted to investigate the other metabolic pathways of FX. A schematic diagram illustrating FX's proposed metabolic pathway is shown in Figure 1.3 (Altamura et al., 1994). Following conversion to NFX it has been reported that FX undergoes further metabolism to form FX carboxylic acid. FX, NFX and FX carboxylic acid all undergo *O*-dealkylation to give *p*-trifluoromethylphenol (TFMP). Following the removal of the TFMP moiety, *O*-dealkylated FX is further metabolized to give benzoic acid, which is then conjugated with

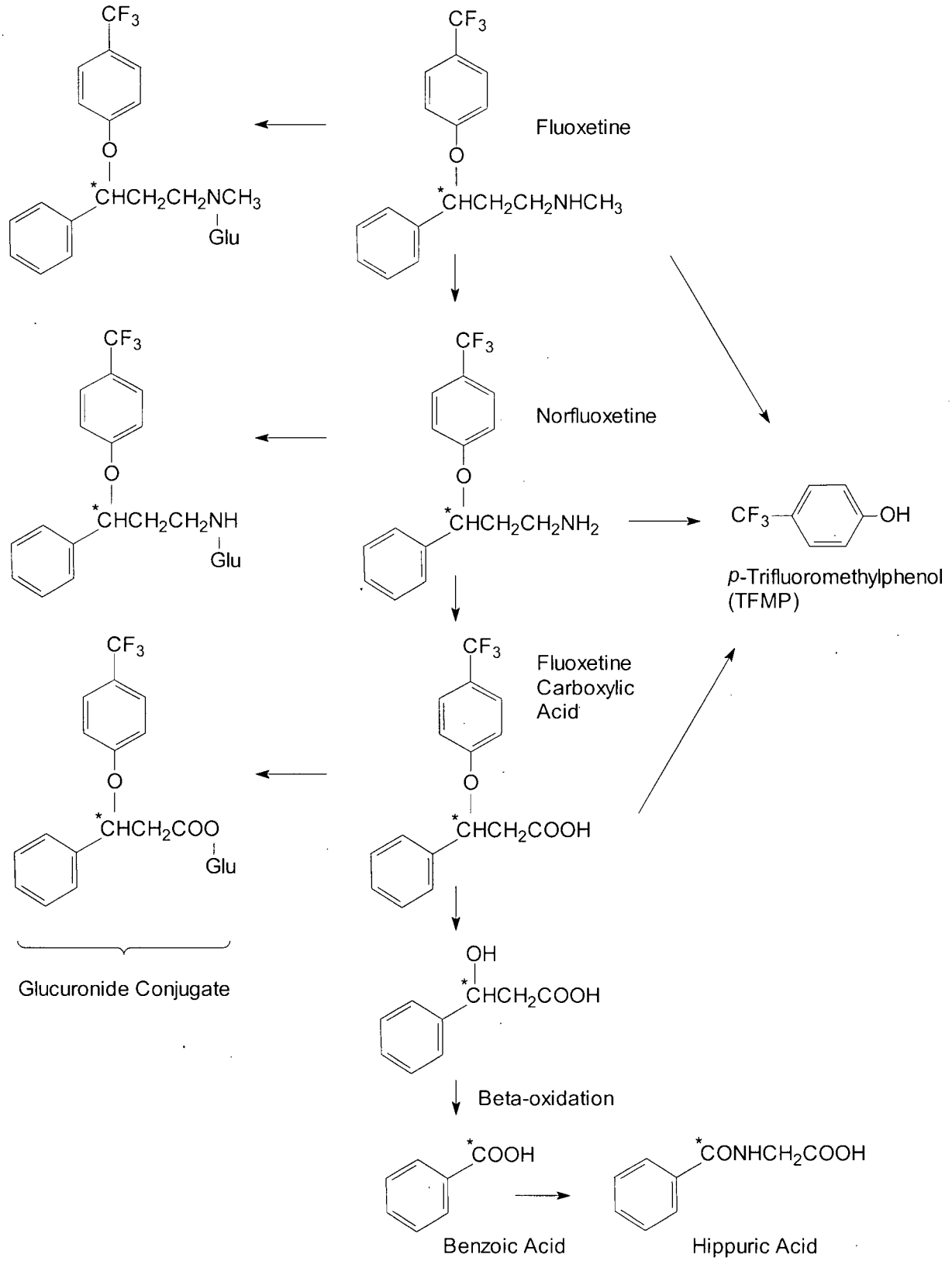


FIGURE 1.3 Proposed schematic diagram of FX metabolic pathway.

glycine to form hippuric acid and excreted into urine. This benzoic acid-glycine conjugate comprises ~20% of the administered FX dose recovered in human urine. About 11% of the administered dose of FX is excreted as free FX, 7% as free NFX, and 7% and 8% as FX and NFX *N*-glucuronides, respectively. TFMP has been quantified in human and rat plasma and urine, with free urine TFMP levels exceeding those in plasma (Urichuk et al., 1997). This metabolite is also observed in adult sheep urine but its contribution to the overall elimination of FX remains to be determined (Chien et al., unpublished data). A fairly recent *in vitro* study reports that CYP2C19 and CYP3A4 are the major cytochrome P450 isoforms responsible for fluoxetine *O*-dealkylation (Liu et al., 2002).

1.1.5 Stereoselective Properties of Fluoxetine and Norfluoxetine

It is important to be aware that though optical isomers share the same physical and chemical properties, they do not necessary have similar pharmacokinetic and pharmacodynamic properties. Table 1.1 summaries the stereoselective properties of fluoxetine and norfluoxetine enantiomers. In terms of pharmacological activity, both S- and R-enantiomers of FX are equally active as serotonin reuptake pump inhibitors (Gram, 1994). However, the pharmacokinetics of the S- and R-enantiomers of FX are significantly different from one another. The clearance of S-FX is significantly lower than that of R-FX resulting in a higher area under the plasma FX concentration-time curve (AUC) and steady-state concentration for S-FX compared to R-FX. Interestingly, while having a smaller clearance value, S-FX has a shorter elimination half-life instead of a longer one compared to R-FX in poor metabolizers (PM) of CYP2D6, and approximately equal in extensive metabolizers (EM) (Fjordside et al., 1999; Eap et al., 2001). A significant difference in the Vd of S-FX and R-

FX should be present in order to explain the difference in elimination half-life based on the theoretical relationship between elimination half-life, clearance and volume of distribution. Unfortunately, information regarding the Vd of FX enantiomers is not available in the literature. FX is highly bound to plasma protein but the stereoselective aspect of its binding characteristics has never been explored.

TABLE 1.1 Stereoselective properties of FX and NFX enantiomers.

PK-PD property	***FX***	***NFX***
Pharmacological activity	S = R	S > R by 20 fold
Clearance (CL) [a]	S < R	N/A
Half-life ($t_{1/2}$) [a]	S < R In PM [c] S = R In EM [c]	S > R in PM S = R in EM
AUC[a]	S > R	S > R
Steady-state conc. (C_{ss}) [b]	S > R	S = R in PM S > R in EM
Volume of distribution (Vd)	N/A	N/A
Protein binding (f_u)	N/A	N/A
CYP2D6 inhibition (K_i)	S < R	S < R

[a] Data based on a single dose study with single oral administration of 60 mg FX to 12 subjects (6 EMs and 6 PMs) (Fjordside et al., 1999).

[b] Data based on a multiple dose study with daily administration of 20 mg FX to 11 subjects (8 EMs and 3 PMs) for up to 23 days (Eap et al., 2001).

[c] PM, CYP2D6 poor metabolizer; EM, CYP2D6 extensive metabolizer.

FX is also known to be a potent inhibitor of CYP2D6 at therapeutic doses, which is of clinical significance in terms of potential drug interactions (Otton et al., 1993). The degree of CYP2D6 inhibition is also different between the FX enantiomers with S-FX a more potent

inhibitor (~6-fold) (inhibition constant (K_i), 0.22 ± 0.11 μM) compared to R-FX (K_i, 1.38 ± 0.48 μM) (Stevens and Wrighton, 1993).

The principal metabolite of FX, NFX is also pharmacologically active in serotonin reuptake inhibition. Unlike the parent drug, pharmacological activity of NFX is significantly different between its enantiomers with S-NFX being equally active compared to the parent drug while R-NFX has only 1/20 of the activity of FX (Fuller et al., 1992; Wong et al., 1993). In PMs, S-NFX has a longer elimination half-life but a similar steady-state plasma concentration (C_{ss}) compared to R-NFX. In EMs, the elimination half-life is the same between NFX enantiomers but C_{ss} is higher for S-NFX compared to R-NFX (Fjordside et al., 1999; Eap et al., 2001). No information is available regarding the clearance, volume of distribution or protein binding of NFX. Nonetheless, NFX has also been shown to be capable of inhibiting CYP2D6 with S-NFX being a stronger enzyme inhibitor compared to R-NFX (K_i: S-NFX = 0.31 ± 0.04 μM versus R-NFX = 1.48 ± 0.27 μM) (Brosen and Skjelbo, 1991; Stevens and Wrighton, 1993).

Pharmacokinetics determines the availability of a drug to its site of action. The site of action can either be an enzyme or a receptor. Fluoxetine and norfluoxetine act by binding to the 5-HT reuptake transporter to elicit their pharmacological effects. Either therapeutic and/or toxic effects will result from this interaction. It is important to point out that this pharmacokinetic-pharmacodynamic (PK-PD) relationship of fluoxetine and norfluoxetine is stereoselective in nature. Under certain circumstances, changes to their stereoselective pharmacokinetic properties may negatively affect their therapeutic and toxicity profiles. Additionally, the stereoselective inhibitory effects of fluoxetine and norfluoxetine enantiomers on CYP2D6 not only complicate their own pharmacokinetic-pharmacodynamic

profiles (i.e. nonlinearity), but also potentially lead to interactions with other drugs, which are substrates of the same enzyme. Inhibitory effects on enzyme activity are frequently concentration dependent, thus pharmacokinetics plays a pivotal role in contributing to this phenomenon. This concentration dependent interaction is often of clinical significance for drugs with a narrow therapeutic index.

In summary, complex stereoselective components exist between the pharmacokinetic-pharmacodynamic relationship of fluoxetine and norfluoxetine. Because of this, changes in their stereoselective pharmacokinetics may have significant clinical implications in their therapeutic effects, toxicity and drug interactions.

1.1.6 Depression and FX in Pregnancy

Depression occurs at a similar rate in pregnant and nonpregnant women, with approximately 20% of women experiencing depressive symptoms during pregnancy (Kessler et al., 1993; Burt and Stein, 2002). Since depressed patients tend to neglect themselves, a depressive episode during pregnancy increases the risk for suicide and poor self-care. Women with depression often present with decreased appetite and consequently lower-than-expected weight gain in pregnancy, factors that have been associated with negative pregnancy outcomes (Bhatia and Bhatia, 1999; Nonacs and Cohen, 2002). In addition, pregnant women with depression are also more likely to smoke and to use either alcohol or illicit drugs, behaviors that further compromise the health of the developing fetus (Zuckerman et al., 1989; Zuckerman et al., 1990; Hoffman and Hatch, 1996). Higher rates of admission to neonatal ICU, lower birth weight and premature delivery have been more commonly associated with women from suffering depression during pregnancy than those

who were not (Steer et al., 1992; Chambers et al., 1996; Grush and Cohen, 1998; Orr et al., 2002). Uncontrolled depression during pregnancy also triples the risk for depression in the postpartum period (O'Hara, 1995; Cooper and Murray, 1998; Newport et al., 2002).

It is generally agreed that drugs of any type should only be taken during pregnancy when the risk to the mother and the fetus of no treatment outweigh the risks of taking the drug (Hendrick and Altshuler, 2002; Nonacs and Cohen, 2002). Thus, when psychotherapy has not been effective, or when the illness can affect fetal prognosis and the future mother-child relationship, pharmacological treatment of depression during pregnancy may be necessary (Robert, 1996; Wisner et al., 1999).

The SSRIs have become the most commonly prescribed class of antidepressants worldwide in the general adult population as well as in pregnant women. The increasing use of SSRIs in pregnant women has raised concerns regarding their safety during pregnancy and resulted in a number of studies to investigate their reproductive risks. The largest amount of information on prenatal SSRI exposures is available for FX (Altshuler, 2002). Neither retrospective nor prospective studies have revealed an increased risk of birth defects, poor perinatal condition, neurodevelopment delay, or poor cognition and language development associated with the use of FX in pregnancy (Chambers et al., 1996; Goldstein et al., 1997; Loebstein and Koren, 1997; Addis and Koren, 2000; Cohen et al., 2000; Nulman et al., 2002). However, several reports of neonatal toxicity, cardiac arrhythmia, and neonatal withdrawal syndrome, characterized by irritability, constant crying, shivering, increased tonus, eating and sleeping difficulties and convulsions, have been reported in newborns exposed prenatally to FX (Spencer, 1993; Mhanna et al., 1997; Mohan and Moore, 2000; Nordeng et al., 2001; Abebe-Campino et al., 2002). These adverse events have been largely attributed to *in utero*

FX exposure in late gestation, which is the period when maternal adaptive changes are most likely to influence drug pharmacokinetics. Furthermore, it was observed that some of these neonates had plasma FX and NFX concentrations that were above the usual adult therapeutic ranges at a given dosage, suggesting that alterations in the pharmacokinetics of FX might be involved during the perinatal period. While the incidence of these adverse events appears to be low, further research should focus on the safety of FX and its pharmacokinetics during the last trimester of pregnancy in order to establish a causal link between drug exposure and toxicity.

1.1.7 Effects of Pregnancy on Pharmacokinetics

During pregnancy, the body undergoes a number of physiological changes, which may modify the pharmacokinetic processes of drug absorption, distribution, metabolism and excretion. Important among these are maternal adaptations that serve to increase the delivery of blood and nutrients to the fetus and to remove metabolic waste products from both mother and fetus (Nation, 1980). These adaptations generally result in a marked increase in both the intravascular and extravascular volumes; the enhancement of cardiac output, renal blood flow, and the glomerular filtration rate; and the augmentation of hepatic and other metabolic activities in the mother, the fetus, and the placenta (Krauer et al., 1980). Consequently, the volume of distribution, biotransformation and renal clearance of xenobiotics can vary significantly in comparison with corresponding values obtained in the nonpregnant state. These pregnancy-associated physiologic changes and their therapeutic implications are summarized in Table 1.2.

TABLE 1.2. Pregnancy-associated physiologic changes and their potential influence on drug pharmacokinetic parameters.

Physiologic change	Kinetic influence(s)	Therapeutic implication(s)
Decreased GI motility Delayed gastric emptying Delayed transit time	Reduced rate of absorption from small bowel Increased absorption from GI tract	Unpredictable absorption of orally administered drugs
Expanded intravascular volume (up by 50% by the eighth month)	Increased Vd (decreased maternal serum concentration) Reduced plasma protein conc. (decreased total drug conc., increased tissue/ plasma distribution, increased drug clearance)	Possible need for larger loading doses Possible underestimation of serum concentration of free or active drug on the basis of total serum drug concentration Possible need for more frequent administration to dampen peak-trough fluctuations
Increased renal blood flow and glomerular filtration rate (up by 50% by fourth month)	Increased drug clearance	Subtherapeutic drug concentrations. Possible need for increased dose
Increased progesterone-activated hepatic metabolism Increased biotransformation due to placental and/or fetal metabolism	Increased rate of biotrans-formation to either active or inactive metabolites	Possible need for increased dose and/or decreased dosing interval
Increased thinning of placenta with advancing gestation	Increased transplacental diffusion (maternal and fetal steady-state concentrations remain unchanged)	Enhanced rate of equilibrium between maternal and fetal circulation

GI, gastrointestinal; Vd, volume of distribution; conc, concentration.

Source: Krauer et al., 1980; Chow and Jewesson, 1985; Reynolds and Knott, 1989.

1.1.7.1 Absorption

During pregnancy, both the tone and motility of the maternal gastrointestinal tract may be reduced as a consequence of physical displacement by the enlarging uterus; these changes may result in delayed gastric emptying and prolonged intestinal transit. The latter effects may in turn influence either the bioavailability and effectiveness of drugs with acid-labile properties or the site-specific absorption of drugs from the intestinal tract (Reynolds and Knott, 1989).

1.1.7.2 Distribution

Several changes in body compartments occur during pregnancy and may have important effects on the distribution of drugs. The normal weight gain in pregnancy of up to 12 kg is a result of the combined increases in total body water, fat stores, and of course, the products of conception (Reynolds and Knott, 1989). The 6-8 liter increase in total body water is accounted for by a rise in plasma volume, by the fetus, uterus, and amniotic fluid, and by a large expansion of the extracellular fluid space. There is a large inter-individual variation in maternal plasma volume as well as in the extracellular accumulation of water. The rise in plasma volume may increase by up to 50 per cent, and this change is dependent on the number and size of the developing fetuses. Extracellular volume may rise by 5 liter or more at the 40^{th} week of gestation in cases of generalized edema (Krauer et al., 1980). The increase in total body water is more profound on the distribution of polar drugs, which are confined to the extracellular space, than on already widely distributed lipophilic drugs, whose distribution is probably more influenced by changes in protein and lipid binding. Body fat increases by 3-4 kg (largely in subcutaneous deposits) during the first two trimesters and this

store is maintained throughout the remaining period of gestation. During the third trimester, plasma concentrations of lipoproteins, glycerol, and non-esterified free fatty acid gradually increase and peak at around delivery (Reynolds and Knott, 1989). Thus, not only will fat-soluble drugs have an increased volume of distribution, but may also compete with other endogenous compounds (e.g. fatty acids) for protein-binding sites. This may result then, in alterations in the unbound fraction of the drug.

1.1.7.3 Protein Binding

Albumin is the major binding protein for acidic drugs such as anticonvulsants, salicylates, and nonsteroidal anti-inflammatory compounds as well as for some neutral drugs including warfarin and diazepam. On the other hand, basic drugs such as local anesthetics, opioids, β-blockers, tricyclic antidepressants, and SSRI antidepressants are principally bound with high affinity to α_1-acid glycoprotein (AAG), which has a lower binding capacity than albumin (Jeffries and Bochner, 1988). During the first half of pregnancy plasma albumin concentrations fall from around 35 to 25 g/l, partly due to plasma dilution (Krauer et al., 1984). Animal and human studies have shown that, in addition to hypoalbuminemia, an increase in plasma concentration of endogenous ligands, including free fatty acids, may compete for albumin binding sites and thus further reduce its binding capacity in late pregnancy (Reynolds and Knott, 1989). Consequently, unpredictable increases in the free fraction have been reported for many drugs and this is often associated with a lower total plasma drug concentration. A serious underestimation of the free, therapeutically active concentration of a drug will result if drug binding is not be accurately predicted. Reduced albumin binding can contribute to the increased distribution volume and clearance for drugs

that undergo restrictive elimination. In contrast to albumin, the concentration of AAG does not vary consistently during pregnancy. Further, the effect of pregnancy on AAG concentrations is poorly documented with no readily apparent trends in drug binding (Wood and Wood, 1981; Krauer et al., 1984).

1.1.7.4 Metabolism

Hepatic metabolism in pregnancy

Smoking, ethanol consumption, and progesterone are potent enzyme inducers and may significantly enhance drug metabolism, while estrogens may competitively inhibit enzyme induction. Theoretically, it is possible that the balance between these increasing hormone levels during pregnancy may dictate the net effect on enzyme activity. A high estrogen/progesterone ratio may lead to enzyme inhibition while a low estrogen/ progesterone ratio may lead to enzyme induction (Krauer, 1984). Progressive changes in this ratio, combined with the influence of environmental agents, may explain the apparently inconsistent findings for individual drugs whose elimination rates may be raised, lowered, or unchanged during pregnancy. Finally, the cholestatic effect of estrogen may interfere with the clearance of drugs that undergo biliary excretion (Harrison and Gibaldi, 1976). While the overall changes of pregnancy on hepatic physiology have the potential to alter drug metabolism, the relevance of this is not presently apparent.

Placental and fetal hepatic metabolism

There is some *in vitro* evidence that both the human placenta and fetal liver are capable of significant drug biotransformation (Pelkonen, 1980). Further, the metabolite profiles appear qualitatively similar to those generated by adult human liver and this may

also be the case in fetal liver. It is thought that differences in the rate of principal metabolite formation between the adult liver and the placenta may relate to differences in microsomal enzyme concentrations as well as to and their specific regulation (Juchau, 1980). Similar to hepatic microsomal enzymes, those in the placenta that are responsible for the metabolic conversion of endogenous substances have also been found to be induced or inhibited by certain drugs (Juchau, 1980; Pasanen 1999). In contrast to oxidative metabolism, the conjugative capabilities of the placenta have not been clearly demonstrated (Juchau, 1980; Pasanen 1999). It is also likely that certain substances may only cross the placenta after biotransformation.

Studies with human fetal liver demonstrate the ability to oxidize endogenous and exogenous substrates, using the cytochrome P450 system, by the end of the first trimester (Loebstein et al., 1997; Hines and McCarver, 2002). In general, the human fetal liver has the adult complement of enzymes, but the activities at term are only about one-half those of the adult and they are not as readily induced (Shimada et al., 1996; Loebstein et al., 1997). In late gestation, the human fetal liver is deficient in UDP-glucuronyltransferase activity (Burchell et al., 1989; de Wildt et al., 1999b), however sulfate conjugation appears to be well developed with the activity of these enzymes even exceeding those in adults (Juchau et al., 1980; McCarver and Hines, 2002). Overall, fetal metabolism contributes very little to metabolite formation and the fetus relies largely on the maternal system for the clearance of parent drug and metabolite(s) (Rane and Tomson, 1980; Ring et al., 1999). However, fetal metabolism may be important when the metabolites produced do not readily cross the placenta as their accumulation in the fetus may have adverse consequences.

In summary, the clinical significance of potential fetal hepatic and placental metabolism remains to be clearly defined. Certainly a more complete understanding of the roles played by enzymatic reactions in both the placenta and fetal liver in fetal parent drug and/or metabolite exposure is required.

1.1.7.5 Excretion

Of all the physiological systems which are modified by pregnancy, renal function perhaps undergoes the greatest change. Within the first few weeks of gestation, the glomerular filtration rate increases by up to 50% and remains elevated until after delivery (Davison and Noble, 1981). Plasma creatinine and urea clearances increase during pregnancy. Creatinine clearance, which closely reflects the glomerular filtration rate, increases to a maximum at 34 weeks, then declines with consequent raised plasma concentrations just prior to delivery. Renal plasma flow starts to rise by about 15 weeks and may have doubled by 26 weeks (Reynolds and Knott 1989). In parallel with these changes, polar, water-soluble drugs, which are predominantly eliminated by renal excretion, are cleared more rapidly during pregnancy and have shorter half-lives (Chow and Jewesson, 1985).

1.1.7.6 Placental Transfer

Movement of drug substances across the placenta is generally bi-directional, although net transfer occurs from mother to fetus in most instances. The transplacental passage of a drug is regulated by many factors, principally the physiological characteristics of the maternal-placental-fetal unit and the physiochemical properties of the drug (Mirkin, 1975). Physiologic factors include 1) hemodynamic changes in either maternal or the fetal

contribution to total placental blood flow, 2) the thickness and maturation of the placental membranes, and 3) the metabolic activity of placental tissues. Although active and facilitated transport of some substances across the placenta has been demonstrated, the transplacental passage of most drugs occurs primarily by simple diffusion (Reynolds and Knott, 1989). The rate of diffusion across the placenta is directly proportional to the maternal-fetal concentration gradient and the surface area of the placenta and is inversely proportional to the thickness of the placental membranes (Mihaly and Morgan, 1984). Early in pregnancy the placental membranes are relatively thick, and this characteristic tends to reduce permeability. The thickness of the trophoblastic epithelium decreases in the last trimester, and any passage of drugs is thereby greatly facilitated (Garland, 1998). This progressive thinning of the placenta as gestation advances results in increased transplacental diffusion and alternative routes of drug elimination from the mother. A drug with a low molecular weight of <600 Da, a high degree of lipid solubility, a low degree of ionization, and a low affinity for protein binding is able to transverse the placenta more readily whereas a drug with a molecular weight of >1,000 Da may be restricted by its size (Reynolds and Knott, 1989). Only the unbound (free) fraction of a drug is subject to placental transfer; therefore, the more a drug is bound to maternal plasma protein, the less it is available for crossing to the fetus. In general, drugs with a low degree of protein binding reach higher total concentrations in the fetus whereas drugs that are highly protein bound achieve higher maternal than fetal total concentrations (Szeto, 1993).

Collectively the physiological changes described above have the potential to alter drug disposition during pregnancy and may therefore have an impact on the pharmacokinetic disposition of FX and NFX in pregnant women. The effect of pregnancy on the clearance of

FX is of particular interest as this is a major factor that needs to be considered for its long-term dosing in the treatment of depression. The high degree of plasma protein binding exhibited by FX is also important since progressive changes in protein binding over the course of pregnancy may also affect its fetal-placental transfer and consequently the overall exposure of the fetus to this drug. Therefore, it is prudent to systemically study these physiological effects of pregnancy on the disposition of FX and NFX to improve our understanding of the appropriate use of FX in the treatment of pregnant women with depression.

1.1.8 Placental Transfer and Pharmacokinetics of FX in Pregnancy: Previous Studies

Earlier studies have been conducted in our laboratory to examine the stereoselective pharmacokinetics of FX placental transfer in chronically-catheterized nonpregnant and late gestational age pregnant sheep following a single *i.v.* dose of the drug (Kim, 2000; Kim et al., 2004). The stereoselective disposition of FX previously described in humans (Fjordside et al., 1999) was also observed in sheep. Differences in plasma concentration, systemic clearance, volume of distribution and free fraction were noted between the FX enantiomers. Based on the estimation of free drug clearance values, it was suggested that differential protein binding of the FX enantiomers appeared to explain the stereoselective disposition of the drug in sheep. In addition, the metabolic profile of FX, in general, was found to be similar between humans and sheep. *N*-demethylation of FX to form NFX, which is a major metabolic pathway of FX in humans, was also observed in sheep. The glucuronide conjugate of FX was also identified in sheep urine although the amount eliminated appeared to be less compared to that reported in human subjects (Altamura et al., 1994).

FX pharmacokinetics in pregnant sheep exhibited a number of statistically significant differences compared to nonpregnant sheep, which included a higher total body clearance, shorter half-life and lower steady-state volume of distribution. It was postulated that these differences could be due to an increased activity of a CYP2D6-like enzyme and/or an increase in the plasma concentration of α_1-acid glycoprotein during pregnancy in sheep. A pregnancy-related induction of CYP2D6 occurs in the human (Wadelius et al., 1997), which suggests that changes in FX pharmacokinetics similar to those in the sheep may occur during pregnancy in human.

FX was found to be extensively transferred across the sheep placenta. The fetal-to-maternal (F/M) AUC ratio in pregnant sheep after maternal *i.v.* bolus administration was 0.57 $\pm$ 0.17 for FX and 0.51 $\pm$ 0.13 for NFX. FX did not appear to accumulate in amniotic and fetal tracheal fluids in contrast to a number of amine drugs studied earlier in sheep (Riggs et al., 1987; Wright et al., 1991; Kumar et al., 1997).

The metabolic capacity of FX in ovine fetuses was also explored. Following fetal *i.v.* bolus administration of FX, no NFX was detected in fetal plasma. *In vitro* microsomal studies using fetal sheep liver also showed no evidence of fetal NFX formation. Thus, metabolic pathway(s) for FX appear to be inactive in the fetal lamb although further studies are required to fully characterize the expression and capacity of the fetal enzymes involved in FX metabolism. The contribution of other routes of elimination of FX in fetal lambs such as renal elimination and placental metabolism, were not determined in these previous studies.

An investigation of the placental transfer of FX in pregnant women during the perinatal period was also conducted as part of this previous work. The average maternal exposure period for FX was 9 months (range 1 to 48 months) at the time of delivery with a

daily dosage of 10-30 mg. The mean serum cord-to-maternal (F/M) ratios of racemic FX and NFX at birth were 1.04 ± 0.29 and 1.04 ± 0.16, respectively (Kim, 2000). Similar umbilical cord to maternal blood concentration ratios of FX and NFX (0.83 ± 0.27 for FX and 0.81 ± 0.24 for NFX) have also been reported by others (Stowe, 2000). The ratios reported by Kim (2000) were significantly higher in the human studies than those observed in the pregnant ewes, where fetal exposure was estimated as the ratio of fetal and maternal plasma AUC following a single FX dose to the ewe. It was suggested that this difference is related to the longer-term of drug administration regimen in the human study, which allows for the attainment of steady-state, compared to the single *i.v.* bolus injection protocol employed in the sheep project. In the same study, a negative correlation was observed between the apparent oral clearance and exposure time for FX in humans. This finding confirms the results by other investigators that the metabolism of FX is mediated by CYP2D6, and also that CYP2D6 is inhibited at drug concentrations in the therapeutic range (Brosen and Skjelbo, 1991; Steven and Wrighton, 1993). In addition, the S/R ratio of steady-state FX concentrations was found to be negatively correlated to the oral clearance of FX but positively correlated to the exposure time to FX in humans during chronic therapy. The relationship of the S/R ratio of steady-state FX concentrations with respect to clearance and time suggests that the stereoselective disposition of FX involves other CYP450 isozyme(s) when the CYP2D6-mediated metabolism is inhibited over time. Interestingly, a similar correlation between the S/R AUC ratio and the systemic clearances of the FX enantiomers was observed in sheep after single *i.v.* bolus FX administration. However, further experiments will be necessary to fully characterize this phenomenon in sheep.

1.2 RATIONALE

Pharmacokinetic evaluations during pregnancy are particularly complex because of the markedly altered and rapidly changing physiological state of the pregnant woman as well as the technical and ethical difficulties associated with assessing fetal drug pharmacokinetics *in utero*. Other issues such as the contributions of placental and fetal tissues as determinants of drug elimination, and the changing relative contributions of fetal-placental and maternal-placental drug clearance during the various stages of pregnancy must also be considered in order to assess the maternal-placental-fetal pharmacokinetic process in a quantitative manner.

Certain conditions such as depression have a high incidence in pregnant women and frequently require pharmacological intervention. A thorough understanding of drug pharmacokinetics in pregnancy is of great importance to allow drugs to be safely used in this special population. Ideally, studies performed in humans will provide us with the most relevant clinical information. However, such data is very limited due to ethical limitations on the design of human placental transfer studies. For the most part such information has usually been obtained from single maternal and umbilical blood samples at delivery following acute single dose administration of drug or chronic dosing to the mother during labor (Bourget et al., 1995; Kim et al., 2004). For these reasons animal species have been commonly employed to examine placental drug transfer and subsequent fetal drug exposure. However, serial determinations of maternal and fetal steady state drug concentrations over time are not practical in small laboratory animals due to limited blood volumes and the inability to chronically implant catheters or monitoring devices in the mother and fetus. As a large farm animal species the chronically instrumented pregnant sheep has been extremely useful in addressing both the effects and kinetics of drugs in the fetus in late gestation (Rurak

et al., 1991). The frequent fetal sampling achieved in the sheep model is not possible in either human or small animal models. Compared to other species (e.g. monkeys), sheep do not often abort or deliver prematurely after intrauterine surgery. The fetus in the last trimester is conveniently large (0.5-5 kg) and the investigator can perform intricate surgeries and obtain serial fetal blood samples large enough for drug analysis. Further, the ewe is a very docile animal and will not interfere with exteriorized indwelling catheters (Simone et al., 1994). While there are clear differences between sheep and humans in placental structure and intrinsic activity of drug metabolizing enzymes, the capability of chronically instrumented sheep studies to provide detailed information on maternal-fetal drug disposition appears to be far more important than any drawbacks.

Previous experiments involving single *i.v.* bolus FX administration to pregnant sheep have contributed much to our understanding of the kinetics of FX transfer to the fetus, as well as the general impact of pregnancy on the disposition of FX. These studies have also shown that the stereoselective disposition of FX is qualitatively similar between sheep and humans. This is very uncommon compared to other species. However, the design of the single dose *i.v.* bolus experiments did not permit a complete assessment of several important pharmacokinetic features of FX such as nonlinearity, time dependent changes of clearance, and inter-relationships in stereoselectivity (S/R ratio). Such information can only be obtained by administering the drug for a longer period of time to steady state. Clinically FX is usually given repeatedly in humans to achieve steady state plasma drug concentrations, hence pharmacokinetic studies of FX at steady state in sheep should provide information of more clinical significance compared to the previous single dose *i.v.* bolus data. Additionally, it was speculated that the discrepancy observed between humans and sheep in the F/M ratio

of FX was attributed to the difference in the duration of drug exposure between the two groups. More experiments involving long-term FX administration in pregnant sheep are necessary to test this assumption. To our knowledge, such experiments have not yet been performed for FX in any species.

Lastly, after a decade of clinical use the pharmacokinetics of FX in pregnancy is still poorly understood. Many articles have been published on the clinical pharmacokinetics and use of FX but only a very few of these address the safety of FX use in pregnancy. With the expiration of the patent on the brand product of fluoxetine, Prozac®, in the United States in September 2001 (Elliott, 2001; Pabst, 2001), the emergence of competing generic substitutes has made this drug more affordable than ever for many patients, hence its use is expected to increase. With the increase of SSRIs in pregnancy it is our hope that through a series of well-designed long-term animal experiments the maternal-fetal disposition and placental transfer of FX can be examined in a more systematic and mechanistic way. Such experiments will tremendously further our understanding of the behavior of FX, and hopefully provide a more rational approach to it use for the treatment of depression and other psychiatric disorders in pregnancy.

1.3 HYPOTHESES

1. Fluoxetine (FX) and norfluoxetine (NFX) enantiomers undergo significant stereoselective pharmacokinetics at steady-state in nonpregnant sheep.

2. Pregnancy-induced physiological changes will alter the stereoselective disposition of FX and NFX at steady-state in sheep.

3. The exposure of the fetus to FX will be greater with steady-state administration, compared to acute single *i.v.* bolus dosing.

4. The clearance of FX and NFX enantiomers in fetal lambs involve both placental and nonplacental routes of elimination.

1.4 OBJECTIVES

1. To examine the stereoselective pharmacokinetics of FX and NFX at steady-state in nonpregnant sheep.

2. To investigate the effect of pregnancy on the stereoselective disposition of FX and NFX at steady-state in pregnant sheep.

3. To study the fetal exposure of FX and NFX enantiomers by examining their placental transfer, and distribution in amniotic and fetal tracheal fluid at steady-state.

4. To study the stereoselective disposition of FX and NFX in fetal lambs, with specific focus on protein binding, hepatic metabolism, and renal excretion.

CHAPTER 2

MATERIALS, INSTRUMENTATION AND ANALYTICAL METHODOLOGIES

2.1 Materials

The reference standards, chemicals, reagents and other material used during the study and sample analysis, along with information on their purity (where applicable) and source, are listed below.

Racemic fluoxetine hydrochloride [(R,S)-*N*-methyl-3-(*p*-trifluoromethylphenoxy)-3-phenylpropylamine (>99% purity)] was generously provided by Novopharm (Toronto, ON, Canada). Racemic norfluoxetine hydrochloride [(R,S)-3-(*p*-trifluoromethylphenoxy) -3-phenylpropylamine (>99% purity)] and (R)-norfluoxetine hydrochloride [(R)-3-(*p*-trifluoromethylphenoxy)-3-phenylpropylamine (>99% purity)] were generously provided by Eli Lilly and Co. (Indianapolis, IN, U.S.A.). The internal standard [2-(diphenylmethoxy)-*N*-methylethylamine hydrochloride] for the gas chromatographic mass spectrometric (GC-MS) assay method was synthesized in our laboratory. Optically pure fluoxetine isomers, R-fluoxetine [(R)-*N*-methyl-3-(*p*-trifluoromethylphenoxy)-3-phenylpropylamine] and S-fluoxetine [(S)-*N*-methyl-3-(*p*-trifluoromethyl-phenoxy)-3-phenylpropylamine] were separated from racemic fluoxetine hydrochloride in our laboratory as described previously (Kim, 2000).

(S)-(−)-trifluoroacetylprolyl chloride (97% purity) was obtained from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Triethylamine (TEA), toluene, isopropanol, acetone, methanol and n-hexane were purchased from Caledon Laboratories (Georgetown, ON, Canada) and were of distilled in glass GC or HPLC grade.

Deionized high purity water was produced on-site by reverse osmosis and subsequent filtration using a Milli-Q® water system (Millipore, Bedford, MA). Ultra high purity (UHP) helium for GC-MS analysis and filtered nitrogen for sample concentration were obtained from Praxair (Mississauga, ON, Canada).

Glassware for extraction and sample preparation (15 mL Pyrex® disposable culture tubes), polytetrafluoroethylene (PFTE) lined crew caps, and 1.0 N sodium hydroxide solution were obtained from VWR Canlab (Vancouver, BC, Canada).

Materials used during the animal experiments were as follows: Veramix® sheep sponges (Tuco Products Co., Orangerville, ON); pregnant mares' serum gonadotropin (Ayerst Laboratories, Montreal, QC); thiopental sodium injectable (1 g/vial) USP (Abbott Laboratories, Montreal, QC, Canada); halothane, USP (Ayerst Laboratories, Montreal, QC, Canada); sterile water for injection, USP; 0.9% sodium chloride injection, USP; 0.9% sodium chloride irrigation, USP (Baxter, Mississauga, ON, Canada); injectable ampicillin (250 mg/vial) (Novopharm, Toronto, ON, Canada); injectable atropine sulfate (0.6 mg/ml) (Glaxo Laboratories, Montreal, QC); heparin (1000 units/mL) (Organon Canada Ltd., West Hill, ON, Canada); lidocaine 2% (Astra Pharma Inc., Mississauga, ON, Canada). All injectable formulations were obtained from BC Children's and Women's Hospital Pharmacy, Vancouver, BC.

Other materials used during the animal experiments included: disposable syringe needles and plastic disposable Luer-Lok® syringes for drug administration and biological fluid sample collection (Becton-Dickinson Canada, Mississagua, ON, Canada); 500-mL sterilized evacuated glass containers for drug solution preparation (Baxter, Mississauga, ON, Canada); silicone or polyvinyl rubber tubing for catheter preparation (Dow Corning,

Midland, MI, U.S.A.); Foley bladder catheters (American Latex Corp., Sullivan, IN, U.S.A.) and urine drainage bags (Bard Medical, Covington, GA, U.S.A.) for urine collection in adult sheep; empty Viaflex® containers for fetal urine collection (Baxter, Mississauga, ON, 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.); and lavender-top K_3EDTA BD Vacutainer™ (Becton Dickinson, Oakville, ON, Canada) for plasma separation.

Materials used for protein binding studies included Plexiglass® dialysis cells and cellophane dialysis membrane sacks with a molecular cutoff of 12,000 daltons (Sigma Chemical Co., St. Louis, MO, U.S.A.). The following reagent-graded chemicals were also used: potassium phosphate monobasic anhydrous, sodium phosphate dibasic heptahydrate (BDH Chemical Co., Toronto, ON, Canada), and sodium chloride (Sigma Chemical Co., St. Louis, MO, 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 HP Chemstation model G1030A workstation software was utilized for the GC-MS analysis of FX and NFX in electron impact (EI) mode (Hewlett-Packard, Avondale, PA, U.S.A.). The gas chromatograph was equipped with a DB5MS (30m x 0.18mm i.d.; film thickness 0.18 μm; 5% phenylmethylsilicone; J&W Scientific, Folsom, CA, U.S.A.) cross-linked fused silica

capillary column for FX and NFX analysis. Samples were injected onto the GC-MS via a deactivated glass single taper inlet liner (4mm i.d., 6.5mm o.d., 900 µl; Agilent Technologies Canada Inc., Mississauga, ON, Canada) and a Thermogreen LC-2 silicone rubber septum (J&W Scientific, Folsom, CA, U.S.A.) in the splitless mode.

2.2.2 Physiological Monitoring

Maternal and fetal blood samples were analyzed for pH, partial pressure of oxygen (pO_2), partial pressure of carbon dioxide (pCO_2), and whole blood base excess (BE) with an IL 1306 pH/blood gas analyser (Allied Instrumentation Laboratory, Milan, Italy) and temperature corrected to 39.5°C for fetal samples and 39°C for maternal samples. Blood Hemoglobin (Hb) and oxygen saturation (O_2 sat) were measured with an OSM-2 Hemoximeter (Radiometer, Copenhagen, Denmark). Glucose and lactate were determined with a glucose/lactate 2306 STAT plus analyser (Y.S.I. Inc., Yellow Springs, OH, U.S.A.).

2.2.3 Other Equipment

The following equipment was used for sample preparation: a vortex-type mixer and incubation oven (Isotemp model 350) (Fisher Scientific, Springfield, MA, U.S.A.); IEC model 2K centrifuge (Damon/IEC, Needham, MA, U.S.A.); rotating mixer (Labquake model 415-110, Lab Industries, Berkeley, CA, U.S.A); Zymak Turbo Vap® LV Evaporator (Zymak Co., Hopkinton, MA, U.S.A.). Other equipment included a syringe pump (Model 944, Harvard Appartus, Millis, MA, U.S.A.) and water bath (Isotemp model 220, Fisher Scientific, Springfield, MA, U.S.A.) for drug infusion and protein binding experiments, respectively.

2.3 Analysis of FX and NFX Enantiomers in Biological Fluids

Enantiomers of FX and NFX in all biological fluids were measured by a chiral assay using GC-MS developed previously in this lab (Kim, 2000). Briefly, appropriate volumes of biological fluids (e.g. plasma, urine) were basified with 0.5 ml of 1.0 N NaOH solution, and extracted for 20 min with 7 ml of 0.05 M TEA in hexane:isopropanol 98:2 v/v mixture on a rotary mixer. The organic extract was separated and dried under a gentle stream of nitrogen at 35°C for 30 min. The residue was reconstituted in 80 µl of a 5% (S)-(−)-*N*-trifluoroacetylprolyl chloride - toluene mixture and derivatized by heating at 65°C for 1 h, which converted the FX and NFX enantiomers into their diastereomers and allowed baseline separation of all peaks using gas chromatography. Following sample cooling a 2 µl aliquot of the final sample was injected into the GC-MS in splitless mode. Chromatographic separations were performed on a DB5MS (30m x 0.18mm i.d.; film thickness 0.18 µm; 5% phenylmethylsilicone) cross-linked fused silica capillary column with helium as the carrier gas at a 15 psi column head pressure. The GC operating conditions were as follows: Injection port temperature 260°C; oven temperature 145°C initially and increased by 40°C/min to 215°C, 1.5°C/min to 239°C and finally by 14.4°C/min to 300°C where it was held for 2.5 min. Total chromatographic run time was 22 minutes. The mass spectrometer was operated in electron impact ionization mode (EI energy = 70 eV) with selected ion monitoring (SIM) at a transfer line temperature of 300°C. Ions monitored for FX, NFX and the internal standard [2-(diphenylmethoxy)-*N*-methylethylamine] were *m/z* 341, 327 and 252 respectively. The method was linear over a concentration range of 1.0-100 ng/ml (r^2>0.99) with intra- and inter-day coefficient of variation (CV) <15%. The limit of quantitation (LOQ) of this assay was 1.0 ng/ml for each FX and NFX enantiomers using a 200 µL

biological fluid sample volume. A representative total ion chromatogram is shown in Figure 2.1.

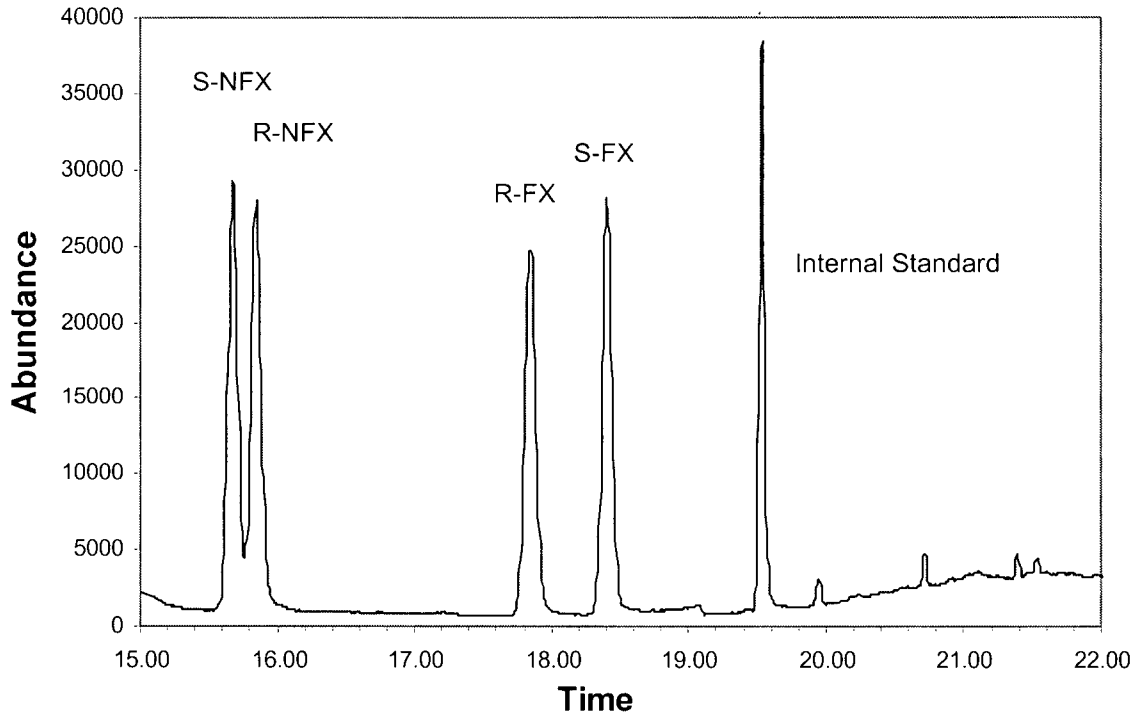


FIGURE 2.1 Representative total ion chromatogram of FX, NFX, and internal standard in SIM mode, showing chromatographic separation of the FX and NFX enantiomers in a run time of 22 minutes.

CHAPTER 3

STEADY-STATE STEREOSELECTIVE PHARMACOKINETICS OF FX AND NFX IN NONPREGNANT SHEEP

Preface

One of the biggest challenges of stereoselective pharmacokinetic studies is the selection of an appropriate animal model. High interspecies variations in the stereoselective disposition of several drugs have been reported (Ruelius, 1987; Jamali et al., 1989). For example, cyclophosphamide did not exhibit stereoselective pharmacokinetic in humans, however, metabolism of the (+)-enantiomer is higher in mice and the opposite is observed in rats and rabbits (Cox et al., 1978). Similar phenomena have been observed with other racemic compounds such as disopyramide, nicotine, oxazepam, fenfluramine, propranolol, and nonsteroidal anti-inflammatory drugs (Cook et al., 1982; Crooks and Godin, 1988; Sisenwine et al., 1982; Jori et al., 1978; Walle, 1985; Jamali, 1988). Human data on the stereoselective pharmacokinetics of FX are very limited in the literature (Fjordside et al., 1999; Eap et al., 2001). Previous studies conducted in this lab revealed significant stereoselective disposition of FX enantiomers in sheep (Kim, 2000), which was similar to that observed in humans. The primary objective of this chapter is to extend our investigation of the stereoselective pharmacokinetics of FX and NFX in sheep under steady-state conditions. Such data will allow us to assess the stereoselective pharmacokinetics of FX and NFX in sheep in greater detail, which is of great importance before further comparisons can be drawn between sheep and humans. Further, it will enable us to decide if extrapolations to the human situation are appropriate or not based on the observations in sheep. The results

from this study will also serve as the foundation to further our studies on the effect of pregnancy on the pharmacokinetics of FX and NFX, as well as the mechanisms behind the stereoselective disposition. Moreover, it has been shown that FX exhibits nonlinear pharmacokinetics upon multiple dosing in humans. The mechanism of this nonlinearity is thought to be related to the inhibition of CYP2D6, which is one of the principal metabolic enzymes responsible for FX metabolism. However, this phenomenon has never been described or characterized in any animal model before. Therefore, the secondary objective of this chapter was to investigate whether or not FX exhibits nonlinear pharmacokinetics in sheep.

3.1 Methods

3.1.1 Animal and Surgical Preparation

Six nonpregnant ewes (Dorset and Suffolk breeds) were used in this study. The study was approved by University of British Columbia Animal Care Committee and the procedures performed on sheep conformed to the guidelines of the Canadian Council on Animal Care. Surgery was performed aseptically under halothane (1-2%) and nitrous oxide (60%) in oxygen anesthesia after induction with intravenous (*i.v.*) sodium pentothal (1 g) and intubation of the ewe. Silicone rubber catheters were implanted in femoral artery and vein for blood sampling and drug administration, respectively. Intramuscular injections of ampicillin (500 mg) were given to the ewe on the day of surgery and for 3 days postoperatively. All catheters were flushed daily with approximately 2 ml of heparinized (12 unit/ml) sterile 0.9% sodium chloride to maintain their patency. After surgery, the ewes were kept in holding pens with other sheep and allowed free access to food and water. A

minimum of 3 days were allowed for the ewes to recover before experimentation. One day before the experiment, a Foley® bladder catheter was inserted via the urethra and attached to a urine drainage bag for cumulative urine collection.

3.1.2 Experimental Protocol

Figure 3.1 is a schematic illustration of the experimental protocol. The infusion study was conducted at two FX dose levels, namely the high dose and the low dose experiments. Racemic FX infusion solution was prepared in sterile water for injection to give a final concentration of 1.523 g/ 550ml (2.77 mg/ml) and 0.762 g/ 550 ml (1.39 mg/ml) for the high dose and low dose experiments, respectively. The resulting solution was sterilized by filtering through a 0.22-μm nylon filter into a capped sterile evacuated glass container before administration. In the high dose experiment (n=6), each animal received a 70 mg *i.v.* bolus loading dose over one minute followed by a continuous *i.v.* infusion of racemic FX via the maternal femoral vein at a rate of 6.92 mg/h. In the low dose experiment (n=5), a 35 mg *i.v.* bolus loading dose over one minute followed by continuous infusion of racemic FX at 3.46 mg/h (half of the rate used in the high dose experiments) was given. In both cases, the infusion continued for 8 days in order to achieve a desired total FX steady-state concentrations (C_{ss}) of 100 ng/ml and 50 ng/ml in high and low dose experiments, respectively. In three cases the same animals (Ewe # 106, 6117, 7107) were used for the high and low dose experiments following an appropriate washout period. Serial blood samples from the maternal femoral arterial catheter (5 ml) were collected at 0, 5, 15, 30 min, 1, 2, 4, 6, 12, 24, 36, 48, 60, 72, 84, 96, 108, 120, 132, 144, 156, 168, 180 h during the

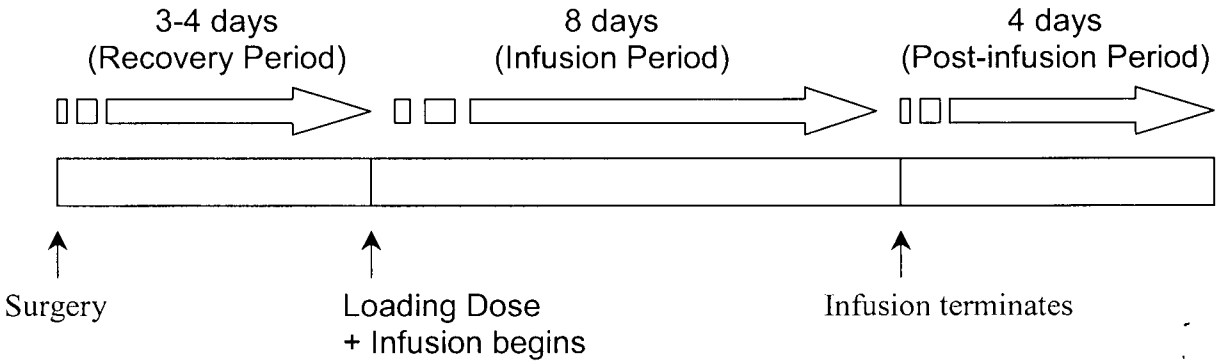


FIGURE 3.1 Schematic diagram of the experimental protocol used in the nonpregnant adult sheep experiments.

infusion, and at 0, 5, 10, 20, 30, 45 min, 1, 2, 3, 4, 6, 9, 12, 24, 36, 48, 60, 72, and 96 h post-infusion. The initial zero time blood sample was collected 10 minutes prior to *i.v.* bolus dose administration. Urine samples were collected immediately before dosing, then at twelve-hour intervals during the drug infusion period, and up to 72 hours after the discontinuation of the infusion.

All blood samples collected for drug and metabolite analysis were placed into K_3EDTA Vacutainer® tubes, gently mixed, and centrifuged at 2000g for 10 min to separate the plasma. The plasma supernatant was transferred into clean borosilicate test tubes with polytetrafluoroethylene (PTFE)-lined caps. The volume of urine collected at each time interval was recorded and a 10 ml aliquot kept and transferred into a clean borosilicate test tube for subsequent FX and NFX measurement. All biological fluid samples were stored at -20°C until the time of GC/MS analysis.

3.1.3 Determination of FX and NFX Plasma Protein Binding

Plasma protein binding of FX and NFX was determined *ex vivo* using the equilibrium dialysis procedure described by Kim (2000). Briefly, dialysis was carried out at 39°C using 1 ml Plexiglass® dialysis cells and cellophane dialysis membranes (MW cutoff of 12,000 Da). The dialysis membrane was boiled in distilled water for 30 minutes and soaked in isotonic phosphate buffer for a minimum of 1 hour prior to use. Aliquots (800 µl) of plasma sample were dialyzed against an equal volume of the isotonic phosphate buffer (pH 7.4), which was prepared by dissolving 1.79 g of KH_2PO_4, 14.35 g of $Na_2HPO_4 \cdot 7H_2O$, and 3.9 g of NaCl in 1 liter of distilled water. The equilibration time was 4 hours after which the

plasma and buffer were analyzed for FX and NFX. Plasma unbound fraction (f_u) was calculated as:

$$\%f_u = \frac{C_b}{C_p} \times 100 \qquad (3.1)$$

where C_b and C_p are drug or metabolite concentrations in buffer and plasma at the end of the dialysis experiment, respectively (Wright et al., 1996).

3.1.4 Pharmacokinetic Analysis

All pharmacokinetic parameters were calculated by standard pharmacokinetic procedures (Gibaldi and Perrier, 1982). Briefly, terminal elimination half-lives ($t_{1/2\beta}$) of the parent drug and metabolite were obtained from model fitting of the post-infusion data using the nonlinear least-squares regression software WinNonlin Version 1.1 (Scientific Consulting, Inc., Apex, NC). Model fittings were carried out using a weighting factor of 1/predicted y. Goodness of fit was evaluated based on Akaike's Information Criterion (AIC) and Schwarz Criterion (SC) values. The model associated with the smallest values of AIC and SC was regarded as giving the best fit. The area under the plasma concentration-time curves ($AUC^{0-\infty}$) was calculated as the sum of area under the curve (AUC^{0-t}) to the last time point sampled (C_t) as determined by the linear trapezoidal rule, and the estimated area to infinity ($AUC^{t-\infty}$) was determined by the relationship C_t/β, where β is the terminal elimination rate constant. The area under the first moment curve ($AUMC^{0-\infty}$) was calculated in a similar fashion with the portion from $t*C_t$ to infinity determined by ($t_{last}*C_t/\beta + C_t/\beta^2$). Total body clearance (CL_{TB}) and renal clearance (CL_{renal}) were determined from CL_{TB} = total dose/$AUC^{0-\infty}$ and $CL_{renal} = X_u/AUC^{0-\infty}$, respectively, where X_u is the cumulative amount of

drug (FX) or metabolite (NFX) collected in urine from 0 h of infusion to 96 h post-infusion (Note: total dose = *i.v.* bolus dose + total amount of FX infused over 8 days). Mean residence times (MRT) of the parent drug (Cheng and Jusko, 1991) and metabolite (Veng-Pedersen and Gillespie, 1987) were calculated as follows:

$$MRT_{\text{parent drug}} = \left(\frac{AUMC^{0-\infty}}{AUC^{0-\infty}} \right)_{\text{parent drug}} - \frac{k_o \cdot \tau^2}{2(k_o \cdot \tau + D_{\text{bolus}})} \qquad (3.2)$$

$$MRT_{\text{metabolite}} = \left(\frac{AUMC^{0-\infty}}{AUC^{0-\infty}} \right)_{\text{metabolite}} - MRT_{\text{parent drug}} \qquad (3.3)$$

where k_o, τ, and D_{bolus} are the infusion rate, infusion duration, and initial bolus loading dose of the parent drug, respectively. Steady-state volume of distribution of the parent drug (Vd_{ss}) was calculated by CL_{TB}*$MRT_{\text{parent drug}}$.

3.1.5 Statistical Analysis

A paired t-test was used for statistical analysis of the pharmacokinetic parameters when comparing 1) R- versus S-enantiomers, and 2) parent drug versus metabolite. Comparisons of the mean values of the parameters from the high and low dose infusion and *i.v.* bolus experiments were performed using one-way ANOVA followed by a post hoc Tukey's test. The significance level chosen was $p<0.05$. The achievement of steady-state for plasma FX concentrations was evaluated by testing whether the slope of plasma concentration versus time curve was significantly different from the value of zero. Unless otherwise specified, all data are reported as the mean ± standard deviation (S.D.).

3.2 Results

The mean body weight of nonpregnant ewes in the high dose and low dose experiments were 57.9 ± 10.3 kg and 59.3 ± 10.0 kg, respectively.

3.2.1 Stereoselective Pharmacokinetics

3.2.1.1 Fluoxetine

Figures 3.2 and 3.3 show the representative plasma concentration versus time profiles of FX and NFX enantiomers in a nonpregnant ewe for a high dose and low dose experiment, respectively. Significant stereoselective pharmacokinetics of the FX enantiomers was observed in both experiments. Differences in the concentrations of FX enantiomers were observed as early as 15 minutes after the start of the infusion. The concentration of S-FX remained higher than that of R-FX throughout the entire duration of the infusion and post-infusion periods. The time course of average total FX plasma concentrations of the high and low dose experiments are shown graphically in Figure 3.4. As illustrated in Figures 3.2, 3.3, and 3.4, FX and its enantiomers progressively declined from peak plasma levels obtained with the initial *i.v.* bolus loading dose and reached a minimum at around 12 hours. Following this they increased gradually and reached steady-state during day-5 to day-8 of the infusion period in both sets of experiments [slopes calculated from the plasma concentration versus time curve between day-5 to -8 were not significantly different from zero (p>0.05)]. The average C_{ss} of total FX was 151 ± 39.2 ng/ml for the high dose experiment and 62.0 ± 30.8 ng/ml for the low dose experiments. The drug infusion was terminated after day-8 and the post-infusion pharmacokinetic profile was followed for 4 days. Upon discontinuation of the infusion, FX exhibited an apparent biphasic log plasma concentration

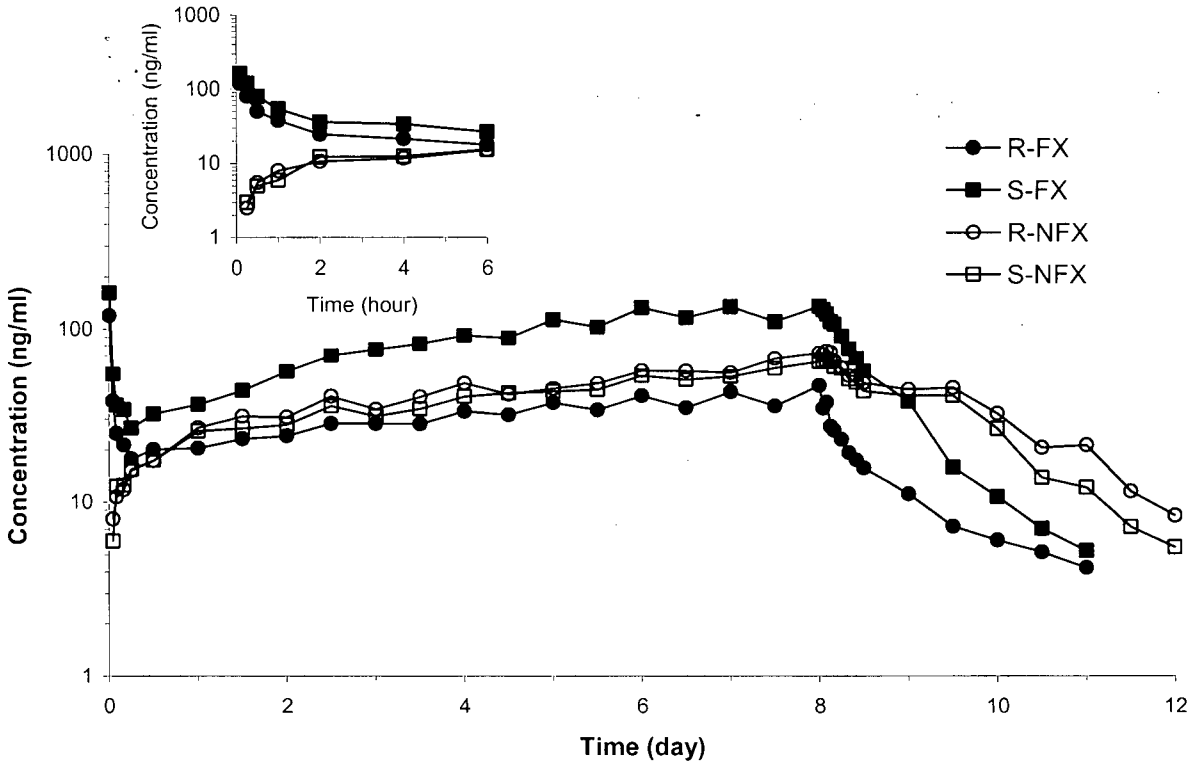


FIGURE 3.2 Representative plasma concentration versus time profiles of FX and NFX enantiomers in a nonpregnant sheep (E7139) for a high dose 8-day *i.v.* infusion experiment. The inset figure shows the concentration-time profile during the first 6 hours of infusion.

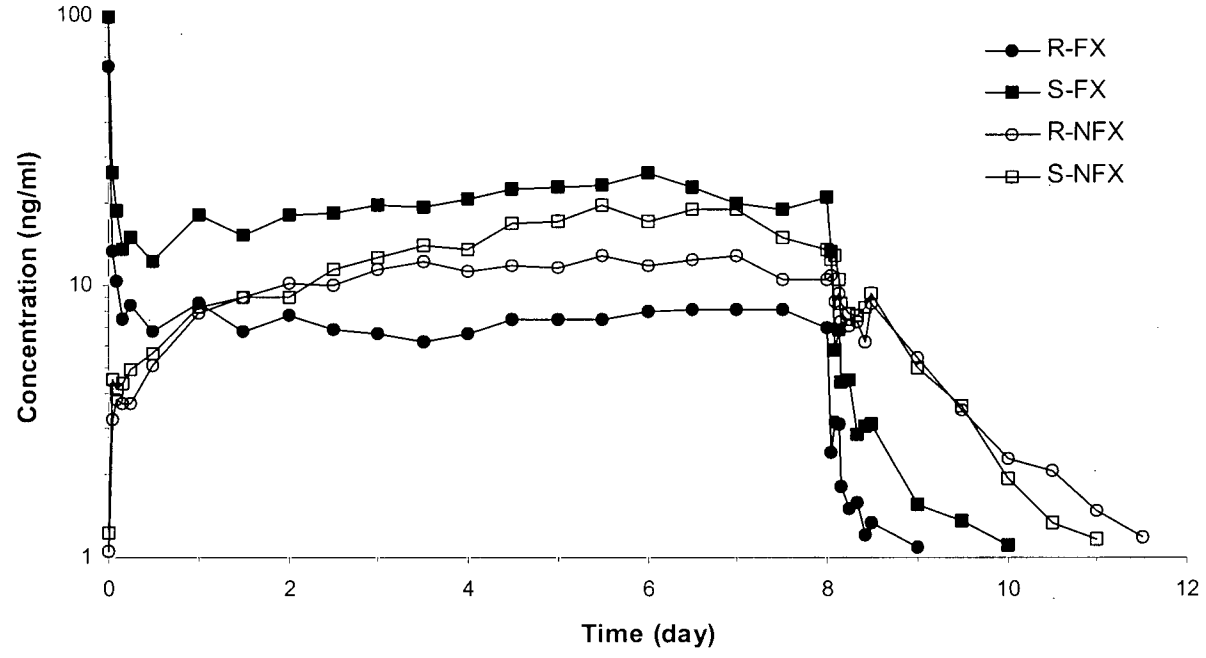


FIGURE 3.3 Representative plasma concentration versus time profiles of FX and NFX enantiomers in a nonpregnant sheep (E8107) for a low dose 8-day *i.v.* infusion experiment.

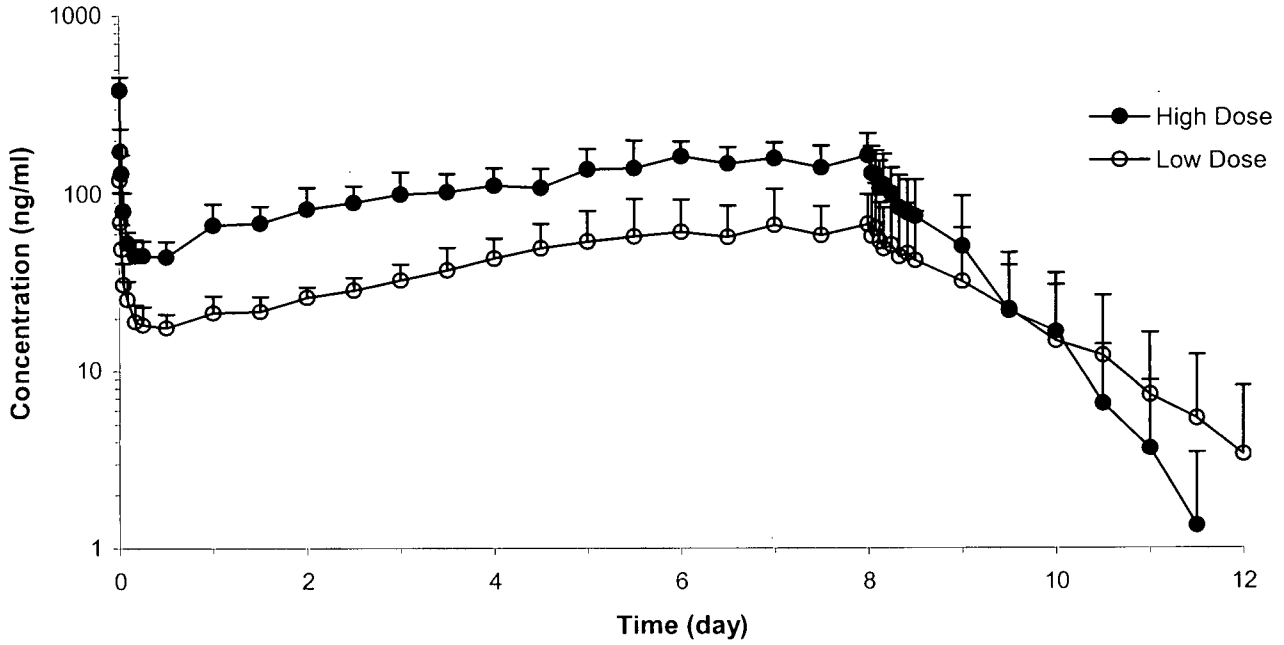


FIGURE 3.4 Comparison of the average plasma concentration versus time profiles for total FX from the high and low dose FX 8-day *i.v.* infusion experiments in nonpregnant adult sheep (n = 6).

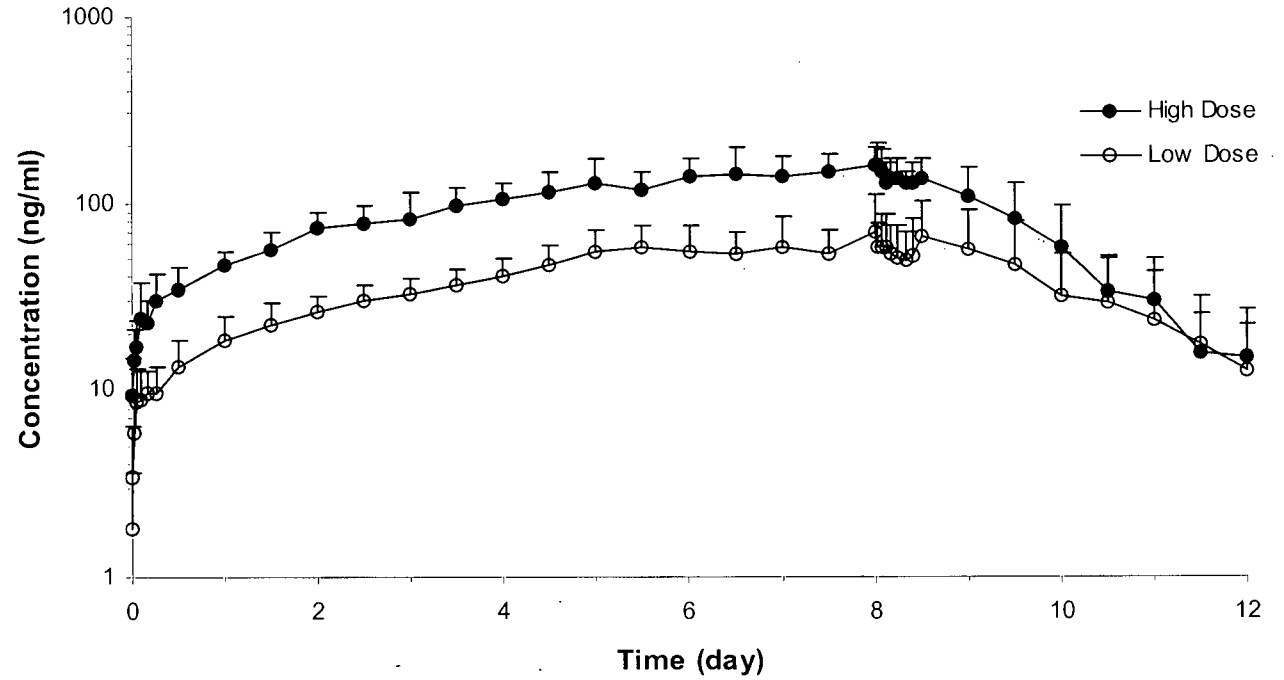


FIGURE 3.5 Comparison of the average plasma concentration versus time profiles of total NFX from the high and low dose FX 8-day *i.v.* infusion experiments in nonpregnant adult sheep (n = 6).

versus time elimination profile, which was best described by a two-compartment pharmacokinetic model based on AIC and SC values.

The pharmacokinetic parameters calculated for the FX enantiomers (mean ± S.D.) and their corresponding S/R ratios (mean ± S.D.) from the high and low dose experiments are summarized in Table 3.1. With the exception of terminal elimination half-life ($t_{1/2\beta}$), significant differences were found in all pharmacokinetic parameters between the FX enantiomers. The elimination half-life of R-FX was similar to that of S-FX in both experiments (High dose: 18.4 ± 7.7 h versus 14.8 ± 3.3 h, $p = 0.20$; Low dose: 20.1 ± 8.5 h versus 19.7 ± 8.8 h, $p = 0.18$). Mean residence time (MRT) of R-FX was significantly shorter than that of S-FX (High dose: 24.6 ± 7.0 h versus 34.9 ± 8.6 h, $p<0.0001$; Low dose: 33.4 ± 19.9 h versus 44.1 ± 22.1 h, $p<0.01$). C_{ss} was significantly lower for R-FX compared to S-FX (High dose: 35.8 ± 7.6 ng/ml versus 115.2 ± 31.6 ng/ml, $p<0.0005$; Low dose: 12.6 ± 4.6 ng/ml versus 49.4 ± 26.7 ng/ml, $p<0.05$). Similarly, area under the plasma concentration versus time curve (AUC) of R-FX was also significantly lower than that of S-FX (High dose: 6086 ± 1311 μg*h/l versus 18056 ± 5448 μg*h/l, $p<0.001$; Low dose: 2289 ± 880 μg*h/l versus 8080 ± 4318 μg*h/l, $p<0.05$). R-FX had a higher volume of distribution at steady-state (Vd_{ss}) per kilogram compared to S-FX (High dose: 44.9 ± 11.3 l/kg versus 22.7 ± 8.3 l/kg, $p<0.001$; Low dose: 76.7 ± 32.6 l/kg versus 31.0 ± 13.6 l/kg, $p<0.01$). The weight-normalized total body clearance (CL_{TB}) of R-FX was significantly higher than the clearance value of S-FX (High dose: 1.91 ± 0.61 l/h/kg versus 0.68 ± 0.28 l/h/kg, $p<0.001$; Low dose: 2.55 ± 0.68 l/kg versus 0.79 ± 0.28 l/kg, $p<0.005$). The free fraction of the FX enantiomers was determined from plasma samples obtained at 144, 168 and 192 h during the

TABLE 3.1 Pharmacokinetic parameters of the FX enantiomers in nonpregnant sheep after high dose (HD) and low dose (LD) 8-day racemic FX infusions and the corresponding *p*-values.

Animal Number	$t_{1/2\beta}$ (h)		MRT (h)		C_{ss} (ng/ml)		AUC (μg*h/l)		Vd_{ss} (l/kg)		CL_{TB} (l/h/kg)		f_u (%)	
	R-FX	S-FX	R-FX	S-FX	R-FX	S-FX	R-FX	S-FX	R-FX	S-FX	R-FX	S-FX	R-FX	S-FX
HD6117	18.4	16.3	21.4	31.5	34.5	119.6	6396	20439	28.1	13.0	1.31	0.41	1.56	0.52
HD7107	15.9	18.5	36.4	49.7	49.2	167.8	7998	26255	48.3	20.0	1.33	0.40	2.27	0.81
HD7236	10.4	10.7	21.3	28.3	34.9	107.7	5902	17790	43.3	19.1	2.03	0.67	1.57	0.44
HD7250	27.9	16.3	16.4	25.7	29.7	92.5	5600	14939	42.5	25.0	2.60	0.97	2.37	0.60
HD7139	27.3	16.4	28.0	38.6	38.5	127.1	6605	18919	44.3	21.3	1.58	0.55	3.59	1.03
HD106	10.7	10.6	23.9	35.4	27.8	76.4	4011	9994	63.1	37.6	2.64	1.06	3.13	0.89
Mean ± S.D.	18.4 ± 7.7	14.8 ± 3.3	24.6 ± 7.0	34.9 ± 8.6	35.8 ± 7.6	115.2 ± 31.6	6086 ± 1311	18056 ± 5448	44.9 ± 11.3	22.7 ± 8.3	1.91 ± 0.61	0.68 ± 0.28	2.42 ± 0.82	0.71 ± 0.23
S/R Ratio	0.87 ± 0.24		1.43 ± 0.09		3.19 ± 0.26		2.92 ± 0.31		0.50 ± 0.08		0.35 ± 0.04		0.30 ± 0.04	
Paired t-test R- vs. S-FX *p*-values	0.20		<0.0001		<0.0005		<0.001		<0.001		<0.001		<0.005	
LD7107	26.7	27.0	49.4	59.6	17.7	92.0	3105	14830	84.3	21.3	1.71	0.36	1.88	0.75
LD106	13.5	12.3	25.5	34.9	9.0	32.0	1598	5089	84.6	36.4	3.32	1.04	4.10	1.71
LD6117	14.4	13.8	9.8	14.6	7.9	22.7	1554	4085	26.4	15.1	2.70	1.03	5.86	4.17
LD5134	14.2	14.0	24.0	40.1	11.2	46.8	1809	6771	72.1	32.1	3.00	0.80	4.93	2.16
LD8107	31.7	31.4	58.2	71.4	17.3	53.5	3377	9625	116.4	50.1	2.00	0.70	4.86	3.04
Mean ± S.D.	20.1 ± 8.5	19.7 ± 8.8	33.4 ± 19.9	44.1 ± 22.1	12.6 ± 4.6	49.4 ± 26.7	2289 ± 880	8080 ± 4318	76.7 ± 32.6	31.0 ± 13.6	2.55 ± 0.68	0.79 ± 0.28	4.33 ± 1.50	2.37 ± 1.30
S/R Ratio	0.97 ± 0.04		1.39 ± 0.19		3.78 ± 0.94		3.44 ± 0.86		0.43 ± 0.11		0.30 ± 0.07		0.52 ± 0.14	
Paired t-test R- vs. S-FX *p*-value	0.18		<0.01		<0.05		<0.05		<0.01		<0.005		<0.005	

Note: Data are expressed as mean ± S.D.

FX constant rate infusion (i.e. at steady-state). R-FX had a higher free fraction compared to S-FX (High dose: 2.42 ± 0.82 % versus 0.71 ± 0.23%, $p<0.005$; Low dose: 4.33 ± 1.50 % versus 2.37 ± 1.30 %, $p<0.005$).

Due to premature detachment of the Foley® catheter during the early experimental period in some animals, complete urine collection was obtained from only three sheep in the high dose experiments (Ewe # 106, 7139, and 7250). All urine was successfully collected from the five sheep in the low dose experiments. Figure 3.6 illustrates a representative plot of the time course of the cumulative amount of FX excreted in the urine of a nonpregnant sheep in a high dose experiment. The cumulative amount of the unchanged FX excreted in urine reached a maximum 2 days after the termination of the drug infusion. The residual amount of drug collected over the last 48 hours period was less than 1% of the total amount excreted. Pharmacokinetic parameters for FX renal elimination obtained in the nonpregnant sheep in the high (n=3) and low dose (n=5) experiments are summarized in Table 3.2. In both sets of experiments, mean weight normalized renal clearance (CL_{renal}) of R-FX appeared to be higher than that of S-FX (High dose: 0.97 ± 0.40 ml/h/kg versus 0.66 ± 0.38 ml/h/kg, $p=0.16$; Low dose: 2.64 ± 3.58 ml/h/kg versus 0.57 ± 0.38 ml/h/kg, $p=0.24$) but the difference was not statistically significant. The percentage of unchanged FX recovered in urine was less than 1% of the total administered dose and did not differ significantly between R-FX and S-FX (High dose: 0.04 ± 0.02 % versus 0.08 ± 0.03 %, $p=0.15$; Low dose: 0.10 ± 0.12 % versus 0.07 ± 0.04 %, $p=0.49$).

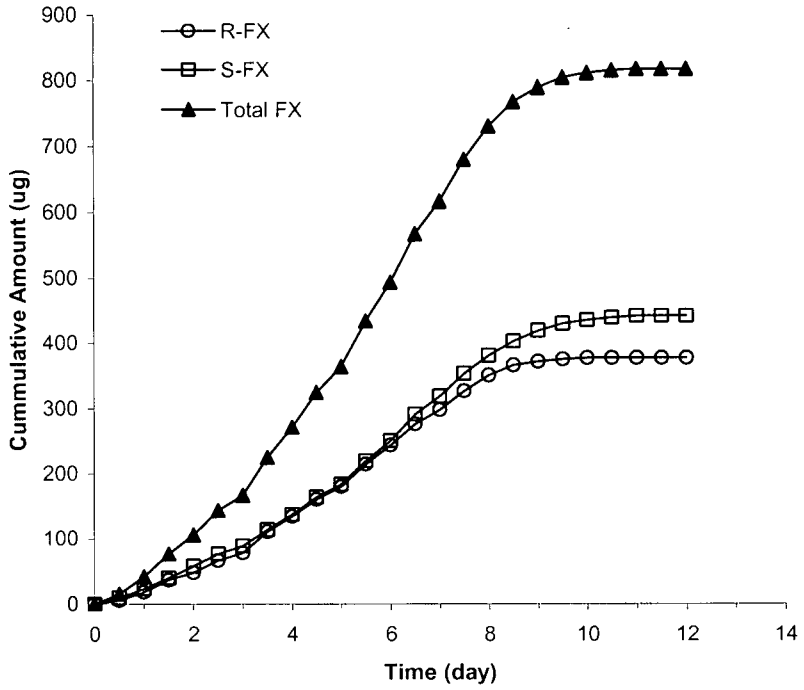


FIGURE 3.6 Representative plot of the cumulative amount of FX excreted in the urine of a nonpregnant sheep (E7139) for a high dose FX 8-day *i.v.* infusion experiment.

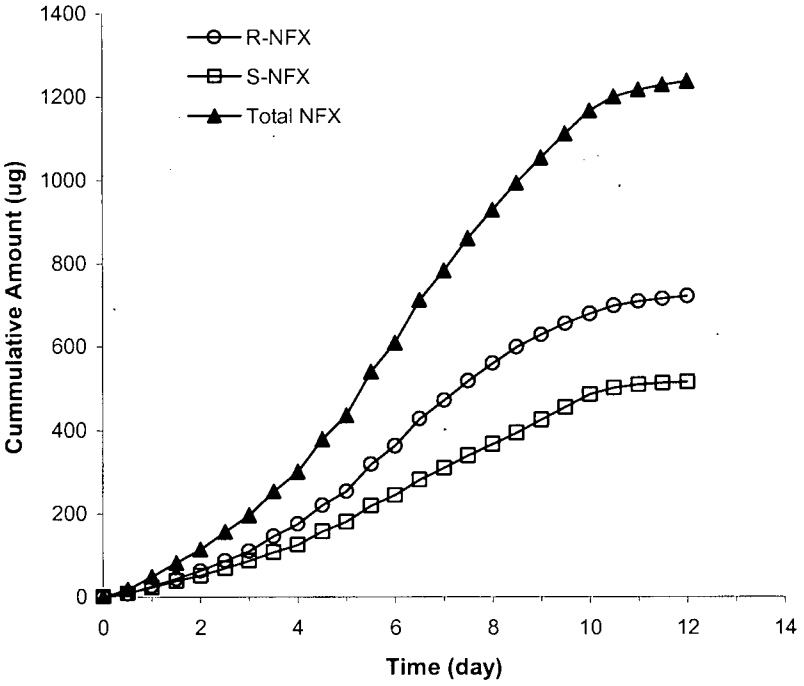


FIGURE 3.7 Representative plot of the cumulative amount of NFX excreted in the urine of a nonpregnant sheep (E7139) for a high dose FX 8-day *i.v.* infusion experiment.

TABLE 3.2 Pharmacokinetic parameters for the renal elimination of FX and NFX in nonpregnant sheep after high dose (HD) and low dose (LD) 8-day racemic FX infusions and the corresponding *p*-values.

Animal Number	CL_{renal} (ml/h/kg)†				Amount excreted as % of FX dose			
	R-FX	S-FX	R-NFX	S-NFX	R-FX	S-FX	R-NFX	S-NFX
HD7250	1.37	1.09	2.64	1.73	0.05	0.11	0.27	0.19
HD7139	0.95	0.39	0.98	0.81	0.06	0.07	0.12	0.08
HD106	0.58	0.50	0.77	0.69	0.02	0.05	0.08	0.07
Mean ± S.D.	0.97 ± 0.40	0.66 ± 0.38	1.46 ± 1.03	1.07 ± 0.57	0.04 ± 0.02	0.08 ± 0.03	0.15 ± 0.10	0.11 ± 0.06
S/R Ratio	0.69 ± 0.25		0.79 ± 0.12		1.82 ± 0.56		0.77 ± 0.11	
Paired t-test R- vs. S-enantiomer *p*-values	0.16		0.28		0.15		0.17	
LD7107	1.38	0.26	0.61	0.44	0.08	0.07	0.11	0.07
LD106	0.89	0.51	0.67	0.57	0.03	0.05	0.05	0.05
LD6117	0.99	0.93	2.90	2.14	0.04	0.09	0.17	0.16
LD5134	9.04	0.99	0.43	0.55	0.30	0.12	0.06	0.05
LD8107	0.91	0.15	0.34	0.32	0.05	0.02	0.05	0.03
Mean ± S.D.	2.64 ± 3.58	0.57 ± 0.38	0.99 ± 1.07	0.81 ± 0.75	0.10 ± 0.12	0.07 ± 0.04	0.09 ± 0.05	0.07 ± 0.05
S/R Ratio	0.40 ± 0.36		0.91 ± 0.22		1.22 ± 0.91		0.77 ± 0.19	
Paired t-test R- vs. S-enantiomer *p*-values	0.24		0.29		0.49		0.08	

Note: Data are expressed as mean ± S.D.

† Complete urine collection was only obtained in 3 adult sheep in the high dose infusions.

3.2.1.2 Norfluoxetine

Representative plasma concentration versus time profiles obtained for the NFX enantiomers in a nonpregnant ewe for the high and low dose experiments are presented in Figures 3.2 and 3.3, respectively. Unlike the parent drug, no apparent differences were observed in the plasma concentration-time profiles of the NFX enantiomers. The mean total NFX plasma concentration versus time profiles obtained for the high and low dose experiments are illustrated in Figure 3.5. NFX appeared rapidly in plasma with quantifiable levels observed at 5 minutes after the beginning of FX infusion in all animals. The concentration of NFX continued to accumulate gradually and reached steady-state between day-6 to day-8 of the infusion period. Mean C_{ss} of total NFX was 143.2 ± 38.4 ng/ml for the high dose experiments and 57.8 ± 23.7 ng/ml for the low dose studies. The concentration of NFX was comparable to that of FX at steady-state. Upon discontinuation of the 8-day FX infusion, NFX levels remained relatively constant for 2 to 3 hours before starting to decline. The elimination of NFX was slower than that of FX with considerable levels of the metabolite measured in all samples collected 4 days post infusion (range 2.4 – 35.7 ng/ml for NFX versus 0 – 10.3 ng/ml for FX). The pharmacokinetic parameters of the R and S NFX enantiomers (mean ± S.D.) and their corresponding S/R ratios (mean ± S.D.) for the high and low dose experiments are summarized in Table 3.3. The terminal elimination half-life was longer for R-NFX compared to S-NFX (High dose: 26.0 ± 5.6 h versus 21.4 ± 4.2 h, $p<0.05$; Low dose: 31.0 ± 3.5 h versus 26.1 ± 4.8 h, $p<0.05$). Similarly, the MRT of R-NFX was also significantly higher than that of S-NFX (High dose: 124.8 ± 5.4 h versus 108.5 ± 3.6 h, $p<0.05$; Low dose: 126.1 ± 16.7 h versus 105.7 ± 10.0 h, $p<0.05$). In contrast to the parent drug, the average C_{ss} of R-NFX and S-NFX were not significantly different from one another

TABLE 3.3 Pharmacokinetic parameters of NFX enantiomers in nonpregnant sheep after high dose (HD) and low dose (LD) 8-day racemic FX infusions and the corresponding *p*-values.

Animal Number	$t_{1/2\beta}$ (h)		MRT (h)		C_{ss} (ng/ml)		AUC (μg*h/l)		NFX/FX AUC ratio		FX+NFX AUC (μg*h/l)		f_u (%)	
	R-NFX	S-NFX	R-NFX	S-NFX	R-NFX	S-NFX	R-NFX	S-NFX	R-NFX	S-NFX	R-NFX	S-NFX	R-NFX	S-NFX
HD6117	24.1	24.6	125.6	109.1	53.4	41.4	10624	8709	1.66	0.43	17021	29148	1.62	1.07
HD7107	23.0	19.8	124.7	104.4	111.7	85.6	21490	16736	2.69	0.64	29488	42991	1.92	1.62
HD7236	20.3	16.5	115.2	104.4	69.1	63.9	12434	11394	2.11	0.64	18337	29184	1.47	0.62
HD7250	28.5	21.6	129.1	113.2	83.5	96.9	14652	15597	2.62	1.04	20252	30535	2.03	0.92
HD7139	36.0	27.7	130.5	111.4	63.5	57.7	12237	10609	1.85	0.56	18842	29528	2.75	1.39
HD106	24.0	18.0	123.5	108.4	65.0	67.2	10508	10543	2.62	1.05	14519	20537	2.31	1.23
Mean ± S.D.	26.0 ± 5.6	21.4 ± 4.2	124.8 ± 5.4	108.5 ± 3.6	74.4 ± 20.7	68.8 ± 19.9	13658 ± 4122	12265 ± 3169	2.26 ± 0.44	0.73 ± 0.26	19743 ± 5152	30321 ± 7206	2.02 ± 0.47	1.14 ± 0.35
S/R Ratio	0.83 ± 0.10		0.87 ± 0.02		0.93 ± 0.15		0.91 ± 0.11		0.32 ± 0.07		1.54 ± 0.11		0.57 ± 0.16	
Paired t-test R- vs. S-NFX *p*-values	<0.05		<0.0001		0.35		0.14		<0.0001		<0.0005		<0.005	
LD7107	24.6	22.9	127.8	102.7	52.3	40.1	9945	7938	3.20	0.54	13050	22767	1.62	1.42
LD106	29.3	22.1	119.3	100.8	20.5	22.8	3971	4488	2.49	0.88	5569	9577	4.21	2.44
LD6117	30.7	29.8	117.5	114.3	12.1	17.4	2489	3090	1.60	0.76	4043	7175	6.04	4.23
LD5134	32.8	23.2	154.2	117.4	35.2	26.5	7367	4667	4.07	0.69	9176	11438	4.02	2.96
LD8107	35.7	32.6	111.9	93.2	39.2	22.8	9060	5467	2.68	0.57	12437	15092	4.85	3.94
Mean ± S.D.	31.0 ± 3.5	26.1 ± 4.8	126.1 ± 16.7	105.7 ± 10.0	31.9 ± 15.8	25.9 ± 8.6	6566 ± 3226	5130 ± 1788	2.81 ± 0.91	0.69 ± 0.14	8855 ± 4015	13210 ± 6076	4.15 ± 1.62	3.00 ± 1.14
S/R Ratio	0.84 ± 0.11		0.84 ± 0.08		0.93 ± 0.34		0.88 ± 0.29		0.28 ± 0.13		1.54 ± 0.28		0.74 ± 0.11	
Paired t-test R- vs. S-NFX *p*-value	<0.05		<0.05		0.23		0.17		<0.01		<0.05		<0.05	

Note: Data are expressed as mean ± S.D.

(High dose: 74.4 ± 20.7 ng/ml versus 68.8 ± 19.9 ng/ml, $p = 0.35$; Low dose: 31.9 ± 15.8 ng/ml versus 25.9 ± 8.6 ng/ml, $p = 0.23$). Similarly, no difference was observed between the AUC values of R-NFX and S-NFX (High dose: 13658 ± 4122 μg*h/l versus 12265 ± 3169 μg*h/l, $p = 0.14$; Low dose: 6566 ± 3226 μg*h/l versus 5130 ± 1788 μg*h/l, $p = 0.17$). The AUC ratio of NFX to FX was significantly higher for the R-enantiomer compared to the S-enantiomer (High dose: 2.26 ± 0.44 versus 0.73 ± 0.26, $p<0.0001$; Low dose: 2.81 ± 0.91 versus 0.69 ± 0.14, $p<0.01$). The combined AUC values of the parent and metabolite showed a significant difference between the R- and S-enantiomers with the R-enantiomers having a lower combined AUC value compared to the S-enantiomers (High dose: 19743 ± 5152 μg*h/l versus 30321 ± 7206 μg*h/l, $p<0.0005$; Low dose: 8855 ± 4015 μg*h/l versus 13210 ± 6076 μg*h/l, $p<0.05$). Similar to the parent compound, the free fraction was significantly higher for R-NFX compared to S-NFX (High dose: 2.02 ± 0.47 % versus 1.14 ± 0.35 %, $p<0.005$; Low dose: 4.15 ± 1.62 % versus 3.00 ± 1.14 %, $p<0.05$).

Figure 3.7 illustrates a representative plot of the time course of the cumulative amount of NFX collected from the urine of a nonpregnant sheep in a high dose experiment. Overall, the excretion of NFX was close to complete 4 days after the termination of the 8-day FX infusion, with the amount of metabolite collected over the last 48 h accounting for less than 5% of the total amount excreted. The renal elimination kinetic parameters for R and S NFX in the nonpregnant sheep are summarized in Table 3.2. No significant difference was observed between the mean weight normalized renal clearance (CL_{renal}) of R-NFX and S-NFX (High dose: 1.46 ± 1.03 ml/h/kg versus 1.07 ± 0.57 ml/h/kg, $p = 0.28$; Low dose: 0.99 ± 1.07 ml/h/kg versus 0.81 ± 0.75 ml/h/kg, $p = 0.29$). Similar to FX, the percentage of total NFX recovered in urine was less than 1% of the total administered FX dose. The percentage

of R-NFX excreted appeared to be higher than that of S-NFX (High dose: 0.15 ± 0.10 % versus 0.11 ± 0.06 %, $p = 0.17$; Low dose: 0.09 ± 0.05 % versus 0.07 ± 0.05 %, $p = 0.08$) but the difference was not statistically significant.

3.2.2 Nonlinear Pharmacokinetics

3.2.2.1 Fluoxetine

The effect of dosing on the pharmacokinetics of FX was examined by statistically comparing the pharmacokinetic parameters of total FX, and the R and S enantiomers determined from the current high and low dose 8-day infusion experiments, as well as from the previous *i.v.* bolus studies (Kim, 2000). The results of these comparisons are summarized in Table 3.4. No significant difference was found in the elimination half-lives values of total FX, R-FX and S-FX obtained from these three different dosing experiments. MRTs of total FX and S-FX were significantly prolonged in the high and low dose infusion experiments when compared to the *i.v.* bolus experiments ($p < 0.01$). Similarly, MRTs of R-FX obtained from the infusion experiments were also longer compared to the *i.v.* bolus experiments although only the low dose infusion and *i.v.* bolus comparison was statistically significant ($p<0.05$). An increase in Vd_{ss} of FX was observed in the infusion experiments compared to the previous *i.v.* bolus studies, however, this was only significant for the low dose infusion experiments ($p<0.01$). Moreover, the Vd_{ss} determined from the low dose infusion experiments appeared to be higher than those from the high dose infusion studies but this was only statistically significant for R-FX ($p < 0.01$). An interesting dose-related change in clearance was observed among different dosing experiments as illustrated in Figure 3.8. The CL_{TB} of total FX, R-FX, and S-FX did not differ significantly between

TABLE 3.4 Comparison of FX pharmacokinetic parameter values determined from different dosing experiments in nonpregnant sheep.

FX Pharmacokinetic Parameter	Total/ Enantiomer	Dosing Regimen†	Mean ± S.D.	*p*-Value	Dosing Regimen Comparison‡		
					High	Low	*i.v.* bolus
$t_{1/2\beta}$ (h)	Total FX	High	14.2 ± 3.2	0.19	-	NS	-
		Low	19.9 ± 9.4		NS	-	-
		i.v. bolus	n/a§		-	-	-
	R	High	18.4 ± 7.7	0.82	-	NS	NS
		Low	20.1 ± 8.5		NS	-	NS
		i.v. bolus	20.2 ± 6.0		NS	NS	-
	S	High	14.8 ± 3.3	0.33	-	NS	NS
		Low	19.7 ± 8.8		NS	-	NS
		i.v. bolus	14.8 ± 5.0		NS	NS	-
MRT (h)	Total FX	High	32.2 ± 8.3	<0.01	-	NS	**
		Low	41.7 ± 21.6		NS	-	**
		i.v. bolus	9.3 ± 3.4		**	**	-
	R	High	24.6 ± 7.0	<0.05	-	NS	NS
		Low	33.4 ± 19.9		NS	-	*
		i.v. bolus	10.0 ± 3.7		NS	*	-
	S	High	34.9 ± 8.6	<0.01	-	NS	**
		Low	44.1 ± 22.1		NS	-	**
		i.v. bolus	8.8 ± 3.2		**	**	-
Vd_{ss} (l/kg)	Total FX	High	30.8 ± 10.0	<0.01	-	NS	NS
		Low	44.6 ± 19.1		NS	-	**
		i.v. bolus	14.4 ± 3.7		NS	**	-
	R	High	44.9 ± 11.3	<0.01	-	**	NS
		Low	76.7 ± 32.6		**	-	**
		i.v. bolus	18.5 ± 4.7		NS	**	-
	S	High	22.7 ± 8.3	<0.01	-	NS	NS
		Low	31.0 ± 13.6		NS	-	**
		i.v. bolus	11.6 ± 2.9		NS	**	-
CL_{TB} (l/h/kg)	Total FX	High	1.00 ± 0.39	0.06	-	NS	NS
		Low	1.19 ± 0.40		NS	-	NS
		i.v. bolus	1.66 ± 0.51		NS	NS	-
	R	High	1.91 ± 0.61	0.19	-	NS	NS
		Low	2.55 ± 0.68		NS	-	NS
		i.v. bolus	1.97 ± 0.48		NS	NS	-
	S	High	0.68 ± 0.28	<0.01	-	NS	**
		Low	0.79 ± 0.28		NS	-	**
		i.v. bolus	1.44 ± 0.51		**	**	-
Dose normalized AUC (h/l per dose unit)	Total FX	High	0.039 ± 0.011	0.05	-	NS	NS
		Low	0.033 ± 0.016		NS	-	NS
		i.v. bolus	0.018 ± 0.008		NS	NS	-
	R	High	0.010 ± 0.002	0.27	-	NS	NS
		Low	0.007 ± 0.003		NS	-	NS
		i.v. bolus	0.007 ± 0.002		NS	NS	-
	S	High	0.029 ± 0.009	<0.05	-	NS	*
		Low	0.026 ± 0.014		NS	-	*
		i.v. bolus	0.011 ± 0.005		*	*	-
f_u (%)	Total FX	High	0.95 ± 0.32	<0.01	-	**	-
		Low	2.76 ± 1.34		**	-	-
		i.v. bolus	n/a§		-	-	-
	R	High	2.42 ± 0.82	<0.05	-	*	*
		Low	4.33 ± 1.50		*	-	NS
		i.v. bolus	4.9 ± 0.6		*	NS	-
	S	High	0.71 ± 0.23	<0.05	-	*	*
		Low	2.37 ± 1.30		*	-	NS
		i.v. bolus	2.3 ± 0.4		*	NS	-

† High, low and *i.v.* bolus refer to the high and low dose FX *i.v.* infusions and the previous *i.v.* bolus experiments (Kim, 2000), respectively.

‡ From one-way ANOVA with post hoc Tukey comparison (* $p<0.05$, ** $p<0.01$, NS = not significant).

§ Could not be determined due to insufficient data.

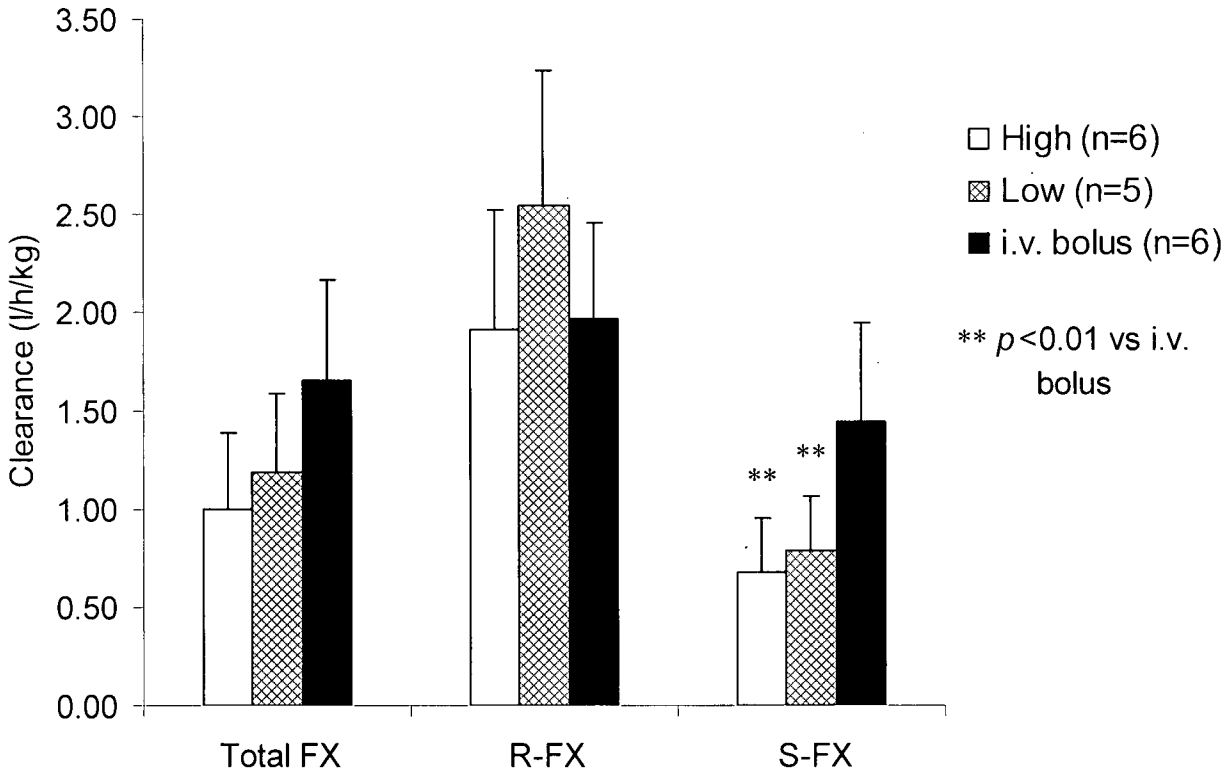


FIGURE 3.8 Total body clearance of Total FX, R-FX, and S-FX following high dose infusion, low dose infusion, and single *i.v.* bolus administration of racemic FX. ** A significant dose related decrease in clearance is observed for S-FX.

the high and low infusion experiments (Figure 3.8, Table 3.4). Decreases of 40% and 20% in CL_{TB} for total FX were observed in the high and low dose infusion experiments, respectively, compared to *i.v.* bolus experiments, however, these differences were not statistically significant (p = 0.06). Interestingly, CL_{TB} of R-FX remained unchanged in all three experiments (p = 0.19) whereas the CL_{TB} of S-FX was significantly decreased by 53% and 45% in the high and low dose infusion experiments, respectively, compared to the *i.v.* bolus experiments (p <0.01) (Figure 3.8, Table 3.4). Corresponding to the change in CL_{TB}, the dose normalized AUC values of S-FX obtained from the infusion experiments were significantly higher than that from the *i.v.* bolus experiment (p<0.05) (Figure 3.9, Table 3.4). The free fraction of total FX determined from the high dose infusion experiment was significantly lower compared to the low dose infusion studies (p<0.01). The free fraction values of R- and S-FX, determined from the high dose infusion experiments, were also significantly lower than those obtained from the low dose infusion and *i.v.* bolus experiments (p<0.05). Free fractions of R-FX and S-FX did not differ significantly between the low dose infusion and *i.v.* bolus experiments.

The comparison of the renal elimination kinetic parameters obtained for FX in the three different studies is summarized in Table 3.5. Total FX CL_{renal} was significantly higher in the *i.v.* bolus experiments compared to both infusion values (p<0.05). In agreement with the lower CL_{renal} values, a relatively lower percentage of the total drug was excreted in urine in the infusion studies compared to the *i.v.* bolus experiments (p = 0.05). CL_{renal} and the % amount excreted for S-FX were also significantly higher in the *i.v.* bolus experiments compared to both infusion studies (p<0.01). On the other hand, no differences were observed for the renal elimination of R-FX among these three different studies (p>0.05).

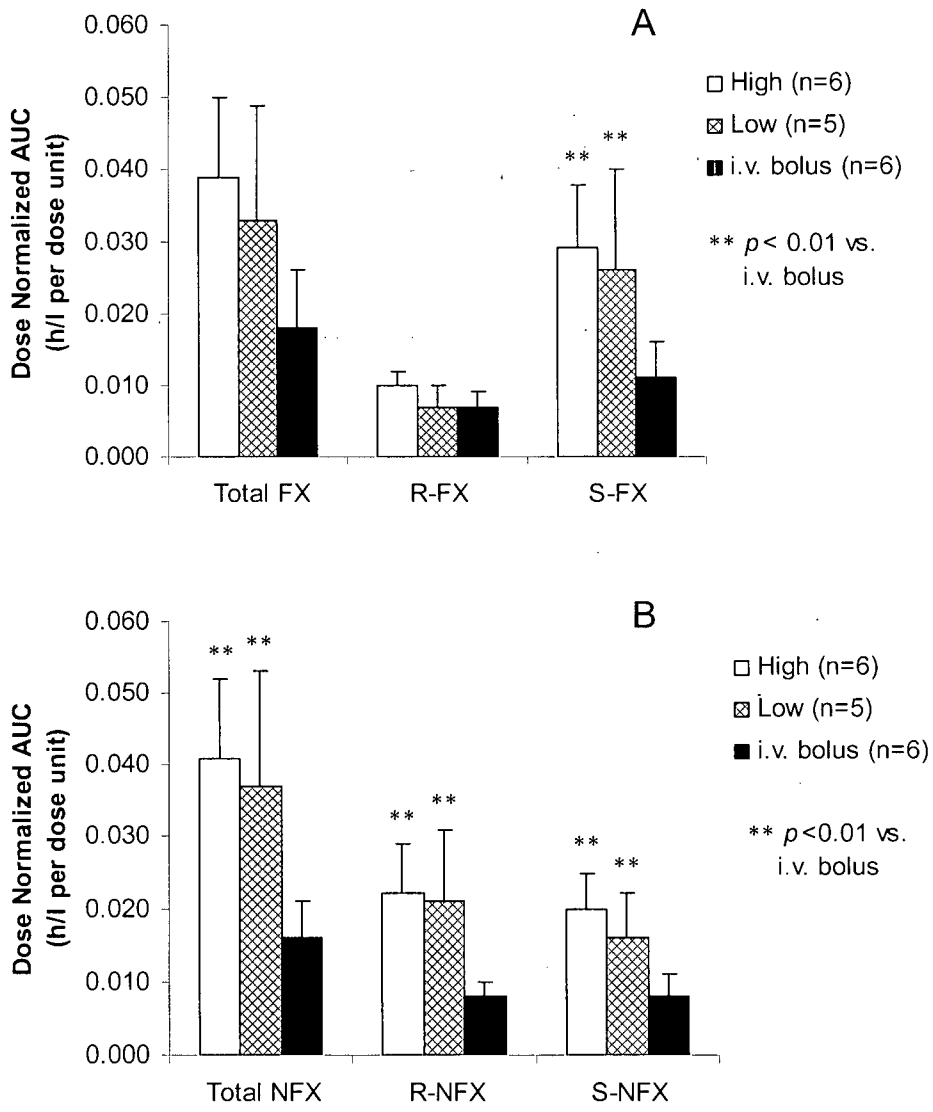


FIGURE 3.9 Dose normalized AUC values of Total FX, R-FX, and S-FX (Chart A), and Total NFX, R-NFX, and S-NFX (Chart B) following high dose infusion, low dose infusion, and single *i.v.* bolus administration of racemic FX. * and ** denote significant differences from the *i.v.* bolus data with *p*-values of <0.01 and <0.05, respectively.

TABLE 3.5 Comparison of renal FX and NFX elimination kinetic parameter values obtained from different dosing experiments in nonpregnant sheep.

Pharmacokinetic Parameter	Total/ Enantiomer	Dosing Group†	Mean ± S.D.	*p*-value	Dosing Group Comparison‡		
					High	Low	*i.v.* bolus
CL_{renal} (ml/h/kg)	Total FX	High	0.74 ± 0.37	<0.05	-	NS	*
		Low	1.01 ± 0.97		NS	-	*
		i.v. bolus	3.24 ± 1.47		*	*	-
	R-FX	High	0.97 ± 0.40	0.68	-	NS	NS
		Low	2.64 ± 3.58		NS	-	NS
		i.v. bolus	2.16 ± 1.01		NS	NS	-
	S-FX	High	0.66 ± 0.38	<0.01	-	NS	**
		Low	0.57 ± 0.38		NS	-	**
		i.v. bolus	4.08 ± 1.83		**	**	-
	Total NFX	High	1.27 ± 0.79	<0.01	-	NS	**
		Low	0.89 ± 0.89		NS	-	**
		i.v. bolus	17.70 ± 8.22		**	**	-
	R-NFX	High	1.46 ± 1.03	<0.01	-	NS	**
		Low	0.99 ± 1.07		NS	-	**
		i.v. bolus	13.14 ± 6.28		**	**	-
	S-NFX	High	1.07 ± 0.57	<0.01	-	NS	**
		Low	0.81 ± 0.75		NS	-	**
		i.v. bolus	23.60 ± 13.01		**	**	-
Amount excreted as % of FX dose	Total FX	High	0.06 ± 0.02	0.05	-	NS	NS
		Low	0.08 ± 0.07		NS	-	NS
		i.v. bolus	0.19 ± 0.09		NS	NS	-
	R-FX	High	0.04 ± 0.02	0.51	-	NS	NS
		Low	0.10 ± 0.12		NS	-	NS
		i.v. bolus	0.11 ± 0.05		NS	NS	-
	S-FX	High	0.08 ± 0.03	<0.01	-	NS	**
		Low	0.07 ± 0.04		NS	-	**
		i.v. bolus	0.27 ± 0.13		**	**	-
	Total NFX	High	0.12 ± 0.10	<0.01	-	NS	**
		Low	0.08 ± 0.05		NS	-	**
		i.v. bolus	0.96 ± 0.58		**	**	-
	R-NFX	High	0.15 ± 0.10	<0.05	-	NS	*
		Low	0.09 ± 0.05		NS	-	*
		i.v. bolus	0.76 ± 0.48		*	*	-
	S-NFX	High	0.11 ± 0.06	<0.01	-	NS	**
		Low	0.07 ± 0.05		NS	-	**
		i.v. bolus	1.15 ± 0.67		**	**	-

† High, low and *i.v.* bolus refers to the high and low dose FX *i.v.* infusion and the previous *i.v.* bolus experiments (Kim, 2000), respectively.

‡ From one-way ANOVA with post hoc Tukey comparison (* $p<0.05$, ** $p<0.01$, NS = not significant).

3.2.2.2 Norfluoxetine

Statistical analysis was also performed to examine the effect of dosing on the pharmacokinetics of NFX by comparing the pharmacokinetic parameters of total NFX, R-NFX, and S-NFX obtained from the high and low dose infusions, and previous *i.v.* bolus experiments; these results are summarized in Table 3.6. The elimination half-life ($t_{1/2\beta}$) of total NFX in the high dose infusion experiments was significantly shorter than that in the low dose infusion studies ($p<0.05$). The elimination half-life ($t_{1/2\beta}$) of total NFX could not be determined in the *i.v.* bolus experiments therefore it is not included in the analysis. Although a significant difference was observed for the $t_{1/2\beta}$ of total NFX, no statistically significant differences were found in the $t_{1/2\beta}$ values of the NFX enantiomers among the high dose infusion, low dose infusion, and *i.v.* bolus experiments. However, the MRTs of total NFX, R-NFX and S-NFX were significantly prolonged by more than 10-fold in the high and low dose infusion experiments compared to the *i.v.* bolus experiments ($p<0.01$). Metabolite to parent area ratios (NFX/FX AUC ratio) for the total drug increased by 27-30% in the infusion experiments compared to *i.v.* bolus experiment but this difference was not statistically significant. The NFX/FX AUC ratios of R-enantiomer were significantly higher (>2-fold) in the high and low dose infusion experiments compared to the *i.v.* bolus studies ($p<0.01$). On the contrary, no statistically difference was found in NFX/FX AUC ratios of S-enantiomer among these experiments. The dose normalized AUC values of total NFX, R-NFX and S-NFX showed significant increases in the high and low dose infusion studies compared to the *i.v.* bolus study ($p<0.01$ in all cases) and these effects are illustrated graphically in Figure 3.9. The free fraction of NFX in the high dose experiments appeared to be the lowest among all free fractions determined in the other experiments. The free fraction

TABLE 3.6 Comparison of pharmacokinetic parameters of NFX between different dosing experiments in nonpregnant sheep.

NFX Pharmacokinetic Parameter	Total/ Enantiomer	Dosing Group†	Mean ± S.D.	*p*-value	Dosing Group Comparison‡		
					High	Low	*i.v.* bolus
$t_{1/2\beta}$ (h)	Total NFX	High	23.7 ± 4.9	<0.05	-	*	-
		Low	32.9 ± 5.9		*	-	-
		i.v. bolus	n/a§		-	-	-
	R	High	26.0 ± 5.6	0.11	-	NS	NS
		Low	31.0 ± 3.5		NS	-	NS
		i.v. bolus	48.8 ± 30.2		NS	NS	-
	S	High	21.4 ± 4.2	0.54	-	NS	NS
		Low	26.1 ± 4.8		NS	-	NS
		i.v. bolus	26.2 ± 14.1		NS	NS	-
MRT (h)	Total NFX	High	114.3 ± 4.2	<0.01	-	NS	**
		Low	113.7 ± 12.5		NS	-	**
		i.v. bolus	8.5 ± 4.0		**	**	-
	R	High	124.8 ± 5.4	<0.01	-	NS	**
		Low	126.1 ± 16.7		NS	-	**
		i.v. bolus	8.5 ± 4.2		**	**	-
	S	High	108.5 ± 3.6	<0.01	-	NS	**
		Low	105.7 ± 10.0		NS	-	**
		i.v. bolus	8.2 ± 3.8		**	**	-
Dose normalized AUC (h/l per dose unit)	Total NFX	High	0.041 ± 0.011	<0.01	-	NS	**
		Low	0.037 ± 0.016		NS	-	**
		i.v. bolus	0.016 ± 0.005		**	**	-
	R	High	0.022 ± 0.007	<0.01	-	NS	**
		Low	0.021 ± 0.010		NS	-	**
		i.v. bolus	0.008 ± 0.002		**	**	-
	S	High	0.020 ± 0.005	<0.01	-	NS	**
		Low	0.016 ± 0.006		NS	-	**
		i.v. bolus	0.008 ± 0.003		**	**	-
NFX/FX AUC ratio	Total NFX	High	1.12 ± 0.31	0.12	-	NS	NS
		Low	1.15 ± 0.18		NS	-	NS
		i.v. bolus	0.88 ± 0.15		NS	NS	-
	R	High	2.26 ± 0.44	<0.01	-	NS	**
		Low	2.81 ± 0.91		NS	-	**
		i.v. bolus	1.08 ± 0.11		**	**	-
	S	High	0.73 ± 0.26	0.91	-	NS	NS
		Low	0.69 ± 0.14		NS	-	NS
		i.v. bolus	0.74 ± 0.19		NS	NS	-
f_u (%)	Total NFX	High	1.64 ± 0.39	<0.01	-	**	-
		Low	3.76 ± 1.45		**	-	-
		i.v. bolus	n/a§		-	-	-
	R	High	2.02 ± 0.47	<0.05	-	*	NS
		Low	4.15 ± 1.62		*	-	NS
		i.v. bolus	3.8 ± 0.4		NS	NS	-
	S	High	1.14 ± 0.35	<0.01	-	**	**
		Low	3.00 ± 1.14		**	-	NS
		i.v. bolus	3.0 ± 0.5		**	NS	-

† High, low and *i.v.* bolus refers to the high and low dose FX *i.v.* infusion and the previous *i.v.* bolus experiments (Kim, 2000), respectively.

‡ From one-way ANOVA with post hoc Tukey comparison (* $p<0.05$, ** $p<0.01$, NS = not significant).

§ Could not be determined due to insufficient data.

of total NFX in the high dose infusion experiments was significantly lower than that in the low dose infusions ($p<0.01$). Similarly, the free fraction for R-NFX in the high dose infusions was significantly lower than that in the low dose infusion experiments ($p<0.05$) while no statistically significant difference was observed compared to the *i.v.* bolus value. For S-NFX, the free fraction in the high dose infusion experiment was significantly lower compared to both the low dose infusion and *i.v.* bolus experiments ($p<0.01$). There were no differences in the free fractions of R-NFX and S-NFX between low dose infusion and *i.v.* bolus experiments.

Renal elimination kinetics parameters from the three different dosing experiments were also compared for total NFX, and R- and S-NFX and the results are summarized in Table 3.5. In general, CL_{renal} and the percentage of dose excreted in urine for total NFX, R-NFX and S-NFX were significantly higher in the *i.v.* bolus experiments compared to the infusions ($p<0.05$), suggesting diminished renal elimination of NFX upon long-term FX administration.

3.3 Discussion

Pharmacokinetic studies in human subjects have shown that FX and NFX exhibit stereoselective disposition, with the S-enantiomers of both FX and NFX having higher plasma concentrations than the R-antipodes (Hiemke and Hartter, 2000). Stereoselective disposition of FX has also recently been demonstrated in sheep following a single *i.v.* bolus injection of FX (Kim, 2000). In the present study, the disposition of the FX and NFX enantiomers was further investigated under steady-state conditions in nonpregnant adult sheep.

3.3.1 Stereoselective Pharmacokinetics

3.3.1.1 Fluoxetine

In agreement with the previous findings, we observed a significant difference in the pharmacokinetic disposition of FX enantiomers in sheep following 8-day continuous racemic FX infusion. In both high dose and low dose infusion experiments, C_{ss} and AUC of S-FX were 3.19 to 3.78- and 2.92 to 3.44-fold higher, respectively, than those for R-FX, and the mean CL_{TB} was 2.81 to 3.23-fold lower than that for R-FX (Table 3.1). The S/R ratios for C_{ss}, AUC, or CL_{TB} were either significantly larger or smaller then the value of one. Interestingly, the degree of pharmacokinetic parameter differences between FX enantiomers observed in the present infusion studies were more dramatic in comparison with the results reported from the previous single *i.v.* bolus studies. Approximately 3-fold differences in AUC and CL_{TB} for R-FX and S-FX were observed in this study whereas only 1.4-fold differences in AUC and CL_{TB} were observed in the *i.v.* bolus experiments. These differences appear to be related to the dose as well as the duration of administration of FX. The effect of dose on the stereoselective pharmacokinetics of FX will be discussed in further detail in a later section of this chapter.

In agreement with the differences in AUC, there was also a significant difference in MRT for FX enantiomers with MRT of S-FX being approximately 1.4-fold longer than that of R-FX. The terminal elimination half-lives in the present infusion study did not differ significantly between the R- and S- FX enantiomers whereas the previous *i.v.* bolus experiment reported a longer $t_{1/2}$ for R-FX compared to S-FX (20.2 h versus 14.8 h). Apparently, the R versus S relationship for elimination half-life does not agree with that for clearance, since the clearance value for R-FX was higher than that of S-FX, and it is

generally assumed that a larger clearance value would be associated with a shorter elimination half-life. However, it is important to realize that elimination half-life is a dependent variable and relates directly to the volume of distribution and inversely to clearance. Volume of distribution and clearance are determined by different physiological factors, and the two parameters are independent of each other (Wilkinson, 1987). The apparent relationship among $t_{1/2}$, Vd, and CL_{TB} can be illustrated by the following equation:

$$t_{1/2} = 0.693 \times \frac{Vd}{CL_{TB}} \qquad (3.4)$$

Drugs with a higher volume of distribution (i.e. exceeding blood/plasma volume) are extensively distributed outside of blood/plasma into other tissues and, therefore, less is available in the systemic circulation for clearance by eliminating organs such as the kidney and liver. Apparently, the lack of a significant difference in $t_{1/2}$ between R-FX and S-FX is due to a larger Vd term for R-FX, which offsets the effect of a higher CL_{TB} value, resulting in a $t_{1/2}$ value that is similar to that for S-FX (Table 3.1).

The stereoselective difference in the distribution of FX enantiomers has not been reported in other species to our knowledge. Protein binding and tissue binding are thought to be the key components in determining the stereoselective difference between R-FX and S-FX in terms of their volume of distribution. In theory (physiological approach), the relationship between Vd, protein binding and tissue binding can be described by the following equation (Wilkinson, 1983):

$$Vd_{total} = V_b + V_T\left(\frac{f_b}{f_T}\right) \qquad (3.5)$$

where V_b is the blood volume, V_T is the volume of the other tissues of the body, and f_T and f_b are fractions of the drug present in the unbound form in tissue and blood, respectively. We

have shown that the volume of distribution differed significantly by about 2-fold between the FX enantiomers, which was paralleled by a 2-3 fold differences in free fraction. Intuitively, the difference in the volume of distribution could be regarded as a result of the difference in protein binding of the R- and S- enantiomers. Due to the unavailability of tissue binding data, the possibility of unequal tissue binding in relation to the stereoselective difference in volume of distribution of the FX enantiomers cannot be ruled out definitively, however, the extent of its contribution will likely be of minor significance.

FX is extensively metabolized in sheep and the urine data showed that less than 1% of the administered dose was excreted as unchanged drug in urine. Renal clearances of the FX enantiomers were approximately one thousandth of the estimates for CL_{TB} (Tables 3.1 and 3.2). Our data confirm the previous findings from the single *i.v.* bolus studies that renal excretion is a minor pathway for the elimination of FX in sheep. The fact that there is no stereoselective difference observed for this pathway indicates that renal elimination mechanism of FX involves only glomerular filtration, a process primarily based on the physiochemical properties of the substance to be eliminated. Nevertheless, the low CL_{renal} may also suggest the presence of tubular reabsorption of FX in the kidney but further study is required. In humans, the primary route of FX elimination is hepatic metabolism to inactive metabolites, which are subsequently excreted by the kidney (Hiemke and Hartter, 2000). A ^{14}C-FX disposition study showed that less than 5% of the total radioactivity was recovered as unchanged drug in urine (Altamura et al., 1994). Our results are in good agreement with the data reported for humans and suggest that FX metabolism in humans and sheep is similar. Nevertheless, it has been shown that sheep are metabolically more efficient than human in terms of drug metabolism. Previous studies on other compounds such as diphenhydramine

(Kumar et al., 1997), valproic acid (Gordon et al., 1995), metoclopramide (Riggs et al., 1990), and labetalol (Yeleswaram et al., 1993a) have shown that the rate of drug elimination in sheep is generally higher than that reported in humans. The $t_{1/2}$ of total FX in sheep, which ranged from 14 to 20 hours, was much shorter than the values of 1-3 days reported for human volunteers taking the drug orally (Hiemke and Hartter, 2000). Apparent oral clearance (CL/F) of total FX in humans averages 36 l/h and 11 l/h after single and multiple dose administration, respectively (Bergstrom et al., 1988). Assuming a bioavailability of 80% and body weight of 70 kg for a normal human subject, the weight normalized total body clearance is about 0.64 l/h/kg and 0.20 l/h/kg after single dose and multiple dose administration, respectively. This compares to clearance values ranging from 1.00 to 1.66 l/h/kg in sheep (Table 3.4), which suggests that the metabolic rate of FX in this species is about 3 to 5-fold higher than that in humans. From a qualitative perspective, the stereoselective pharmacokinetics of FX in sheep resembles that in humans as summarized in Table 3.7. Similar R versus S-FX enantiomers relationships between sheep and humans are observed for most major pharmacokinetic parameters such as $t_{1/2}$, AUC, C_{ss}, and CL_{TB}. Other kinetic parameters such as MRT, Vd, protein binding, and renal excretion have not been determined for FX enantiomers in humans, therefore these comparisons cannot be performed. Knowing the fact that there are high interspecies variations in the stereoselective disposition of drugs (Ruelius, 1987; Jamali et al., 1989), the striking similarity observed for the FX enantiomers between sheep and human is exceptional and deserves further study to allow a more thorough comparison between these two species.

TABLE 3.7 Comparison of R versus S relationships of FX and NFX enantiomers in sheep and humans.

Compound	Pharmacokinetic Parameter	Sheep (High & low dose infusion data)	Humans	Similarity?
FX	Elimination $t_{1/2}$ (h)	R = S	R = S in EM R > S in PM	Yes
	MRT (h)	R < S	N/A	-
	AUC (μg*h/l)	R < S	R < S	Yes
	C_{ss} (ng/ml)	R < S	R < S	Yes
	Vd_{ss} (l/kg)	R > S	N/A	-
	CL_{TB} (l/h/kg)	R > S	R > S	Yes
	Protein binding (f_u)	R > S	N/A	-
	% excreted in urine	R = S	N/A	-
	CL_{renal} (l/h/kg)	R = S	N/A	-
NFX	Elimination $t_{1/2}$ (h)	R > S	R < S in EM* R = S in PM*	No, but debatable
	MRT (h)	R > S	N/A	-
	AUC (μg*h/l)	R = S	R < S*	No, but debatable
	C_{ss} (ng/ml)	R = S	R < S in EM** R = S in PM**	Yes
	NFX/FX AUC ratio	R > S	R ≈ S in EM* R > S in PM*	Yes
	FX+NFX AUC (μg*h/l)	R < S	R < S	Yes
	Protein binding (f_u)	R > S	N/A	-
	% excreted in urine	R = S	N/A	-
	CL_{renal} (l/h/kg)	R = S	N/A	-

Note: EM, PM = CYP2D6 extensive and poor metabolizers, respectively.

* Data based on single oral dose study (Fjordside et al., 1999).

** Data based on multiple oral dose study (Eap et al., 2001).

3.3.1.2 Norfluoxetine

There was a relatively small difference between R- and S-NFX in terms of their plasma concentration versus time profiles (Figures 3.2 and 3.3). The S/R ratios for both C_{ss} and AUC were close to unity in both the high and low dose infusions. Nevertheless, statistically significant differences in other parameters were found between NFX enantiomers (Table 3.3). The longer $t_{1/2}$ and MRT of R-NFX indicates that the pharmacokinetics of R-NFX is different from those of S-NFX, and hence a stereoselective process is involved in NFX disposition. In addition, the metabolite to parent drug AUC ratio (NFX/FX AUC ratio) was also found to be significantly higher for the R-enantiomer. It has been shown that this ratio approximates the ratio of formation clearance (CL_f) to elimination clearance of the metabolite (CL_m) under steady-state conditions (Lane and Levy, 1981). The difference in this ratio for the NFX enantiomers therefore suggests that stereoselectivity was involved in the formation and elimination processes of NFX. When a drug is given by continuous *i.v.* infusion, the steady-state concentration of the metabolite can be described by the following equation (Houston, 1981):

$$C_{ss(m)} = \frac{f_m \times k_o}{CL_m} \qquad (3.6)$$

According to Equation 3.6, $C_{ss(m)}$, the steady-state plasma concentration for a metabolite is a function of both f_m, the fraction of drug converted to metabolite, and CL_m, the elimination clearance of metabolite; k_o is the rate of infusion for the parent drug. The determination of f_m and CL_m for the NFX enantiomers requires administration of preformed metabolites therefore these parameters are not quantifiable in this study. However, the R versus S relationship for these parameters can be estimated indirectly. Based on the understanding that 1) *N*-demethylation of FX to NFX is the major metabolic pathway for FX elimination and 2) there

is negligible renal elimination of the unchanged drug, the higher CL_{TB} value determined for R-FX suggests a higher f_m value for R-NFX relative to S-NFX. If this is true, CL_m of R-NFX must be higher than that of S-NFX in order to allow both enantiomers of NFX to achieve a similar concentration at steady-state, as was observed in our study. A panel of simulated NFX concentration-time profiles illustrating the relationships among $C_{ss(m)}$, f_m and CL_m are presented in Figure 3.10. Panels A to C suggest that enantiomers of metabolite with different f_m and CL_m values may result in either different or similar $C_{ss(m)}$ levels, depending on the magnitude of their differences. In addition, it is worth mentioning that the Vd of R-NFX is assumed to be higher than that of S-NFX based on the differences observed between R- and S-NFX in their free fractions (Table 3.3). Although Equation 3.6 suggests that Vd does not affect the ultimate level of $C_{ss(m)}$, it does play an important role in determining the elimination half-life which eventually affects the time required to attain $C_{ss(m)}$ as illustrated in Panel D. Among the four panels, Panels C and D mimic the concentration time-profile of NFX in our infusion studies where similar steady-state levels of NFX enantiomers were observed (Figures 3.2 and 3.3). Since no apparent difference was detected for the time to achieve C_{ss} for R-NFX and S-NFX, Panel D may not be the best scenario to describe the pharmacokinetic process of the NFX enantiomers. Therefore we speculate that Panel C most closely reflects our results and suggests that R-NFX has f_m and CL_m values higher than those of S-NFX.

The sum of FX and NFX AUCs provides a measurement of the total body exposure to the pharmacologically active species that contribute to the overall pharmacodynamics of FX. In the case of NFX, the S-enantiomer retains pharmacological activity equal to that of the parent drug. Our data show that the S-enantiomer had a higher summed FX and NFX AUC

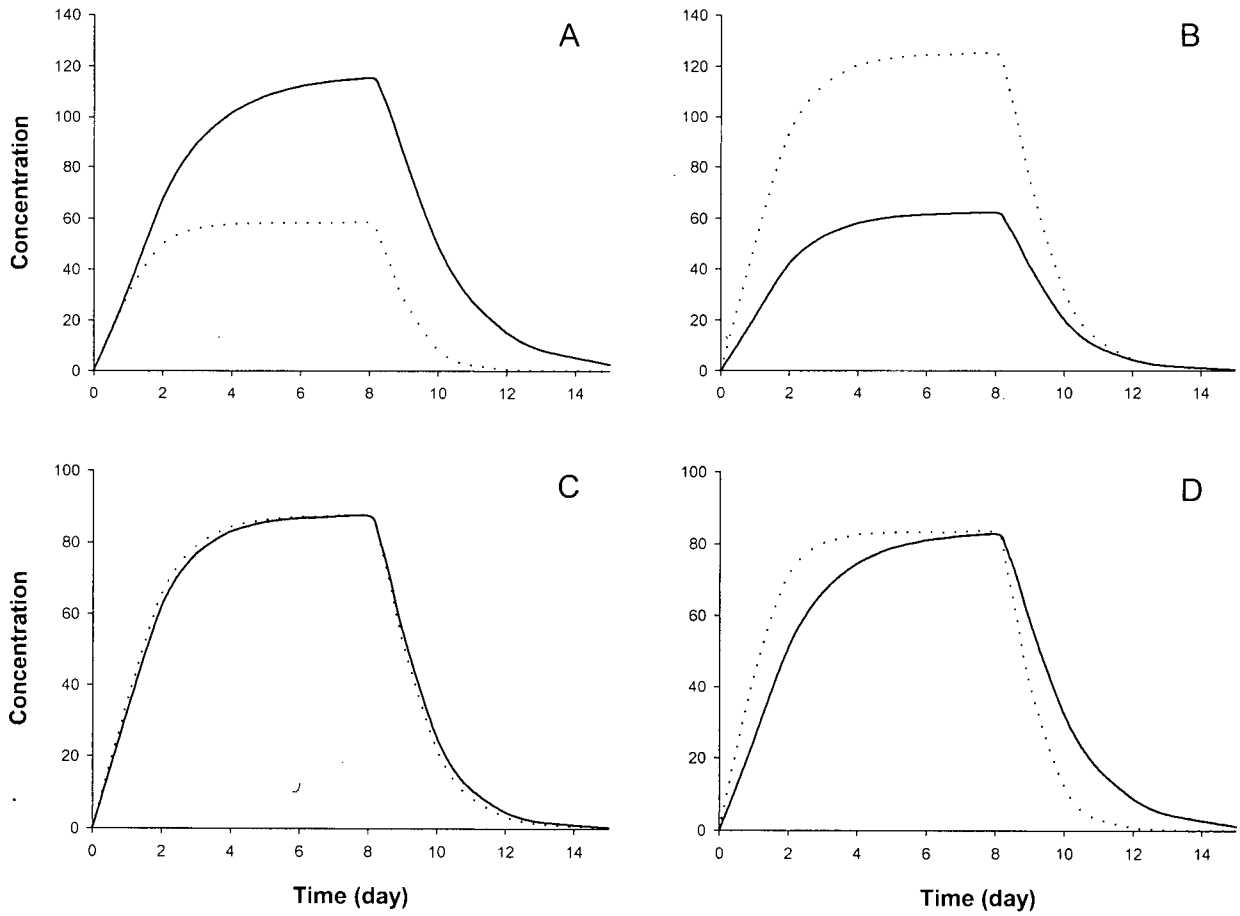


FIGURE 3.10 Simulations of NFX enantiomer concentration-time profiles following an 8-day continuous *i.v.* infusion of FX. The solid (—) and dotted lines (---) denote the concentration-time curves of R-NFX and S-NFX, respectively. The S/R ratio for Vd was fixed at 0.5 in all cases. In Panel A, the S/R ratios of f_m and CL_m were 0.5 and 1, respectively. In Panel B, the S/R ratios of f_m and CL_m were 1 and 0.5, respectively. In Panel C, the S/R ratios of f_m and CL_m were 0.5 and 0.5, respectively. In Panel D, the S/R ratios of f_m and CL_m were 1 and 1, respectively. Panel C closely resembles the concentration-time profiles observed for the NFX enantiomers in the present 8-day FX infusion experiments.

ratio and therefore contributes significantly to the overall pharmacological profile of FX relative to the R-antipode. It is important to realize that factors such as protein binding displacement and stereoselective inhibition of FX metabolism may affect the magnitude of this combined S/R AUC ratio, thereby altering the stereoselective pharmacodynamic and toxicological profile of FX.

In comparison to humans, the elimination half-life of NFX was much shorter in sheep (23.7-32.9 h versus 7-15 days), indicating a mark interspecies difference in terms of the rate of metabolism. Similar to the parent drug, the R versus S relationships of the NFX enantiomers between sheep and humans are generally similar but a few exceptions are noted (Table 3.7). The elimination half-life of R-NFX was longer than that of S-NFX in sheep whereas the opposite was observed in humans. Similar R- and S-NFX AUC values were found in sheep while the AUC of R-NFX was smaller than that of S-NFX in humans. The discrepancies in the R versus S relationships for $t_{1/2}$ and AUC may or may not be attributable to interspecies differences in the underlying pharmacokinetic processes for these enantiomers. The AUC of a metabolite (AUC_m) can be defined by the following equation (Houston, 1981):

$$AUC_m = \frac{f_m \times Dose}{CL_m} \qquad (3.7)$$

As discussed previously, we have rationally predicted that the f_m and CL_m values were higher for R-NFX compared to S-NFX. Making this assumption, the relationships between the S/R ratio of AUC_m versus the S/R ratios of f_m and CL_m based on Equation 3.7 are depicted in Figure 3.11. As shown in Panel A when the S/R ratio of f_m is equal to 0.5, the S/R ratio for AUC_m is approximately equal to 1, which is the case being observed in sheep. When the f_m S/R ratio is changed from 0.5 to 1, and from 1 up to infinity, the S/R AUC_m ratio will be

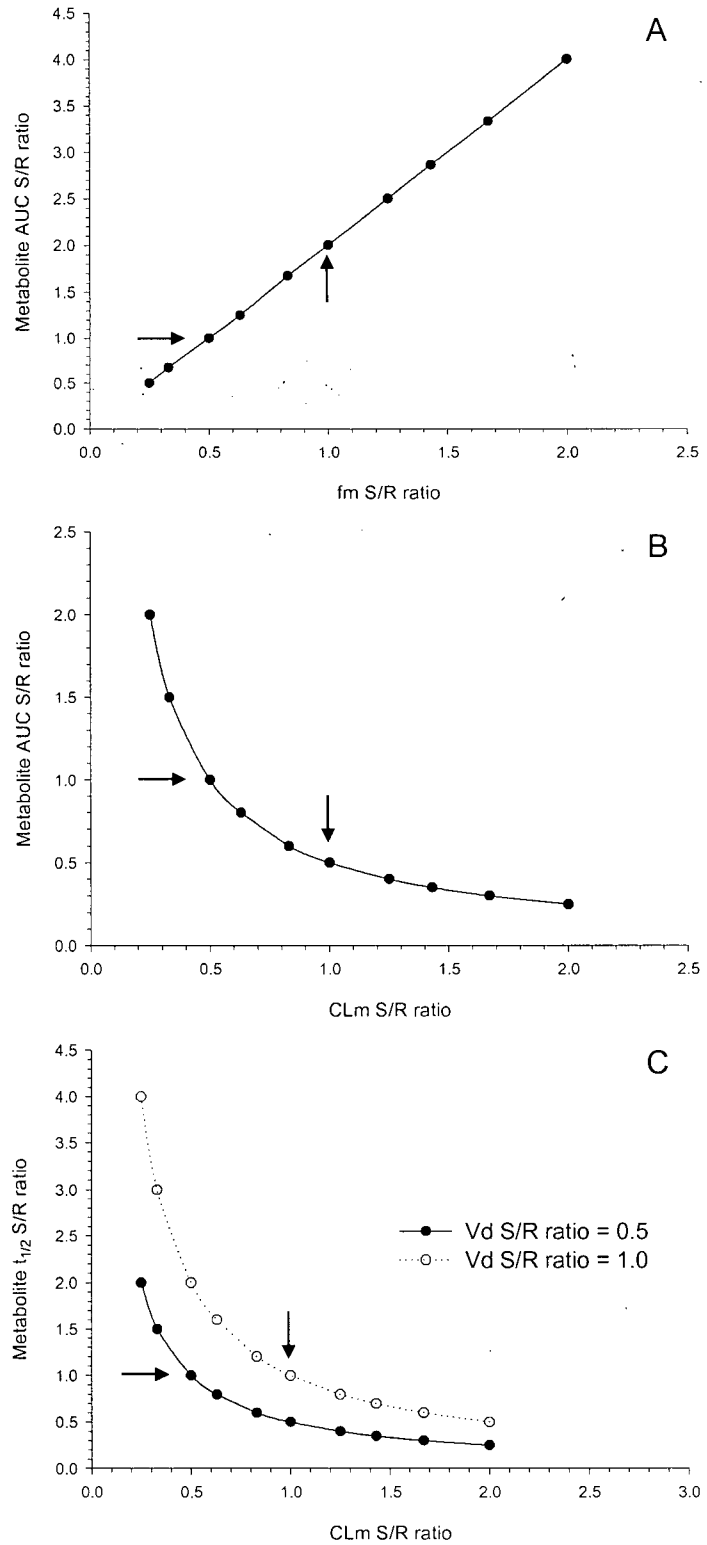


FIGURE 3.11 Interrelationships between the S/R ratios of f_m and AUC (Panel A); S/R ratios of CL_m and AUC (Panel B); S/R ratios of CL_m and $t_{1/2}$ (Panel C). The S/R ratio of CL_m is fixed at 0.5 in (A); S/R ratio of f_m is fixed at 0.5 in (B) and (C). The arrow sign (→) denotes the point when a change in the direction of stereoselectivity occurs.

larger than 1 (i.e. AUC_m of R-NFX is smaller than the AUC_m of S-NFX). From the literature data, the S/R ratio of NFX AUC in humans ranges from 1 to 2 only (Fjordside et al., 1999; Eap et al., 2001), suggesting that the f_m S/R ratio will be less than 1 in humans. Apparently, the direction for the R versus S relationship for f_m remains the same (R > S) between sheep and humans. It is the difference in the magnitude of the S/R ratio that leads to a difference in the AUC_m S/R ratio. The same logic applies to the R versus S relationship of CL_m, which is illustrated in Panel B of Figure 3.11. When CL_m of R-NFX is greater than S-NFX, depending on the degree of their difference, the S/R ratio of AUC_m will be equal to 1 (sheep) or larger than 1 (human). Again, the R versus S relationship of CL_m remains the same in sheep and human (where R > S in both species) and it is the deviation in the magnitude of their S/R ratios that leads to the difference in AUC (Table 3.7). The relationship between CL_m and metabolite $t_{1/2}$ is depicted in Panel C based on Equation 3.4, and the same assumptions and logic are applied to explain the difference in $t_{1/2}$ S/R ratio between sheep and humans. Although little is known about the stereoselective relationship of the Vd of the NFX enantiomers, previous protein binding data suggest that the R versus S relationship for Vd is similar between sheep and humans (Kim, 2000).

Another interesting observation that is found between sheep and human is that the R versus S relationship of NFX in sheep closely resembles that in human subjects with PM CYP2D6 status (see NFX $t_{1/2}$ and C_{ss} comparison in Table 3.7). This finding brings up a series of questions regarding the metabolic status of sheep in relation to the impairment of CYP2D6. Two possibilities can be generated from the above observations: 1) sheep do not have CYP2D6-like enzymatic activity and therefore, behave like PM of CYP2D6 in humans; or 2) sheep have an orthologous enzyme that processes CYP2D6-like activity which is

similar to that in humans, but this enzyme is inhibited upon long-term FX administration thereby converting the animal to a metabolizing status similar to PMs of CYP2D6. The key to the above possibilities can be found by examining the nonlinear pharmacokinetics of FX in sheep.

3.3.2 Nonlinearity

Data in the literature indicates that FX exhibits nonlinear pharmacokinetics in humans based on a comparison of single-dose versus steady-state pharmacokinetics in volunteers (Altamura et al., 1994). These studies showed that the elimination $t_{1/2}$ was prolonged from 1.9 to 5.7 days and CL_{TB} decreased from 35.5 to 10.8 l/h after multiple-dose administration. This change in pharmacokinetics leads to a larger AUC at steady-state than is observed after a single dose. It has been shown that such nonlinearity is largely attributed to FX inhibitory effects on CYP2D6, which is the major metabolizing enzyme responsible for its biotransformation (Ring et al., 2001) i.e. a phenomenon of self-inhibition. It is assumed that FX is not likely to exhibit nonlinear pharmacokinetics in a species that lacks the CYP2D6 enzyme or CYP2D6-like activity. Based on this assumption, it is tempting to examine whether FX exhibits nonlinear pharmacokinetics in sheep in order to assess whether this species has a CYP2D6-like enzyme that possesses activity similar to that found in humans. The first sign of nonlinearity in FX pharmacokinetics in sheep, relates to the deviations observed between the FX C_{ss} values (High dose: 151 ng/ml, Low dose: 62 ng/ml) and the targeted C_{ss} (High dose: 100 ng/ml, Low dose: 50 ng/ml) that we originally planned to achieve in the 8-day infusion experiments. This target C_{ss} was determined based on the total body clearance value obtained from our previous *i.v.* bolus studies (Kim, 2000):

$$C_{ss(target)} = \frac{k_o}{CL_{TB}\ i.v.} \qquad (3.8)$$

where k_o is the constant *i.v.* infusion rate and CL_{TB} *i.v.* is the total body clearance value obtained with *i.v.* bolus dosing.

From our current studies, it is apparent that there was a change in the pharmacokinetics of FX upon long-term administration compared to acute *i.v.* bolus dosing. ANOVA analyses comparing the pharmacokinetic parameters of FX following high or low dose 8-day continuous infusions or single bolus administration of FX were performed to further examine this phenomenon. As illustrated in Figure 3.8, a progressive decrease in CL_{TB} of total FX was noted in the high dose infusion studies compared to the low dose infusion and single *i.v.* bolus experiments. This suggests inhibition of FX metabolism upon long-term dosing, which is believed to be the cause of the discrepancy between the observed C_{ss} and target C_{ss} in our current infusion studies. Interestingly, upon closer examination of the clearance of individual FX enantiomers, a significant reduction in clearance was found for S-FX but not R-FX, indicating a stereoselective mechanism behind this nonlinear kinetic process. Moreover, the significantly prolonged MRT observed in the infusion studies, suggests that persistence of the drug upon long-term administration parallels its decrease in clearance. Renal clearance of FX also appeared to be decreased in the infusion studies compared to the single bolus data, suggesting that renal elimination of FX may involve a carrier mediated process (such as tubular reabsorption) that is saturable at higher drug concentrations. However, the reduction of renal clearance will not have any significant impact on the pharmacokinetics of FX due to its low contribution to the overall total body clearance (i.e. CL_{TB}). The distribution characteristics of FX also appeared to be altered during the infusion studies. Volume of distribution increased by 2-3 fold in the infusion

experiments compared to the single bolus studies. Concentration-dependent protein binding, characterized by an increase unbound free fraction due to the saturation of binding sites, is the most common phenomenon that causes an increase in Vd at high drug concentration. However, we observed an unusual decrease in unbound fraction in the high dose infusion experiment compared to the low dose infusion and single bolus experiments. Protein binding of FX appears to follow an atypical dose dependent phenomenon with more drug binding to plasma protein at higher concentrations. This is an unusual finding because it is against conventional saturable protein binding theory, which states that binding decreases as concentration of the substrate increases. It is difficult to judge whether this is an experimental artifact because a downward tendency for Vd, which is consistent with the increase in protein binding, was found in the high dose infusion experiments compared to the low dose infusions. Previous *in vitro* protein binding experiments using spiked sheep plasma at drug concentrations ranging from 100 to 10,000 ng/ml did not reveal this concentration-dependent change in binding (Kim, 2000). Concurrently, a substantial increase in Vd was observed upon long-term FX dosing, which may be attributable to an increase in lysosomal trapping of FX in lungs. Drug accumulation in tissue involving lysosomal trapping has been reported for numerous basic drugs including promazine and amitriptyline (Daniel and Wojcikowski, 1997). This trapping mechanism serves as a depot for extravascular distribution of FX in a time dependent manner where drug accumulation occurred progressively over time. Given the finite availability of lyzosomal vesicles, saturation of this uptake mechanism may happen at high drug concentration. Upon saturation, unbound drugs that are excluded from the lyzosomal vesicles diffuse back into the vascular system and become available for binding with plasma protein, which might explain why there was a

decrease in the free fraction observed at high FX concentration *in vivo*. And because of the involvement of peripheral tissues, this type of redistribution event is not expected to happen in an *in vitro* situation.

The comparison of NFX data between different dosing experiments in sheep yielded some interesting results. Elimination half-life, due to its hybrid characteristics with Vd and CL_{TB}, did not differ significantly between different dosing experiments. However, significant accumulation of NFX was observed with a disproportionate increase in AUC and a significant prolongation of MRT in the infusion studies relative to the *i.v.* bolus experiments. The accumulation of NFX led to an increase in overall total metabolite to parent (NFX/FX) AUC ratio by about 25-30% upon long-term FX dosing. Interestingly, a significant increase in this ratio was observed for the R-enantiomers but not the S-antipodes. This difference is due to a similar percentage increase in dose normalized AUC of R- and S-NFX but a non-parallel rise in dose normalized AUC for FX with a larger increase for the S-enantiomer compared to the R-enantiomer (Figure 3.9). The above observations suggest nonlinearity in NFX disposition, which has never been described or characterized before in the literature. It is proposed that the major metabolic pathways of NFX involve deamination and *O*-dealkylation to FX carboxylate and *p*-TFMP, respectively, yet the metabolic enzymes responsible for the biotransformation of NFX remain poorly understood (Altamura et al., 1994). It is speculated that the nonlinear pharmacokinetic characteristics of NFX may be attributed to the inhibition of CYP2D6 by both FX and NFX. Fjordside et al., 1999 have reported smaller AUC values of NFX in PMs of sparteine compared to their EM counterparts after a single oral dose of FX in human volunteers. These results suggest a link between CYP2D6 and NFX metabolism. Nevertheless, more experiments such as *in vitro* metabolism

studies of NFX and *in vivo* inhibition studies (i.e. co-administration of NFX with known cytochrome P-450 inhibitors) will be necessary to systemically delineate the exact mechanism of NFX metabolism.

While inhibition of metabolism is considered to be the most likely reason to explain nonlinearity, nonlinear protein binding may also play a role in the nonlinear pharmacokinetics of NFX. Similar to FX, an unusual decrease in unbound fraction was observed at high drug concentrations. Drugs that are known to exhibit nonlinear pharmacokinetics due to nonlinear protein binding include disopyramide and valproic acid (Upton and Williams, 1986; Gomez Bellver et al., 1993; Wong et al., 2001). Obviously, the protein binding of NFX should be investigated in greater detail in a mechanistic manner in order to help us better understand its role in the overall pharmacokinetics and pharmacodynamics of this active and long half-life FX metabolite.

3.3.3 Mechanism of Stereoselective Pharmacokinetics of FX

The mechanism responsible for the stereoselective disposition of FX in humans has been studied (Stevens and Wrighton, 1993; von Moltke et al., 1997; Margolis et al., 2000; Ring et al., 2001). Yet, most of these studies have focused on the differential biotransformation of FX enantiomers to their respective N-desmethyl metabolites. Previous *in vitro* studies performed with human liver microsomes and cDNA-expressed CYPs suggest that CYP2D6 and CYP2C9 play a pivotal role in the formation of NFX (Margolis et al., 2000; Ring et al., 2001). It has been shown that both CYP2D6 and CYP2C9 contribute to the formation of R-NFX, whereas only CYP2D6 is responsible for the conversion to S-NFX. Upon chronic dosing, the CYP2D6-mediated metabolism of the FX enantiomers is likely to

be inhibited by their counterparts (i.e. one enantiomer inhibiting metabolism of the other enantiomer) as well as by NFX. As a consequence, the formation of R-NFX is mediated by CYP2C9 while the formation of S-NFX involves multiple high K_m enzymes (e.g. CYP3A4 and CYP2C19), resulting in a lower clearance value of S-FX (Ring et al., 2001). *O*-dealkylation to *p*-TFMP has been identified as an alternate route of elimination for FX (Altamura et al., 1994; Urichuk et al., 1997). Studies performed *in vitro* using human liver microsomes have shown that the formation of *p*-TFMP mainly involves CYP2C19 and CYP3A4 (Liu et al., 2001), but the stereoselective aspect of these metabolic pathways is not well understood.

In sheep, the observed stereoselective disposition of FX after a single *i.v.* bolus injection of racemic FX seems to be related to differential protein binding of the FX enantiomers (Kim, 2000). R-FX was found to be less protein bound compared to S-FX, therefore having a higher free fraction and being more readily available for clearance by the liver. Kim (2000) also suggested that the difference in clearance of the FX enantiomers was largely due to differences in their degree of plasma protein binding since free intrinsic clearances (CL_{int}) between the FX enantiomers did not differ significantly. In fact, the reported value for CL_{int} of R-FX is actually lower than that of S-FX (40.2 vs. 62.6 l/h/kg), which is contrary to what was described previously in humans (Stevens and Wrighton, 1993), but the author did not attempt to address this discrepancy at all. Upon closer examination of his report, the method that Kim (2000) employed to estimate CL_{int} seems to be problematic. CL_{int} was determined based on the assumption that FX is a low clearance drug with a low extraction ratio ($E < 0.3$). According to the well-stirred model (Wilkinson, 1987) of hepatic drug elimination, hepatic clearance (CL_H) is defined as:

$$CL_H = Q_H \times E \quad (3.9)$$

where Q_H is the hepatic blood flow, and E is the extraction ratio which can be further defined as (Wilkinson, 1987):

$$E = \frac{f_u CL_{int}}{(Q_H + f_u CL_{int})} \quad (3.10)$$

For a drug that has a low extraction ratio and is predominately eliminated by the hepatic metabolism with negligible renal excretion, CL_{TB} approximates the product of the unbound fraction in plasma (f_u) and CL_{int} as shown in Equation 3.11:

$$CL_{TB} \cong CL_H \cong f_u CL_{int} \quad (3.11)$$

The assumption of FX as a low extraction ratio drug without knowing its actual value is subject to error because Equation 3.11 will underestimate the true CL_{int} value for drugs that have either a medium (E = 0.3-0.7) or high (E > 0.7) extraction ratio. Such a deviation from the true value will amount to 30% even when E = 0.3 (Figure 3.12). Because of this, Equation 3.11 should not be used to calculate CL_{int} when extraction ratio is greater than 0.3. Although the extraction ratio of FX in human has never been published, the literature reported values for apparent clearance (CL_{TB}/F = 36 l/h) and systemic bioavailability (F = 0.7) obtained following a single oral dose administration of FX in human (van Harten, 1993) allow us to estimate extraction ratio using the following equation (assume $CL_{TB} \approx CL_H$, Q_H = 1.5 l/min; Wilkinson and Shand, 1975):

$$E = \frac{CL_H}{Q_H} \cong \frac{CL_{TB}}{Q_H} \quad (3.12)$$

We found that the extraction ratio of total FX was approximately 0.28, therefore fulfilling Kim's assumption that FX is a low extraction ratio drug. However, it is questionable if this assumption truly applies to sheep. First, our data have shown that sheep are more efficient at

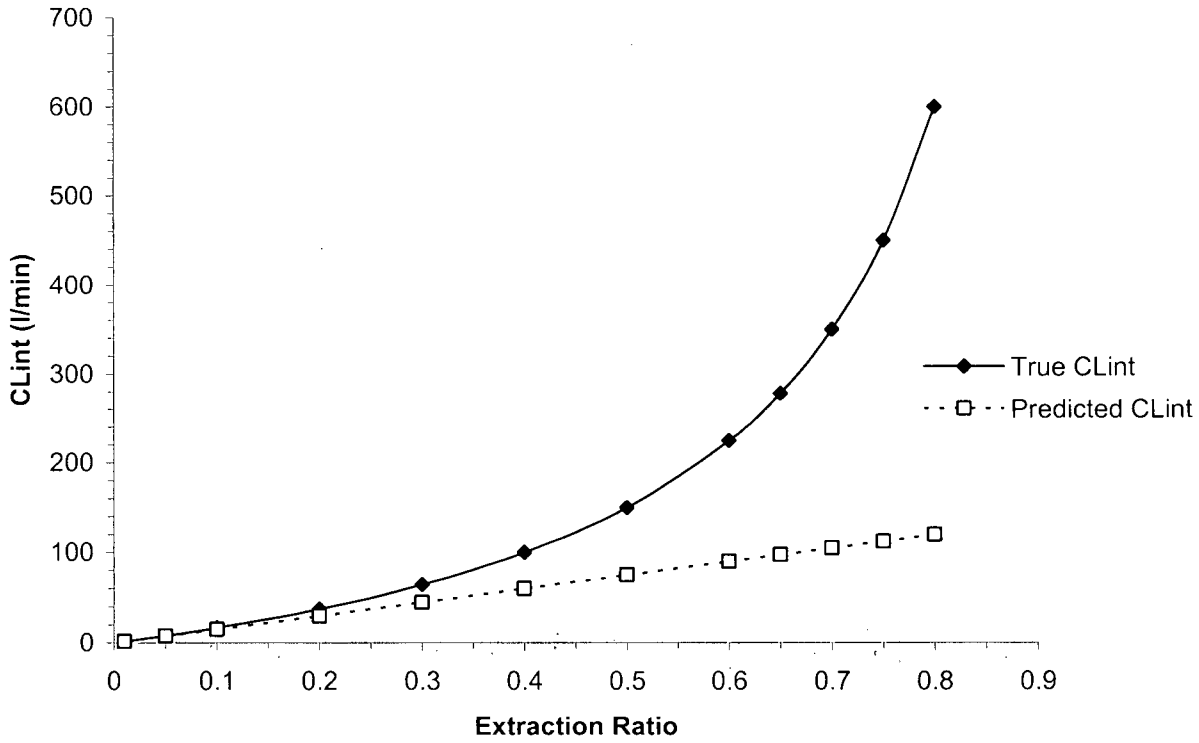


FIGURE 3.12 Plot of extraction ratio versus predicted CL_{int} value calculated from equation 3.11 and the true CL_{int} value calculated using equation 3.13. Q_H and f_u were assumed to be 1.5 l/min and 0.01, respectively, in both cases. A significant deviation (>30%) between the predicted and true value occurred when the extraction ratio was greater than 0.3.

eliminating drugs than humans (see earlier discussion in Section 3.3.1.1); hence it is likely that the extraction ratio of FX in sheep will be different from that in humans. Second, hepatic blood flow in sheep is reported to be approximately 60% higher than that in the human (2.4 l/min vs. 1.5 l/min) (Apatu and Barnes, 1991; Katz and Bergman, 1969). As illustrated above in Equations 3.9 and 3.10, hepatic blood flow is one of the important components in the estimation of a drug's extraction ratio, a difference in hepatic blood flow between sheep and human would be expected to influence the estimation of this ratio in sheep. Third, *in vitro* metabolism data indicated that CL_{int} of R-FX (ranged 0.41 – 5.54 μl/min/mg) was higher than that of S-FX (ranged 0.25 – 0.84 μl/min/mg) (Stevens and Wrighton, 1993). Although we have just calculated that racemic FX has a low extraction ratio of 0.28 based on the *in vivo* literature data, this value may not be applicable to the individual enantiomers due to large stereoselective differences in their pharmacokinetics. Indiscriminant use of racemic data to interpret stereoselective data will lead to a false estimation of the true CL_{int} if one of the enantiomers in fact has an extraction ratio larger than 0.3. It is therefore, imperative to appropriately evaluate FX's pharmacokinetic data in sheep before a rational conclusion can be reached regarding its mechanism(s) of stereoselectivity. Since hepatic blood flow was not measured in these current studies, the literature value of hepatic blood flow of 2.9 l/h/kg in sheep (Apatu and Barnes, 1991) was used to calculate the extraction ratio using Equation 3.12. CL_{int} was determined from Equation 3.13 by rearranging Equation 3.10:

$$CL_{int} = \frac{EQ_H}{f_u(1-E)} \qquad (3.13)$$

The results are summarized in Table 3.8. Similarly, extraction ratio and CL_{int} values for the FX enantiomers in humans calculated based on literature values from various reports

TABLE 3.8 Comparative extraction ratio (E) and intrinsic clearance (CL_{int}) values for the FX enantiomers in sheep and humans.

Pharmacokinetic Parameter	Sheep*				Human**			
	8-day Infusion†		Single *i.v.* Bolus‡		Poor Metabolizer§		Extensive Metabolizer§	
	R-FX	S-FX	R-FX	S-FX	R-FX	S-FX	R-FX	S-FX
E¶	0.69	0.25	0.68	0.50	0.14	0.03	0.36	0.29
CL_{int} (l/h/kg)#	449.8	102.1	266.2	169.0	4.30	0.67	14.3	10.6

* A Literature value of Q_H=2.9 l/h/kg (hepatic blood flow) was used in all cases (Apatu and Barnes, 1991).

** Literature values of Q_H=1.5 l/min (hepatic blood flow), F=0.7 (systemic availability), f_u=0.05 (unbound drug fraction), BW=70 kg (body weight) were used in all cases (Wilkinson, 1987; van Harten, 1993).

† Data based on the high dose and low dose racemic FX 8-day infusion experiments.

‡ Data based on the single *i.v.* bolus dosing experiments (Kim, 2000).

§ Data based on single oral dose study in poor and extensive metabolizers of sparteine (Fjordside et al., 1999).

¶ E (extraction ratio) was calculated using Equation 3.12.

CL_{int} (intrinsic clearance) was calculated using Equation 3.13.

(van Harten, 1993; Fjordside et al., 1999) are also included for purposes of comparison. It was observed that extraction ratios for both FX enantiomers are > 0.3 in the single *i.v.* bolus experiments in sheep. This indicates that FX is a medium extraction ratio drug in sheep as opposed to the previous assumption that the drug has the same extraction ratio in both sheep and humans. In addition, CL_{int} of R-FX was found to be more than 50% higher than that of S-FX, suggesting that, in addition to differential protein binding, stereoselective metabolism is involved in the stereoselective disposition of FX. In contrast to R-FX the extraction ratio and CL_{int} values of S-FX were observed to be lower in the infusion study compared to the single *i.v.* bolus study, resulting in a larger difference between their metabolic rate and more prominent stereoselective differences in their disposition at steady-state. This observation is important in supporting the hypothesis that inhibition of the metabolism of S-FX occurred upon long-term FX administration in sheep, suggesting the presence of a CYP2D6-like enzyme. In humans, a stereoselective difference in the extraction ratio between FX enantiomers is observed and such a difference is more apparent for PMs compared to EMs. Due to a lack of CYP2D6 activity, PMs will have a lower extraction ratio compared to the EM counterparts (Table 3.8). Interestingly, the extraction ratio of R-FX (0.36) in EM is within the middle part of the spectrum (E = 0.3 – 0.7) while S-FX (0.29) falls into the lower range of the spectrum (E < 0.3) and the racemic drug also has an extraction ratio value of less than 0.3. It can be concluded then, that one has to be cautious in the interpretation of the disposition of a racemic drug that exhibits stereoselective pharmacokinetics. Further, the pharmacokinetics of enantiomers should be evaluated individually rather than making generalizations based on racemic data.

3.4 Summary

In summary, we have shown that the enantiomers of FX and NFX exhibit significant pharmacokinetic differences in sheep under steady-state conditions, confirming the observations made in previous *i.v.* bolus experiments (Kim, 2000). In comparison to humans, quantitative differences exist between the two species, however qualitatively the overall stereoselective disposition of FX and NFX is similar. Our data revealed a dose-dependent decrease in clearance upon long term dosing in sheep, suggesting the presence of an orthologous enzyme(s) comparable to human's CYP2D6. From our combined studies the stereoselective disposition of FX involves the following mechanisms: 1) differential protein binding, 2) stereoselective metabolism and 3) stereoselective enzyme inhibition. As mentioned earlier, selection of an appropriate animal model for stereoselective pharmacokinetic drug studies is a challenge given that substantial interspecies variability is usually present in stereoselective drug disposition. Given the similarity between our sheep FX data and that available for humans, sheep may be an animal model of choice for other stereoselective drug disposition studies. There is no doubt, however, that this requires further testing, particularly with respect to metabolic enzyme identification, enzyme kinetics and protein binding mechanisms. Nevertheless, it opens up new opportunities for allometric pharmacokinetic studies and drug metabolism research by including sheep as a suitable model in addition to those that are commonly used for drug research at present (i.e. mice, rats, dogs, monkeys, and rabbits). Finally, our results provide an important framework for further investigations into the effect of pregnancy on the stereoselective pharmacokinetics of FX.

CHAPTER 4

AN INVESTIGATION OF THE EFFECTS OF PREGNANCY ON THE STEREOSELECTIVE DISPOSITION OF FX AND NFX IN PREGNANT SHEEP

Preface

As discussed in the general introduction of this thesis, changes in maternal physiology occur normally during pregnancy and have the potential to alter the absorption, distribution, and elimination of drugs used therapeutically in pregnant women. These physiological changes include: plasma volume expansion and increases in extracellular fluid space and total body water; decreased plasma albumin concentration; a compensated respiratory alkalosis; increased cardiac output with regional blood flow changes; increased renal blood flow associated with increased glomerular filtration; changes in hepatic drug metabolizing enzymes; and changes in gastrointestinal function. These changes begin in early gestation but are most pronounced in the third trimester. Further maternal physiological changes occur intrapartum with some normalizing themselves within 24 hours of delivery, while others are sustained and return to normal some 12 weeks postpartum.

The purpose of this chapter is to examine the effects of pregnancy on the stereoselective disposition of FX and NFX during the last trimester of pregnancy in chronically instrumented sheep. Pharmacokinetic comparisons are made between this data and that obtained for nonpregnant sheep (Chapter 3). Mechanisms of some pregnancy-induced changes on stereoselective drug disposition are also explored based on the current literature data available for other racemic drugs.

4.1 Methods

4.1.1 Animal and Surgical Preparation

Five pregnant ewes (Dorset and Suffolk breeds) at gestational ages ranging from 123 to 125 days and term ~145 days were used in these experiments. The studies were approved by the University of British Columbia Animal Care Committee and the procedures performed conformed to the guidelines of the Canadian Council on Animal Care. To obtain time-dated ewes, the estrus cycle was synchronized by inhibition of spontaneous ovulation. This was accomplished by the administration of a medroxyprogesterone acetate pessary (Vermix Sheep Sponge) intra-vaginally for two weeks. Ovulation was then, induced by removal of the pessary and intramuscular injection of 500 I.U. of pregnant mare's serum gonadotropin. The ewes were subsequently placed with the ram for 1-2 days to result in time-dated pregnancies. Pregnancy was confirmed by ultrasound at ~70-120 days gestation. The general surgical procedure was similar to that described previously in Chapter 3, Section 3.11 for nonpregnant ewes. Blood collection and drug administration were performed via silicone rubber catheters implanted in maternal femoral artery and vein, respectively. A minimum of 3 days was allowed for the ewes to recover before experimentation. Due to the increased risk of infection in the pregnant ewe and/or fetus secondary to long-term Foley catheter placement, urine collection was not performed in these experiments.

4.1.2 Experimental Protocol

The dosing and sampling protocols used were identical to for the nonpregnant sheep experiments (Figure 3.1) except that the FX infusion was conducted only at the high dose level. Each ewe received a 70 mg *i.v.* bolus loading dose over one minute followed by a

continuous *i.v.* infusion of racemic FX via the maternal femoral vein at a rate of 6.92 mg/h for 8 days to achieve steady-state concentrations. As in the nonpregnant ewes, serial blood samples (5 ml) were collected from the maternal femoral arterial catheter at 0, 5, 15, 30 min, 1, 2, 4, 6, 12, 24, 36, 48, 60, 72, 84, 96, 108, 120, 132, 144, 156, 168, 180 h, 192 h during the infusion, and at 5, 10, 20, 30, 45 min, 1, 2, 3, 4, 6, 9, 12, 24, 36, 48, 60, and 72 h post-infusion. The zero time blood samples were collected within 10 minutes prior to loading dose administration.

All blood samples collected for drug and metabolite analysis were placed into K_3EDTA Vacutainer® tubes, gently mixed, and centrifuged at 2000g for 10 min to separate the plasma. The plasma supernatant was transferred into a clean borosilicate test tube with a polytetrafluoroethylene (PTFE)-lined cap. All samples were stored at -20°C until the time of analysis by GC/MS.

4.1.3 Determination of FX and NFX Plasma Protein Binding

Plasma protein binding of FX and NFX was measured *ex vivo* using the equilibrium dialysis procedure as described in Chapter 3, Section 3.1.3. Plasma unbound fraction (f_u) was calculated as described in Chapter 3, Section 3.1.3, Equation 3.1.

4.1.4 Pharmacokinetic Analysis

All pharmacokinetic parameters were calculated by standard pharmacokinetic procedures as described in Chapter 3, Section 3.1.4. Since no urine was collected in these experiments, renal clearance (CL_{renal}) and the corresponding pharmacokinetic parameters were not determined.

4.1.5 Statistical Analysis

Paired t-tests were used for statistical analysis of the pharmacokinetic parameters when comparing R- versus S-enantiomers in the pregnant ewes. Comparisons of the mean values of the parameters between the pregnant and nonpregnant infusion experiments were performed using two-sample t-tests. The significance level used was $p<0.05$. The achievement of steady-state for plasma FX and NFX concentrations was evaluated by testing whether the slope of plasma concentration versus time curve was significantly different from the value of zero. Unless otherwise specified, all data are reported as the mean ± standard deviation (S.D.).

4.2 Results

The mean body weight of pregnant and nonpregnant sheep in the high dose FX infusion experiments were 71.7 ± 9.9 kg and 57.9 ± 10.3 kg, respectively. The mean body weight of pregnant ewes was significantly higher than that of nonpregnant sheep ($p<0.05$).

4.2.1 Fluoxetine

Representative plasma concentration versus time profiles of the R and S enantiomers of FX enantiomers obtained in pregnant sheep are illustrated in Figure 4.1. A comparison of the average plasma concentration versus time profiles of total FX and NFX is shown in Figure 4.2. As in the nonpregnant studies a two-compartment model best described the post-infusion concentration time data based on AIC and SC values using a weighting factor of 1/predicted y. Similar to the findings in the nonpregnant animals, FX exhibited significant stereoselective pharmacokinetics in pregnant sheep. FX reached steady-state plasma

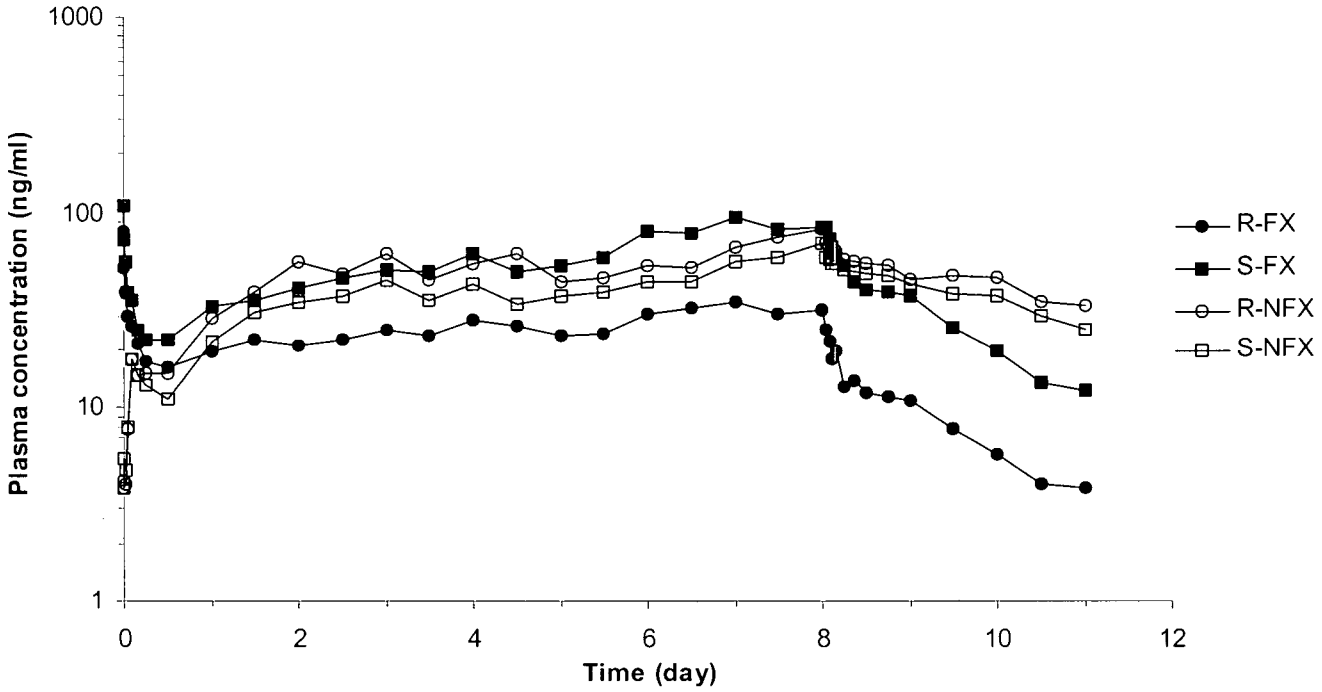


FIGURE 4.1 Representative plasma concentration versus time profiles of FX and NFX enantiomers in a pregnant ewe (E4237) obtained for an 8-day maternal *i.v.* infusion of racemic FX.

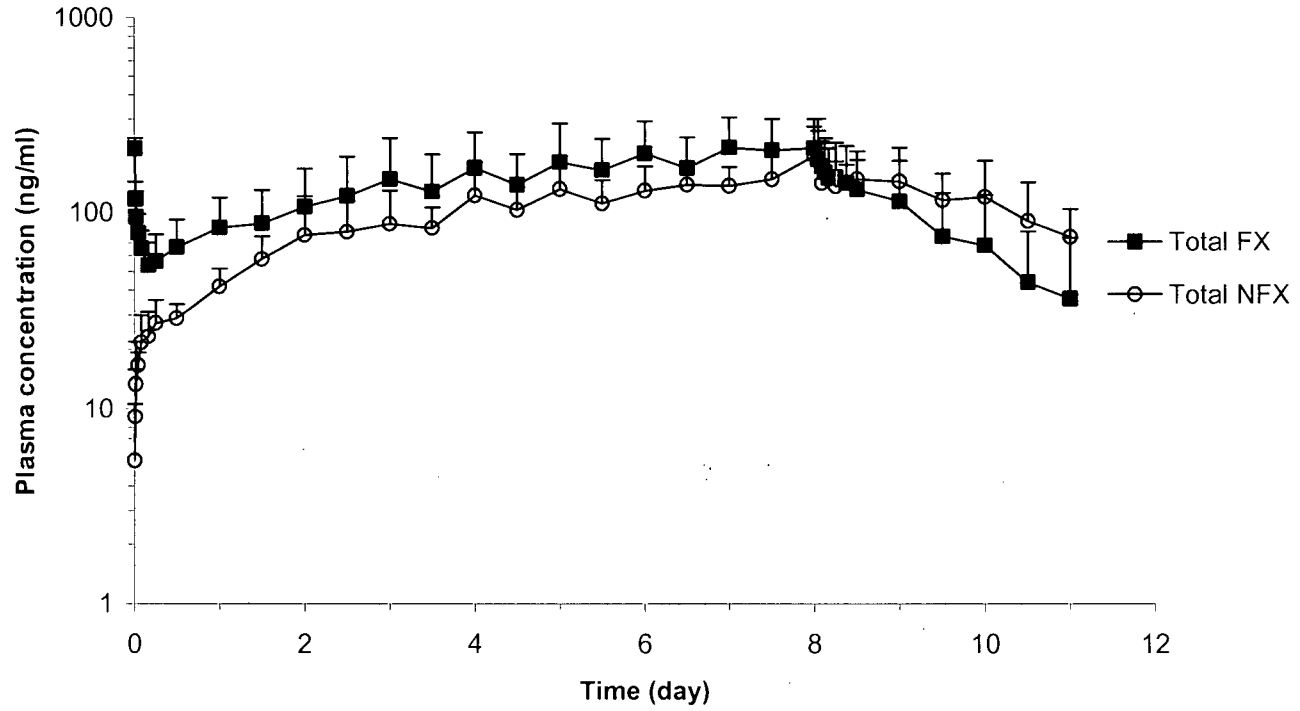


FIGURE 4.2 Comparison of average plasma concentration versus time profiles of total FX and NFX in pregnant sheep after 8-day maternal *i.v.* infusion of racemic FX (n=5).

concentrations during day-6 to day-8 of the infusion period compared to day-5 to -8 in nonpregnant ewes. A comparison of the average plasma total FX and FX enantiomers concentration-time profiles in pregnant and nonpregnant sheep is presented in Figures 4.3 and 4.4, respectively. Steady-state total FX concentrations (C_{ss}) averaged 208.2 ± 85.0 ng/ml and 151.0 ± 39.2 ng/mL in the pregnant and nonpregnant ewes, respectively, and were not significantly different. Similarly there were no significant differences between the two groups in the steady-state concentrations of the R and S enantiomers (Figure 4.4). The pharmacokinetic parameters calculated for the FX enantiomers (mean ± S.D.) from the pregnant ewe experiments are summarized in Table 4.1. Also included in this table are the S/R ratios for the various pharmacokinetic parameters as well as the mean data used for statistical comparisons between pregnant and nonpregnant sheep.

The relationship between the S and R -FX enantiomers in the pregnant ewes was very similar to that observed in nonpregnant sheep (Figure 4.4). No difference was found in the terminal elimination half-life ($t_{1/2\beta}$) while significant differences were observed in all other pharmacokinetic parameters (i.e. MRT, C_{ss}, AUC, Vd_{ss}, CL_{TB}, and f_u) between R-FX and S-FX (Table 4.1). A comparison of the individual enantiomers between the two study groups (i.e. $R\text{-}FX_{(pregnant)}$ versus $R\text{-}FX_{(nonpregnant)}$; $S\text{-}FX_{(pregnant)}$ versus $S\text{-}FX_{(nonpregnant)}$) revealed tendencies for increases in $t_{1/2\beta}$, mean residence time (MRT), C_{ss}, and AUC in pregnant sheep compared to the nonpregnant animals, but the difference was only significant for the $t_{1/2\beta}$ of S-FX (35.9 ± 18.3 h versus 14.8 ± 3.3 h, $p<0.05$). Likewise, the MRTs of R-FX and S-FX also tended to be longer in pregnant than nonpregnant sheep but the differences were not statistically significant (R-FX: 40.2 ± 21.0 h versus 24.6 ± 7.0 h, $p = 0.12$; S-FX: 57.8 ± 24.3 h versus34.9 ± 8.6 h, $p = 0.06$). As a result of the $t_{1/2\beta}$ prolongation, the time to steady-state

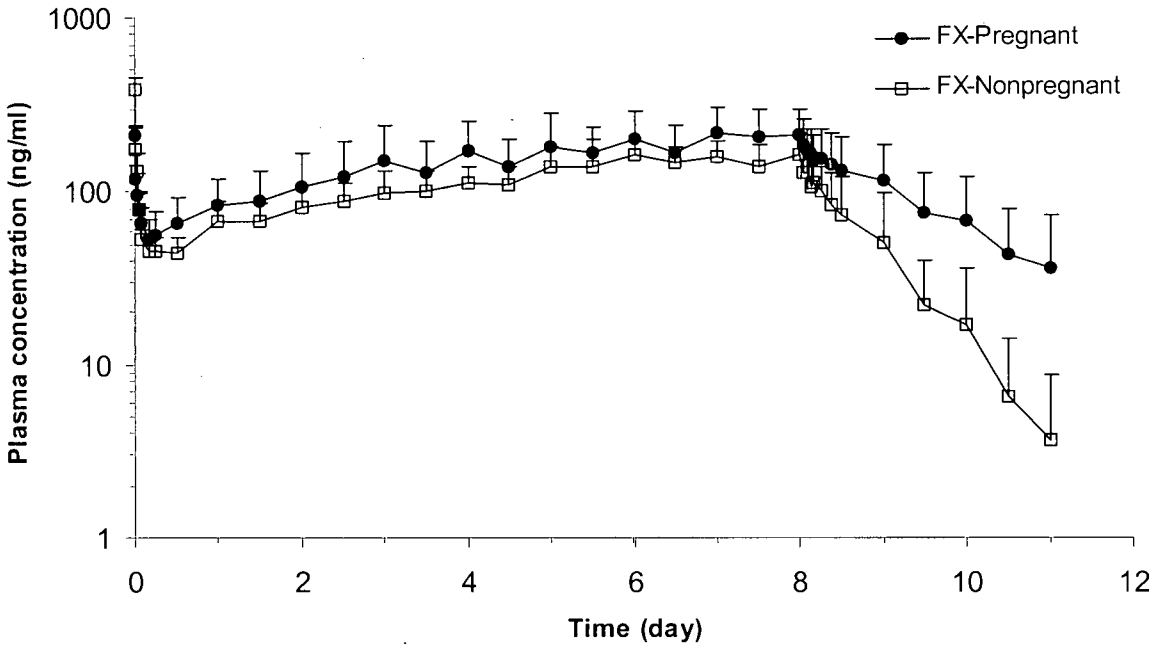


FIGURE 4.3 Comparison of average plasma concentration versus time profiles of total FX of pregnant and nonpregnant sheep after 8-day *i.v.* infusion of racemic FX.

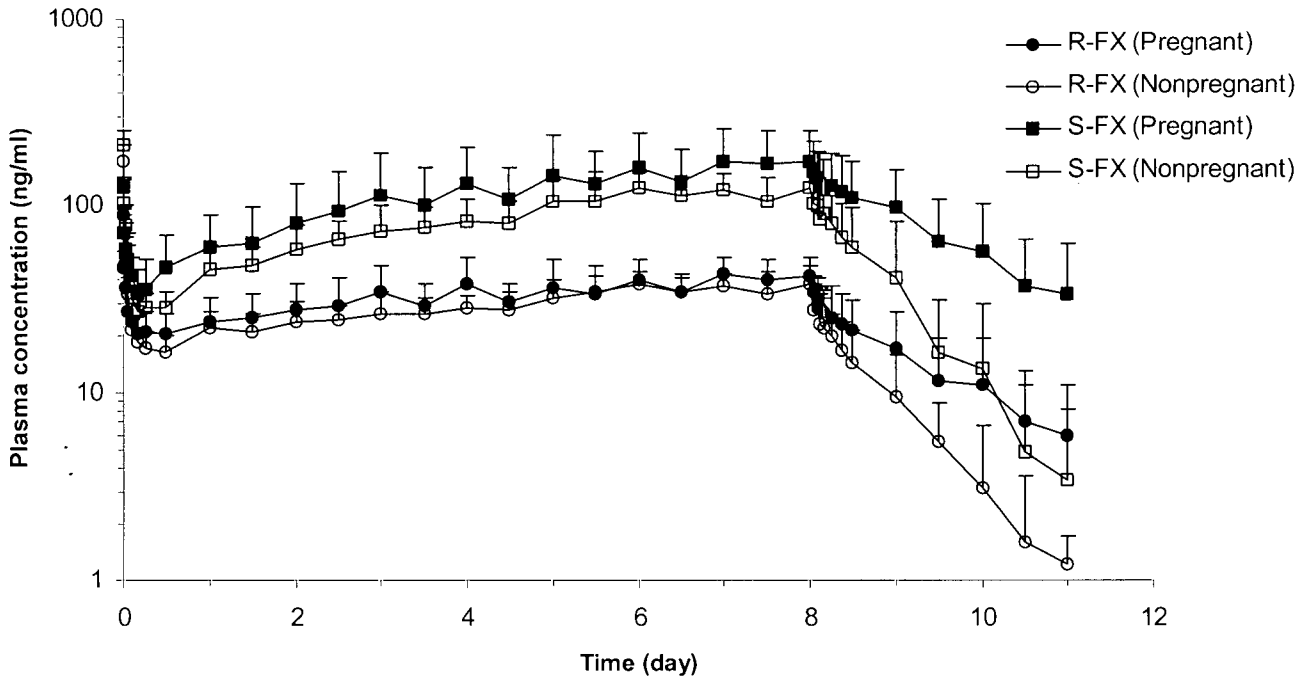


FIGURE 4.4 Comparison of average plasma concentration versus time profiles of FX enantiomers in pregnant and nonpregnant sheep after an 8-day *i.v.* infusion of racemic FX.

TABLE 4.1 Comparison of pharmacokinetic parameters and S/R ratios for FX enantiomers in nonpregnant and pregnant sheep and the corresponding *p*-values.

Animal Number	$t_{1/2\beta}$ (h)		MRT (h)		C_{ss} (ng/ml)		AUC (μg*h/l)		Vd_{ss} (l/kg)		CL_{TB} (l/h/kg)		f_u	
	R-FX	S-FX	R-FX	S-FX	R-FX	S-FX	R-FX	S-FX	R-FX	S-FX	R-FX	S-FX	R-FX	S-FX
NE†	Refer to Table 3.1 for Individual Values													
Mean ± S.D.	18.4 ± 7.7	14.8 ± 3.3	24.6 ± 7.0	34.9 ± 8.6	35.8 ± 7.6	115.2 ± 31.6	6086 ± 1311	18056 ± 5448	44.9 ± 11.3	22.7 ± 8.3	1.91 ± 0.61	0.68 ± 0.28	2.42 ± 0.82	0.71 ± 0.23
S/R Ratio	0.87 ± 0.24		1.43 ± 0.09		3.19 ± 0.26		2.92 ± 0.31		0.50 ± 0.08		0.35 ± 0.04		0.30 ± 0.04	
R- vs. S-FX *p*-values‡	0.20		<0.0001		<0.0005		<0.001		<0.001		<0.001		<0.005	
PE7103†	34.8	43.5	52.6	76.3	45.8	203.2	8277	34956	55.5	19.0	1.05	0.25	2.20	0.72
PE7132	52.2	60.3	70.5	89.5	35.9	161.8	7191	32484	81.4	22.9	1.15	0.26	3.34	1.25
PE5127	13.7	11.5	20.0	31.9	37.6	110.6	6598	17095	21.9	13.5	1.10	0.42	2.46	0.74
PE7250	22.6	26.4	24.6	41.3	53.9	276.7	10308	48553	23.5	8.4	0.95	0.20	3.10	0.56
PE4237	36.3	37.8	33.5	49.7	32.2	83.6	5777	13628	58.7	37.0	1.75	0.74	2.45	0.86
Mean ± S.D.	31.9 ± 14.6	35.9 ± 18.3	40.2 ± 21.0	57.8 ± 24.3	41.1 ± 8.7	167.2 ± 76.7	7630 ± 1753	29343 ± 14207	48.2 ± 25.3	20.2 ± 10.9	1.20 ± 0.32	0.38 ± 0.22	2.71 ± 0.48	0.83 ± 0.26
S/R Ratio	1.09 ± 0.16		1.50 ± 0.16		3.92 ± 1.09		3.68 ± 1.12		0.45 ± 0.16		0.30 ± 0.10		0.31 ± 0.08	
R- vs. S-FX *p*-value‡	0.12		<0.001		<0.05		<0.05		<0.05		<0.001		<0.0001	
PK Parameter Comparison NE vs. PE *p*-value§	0.08	<0.05	0.12	0.06	0.31	0.16	0.11	0.10	0.78	0.66	<0.05	0.08	0.50	0.47
S/R Ratio Comparison NE vs. PE *p*-value§	0.12		0.42		0.14		0.14		0.51		0.28		0.83	

Note: Data are expressed as mean ± S.D.

† NE and PE refer to nonpregnant ewe and pregnant ewe, respectively.

‡ Paired t-test, significance level $p < 0.05$.

§ Two-sample t-test, significance level $p < 0.05$.

was delayed by an additional ~24 hours in the pregnant animals. The C_{ss} of R- and S-FX also tended to be higher in the pregnant animals compared to nonpregnant ewes but again the differences were not statistically significant (R-FX: 41.1 ± 8.7 ng/mL versus 35.8 ± 7.6 ng/mL, p = 0.31; S-FX: 167.2 ± 76.7 ng/mL versus 115.2 ± 31.6 ng/mL, p = 0.16). Similarly, the AUC of R- and S-FX also tended to be higher in the pregnant animals, but again there were no significant differences (R-FX: 7630 ± 1753 μg*h/l versus 6086 ± 1311 μg*h/l, p = 0.11; S-FX: 29343 ± 14207 μg*h/l versus 18056 ± 5448 μg*h/l, p = 0.10). These changes appeared to be consistent with a lowering of the weight-normalized total body clearance (CL_{TB}) of FX observed in pregnant sheep compared to nonpregnant sheep (R-FX: 1.20 ± 0.32 l/h/kg versus 1.91 ± 0.61 l/h/kg, p<0.05; S-FX: 0.38 ± 0.22 l/kg versus 0.68 ± 0.28 l/kg, p = 0.08). Despite a generally known increase in plasma volume and altered plasma protein concentrations during pregnancy, no significant difference was found in Vd_{ss} (R-FX: 48.2 ± 25.3 l/kg versus 44.9 ± 11.3 l/kg, p = 0.78; S-FX: 20.2 ± 10.9 l/kg versus 22.7 ± 8.3 l/kg, p = 0.66) or the steady-state unbound fraction (f_u) of the FX enantiomers (R-FX: 2.71 ± 0.48 % versus 2.42 ± 0.82 %, p = 0.50; S-FX: 0.83 ± 0.26 % versus 0.71 ± 0.23 %, p = 0.47) between the pregnant and nonpregnant animals.

The S/R ratios of FX pharmacokinetic parameters were comparable between pregnant and nonpregnant animals with no significant differences noted. Outwardly, at least, pregnancy did not cause any remarkable changes in the S/R ratios of the FX enantiomers. Nevertheless, subtle changes in the S/R ratios were observed, particularly with C_{ss}, which appeared to be positively correlated with time and total FX concentrations as illustrated in Figures 4.5 and 4.6. The average C_{ss} of FX in the pregnant animals was higher than in the nonpregnant animals, resulting in a generally higher S/R C_{ss} ratio in pregnant sheep. A higher degree of

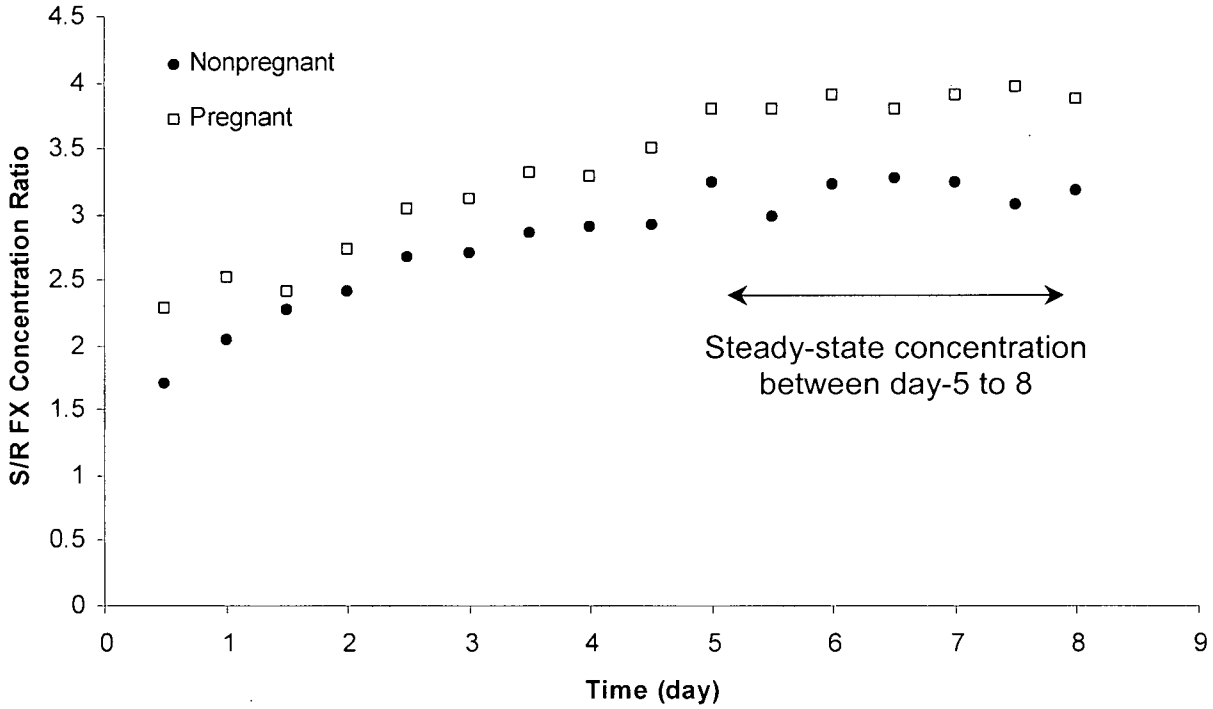


FIGURE 4.5 Schematic diagram of average S/R FX concentration ratio versus time profiles from the nonpregnant and pregnant studies illustrating the dependency of the S/R ratio to time to steady-state in all cases (error bars have been omitted for clarity).

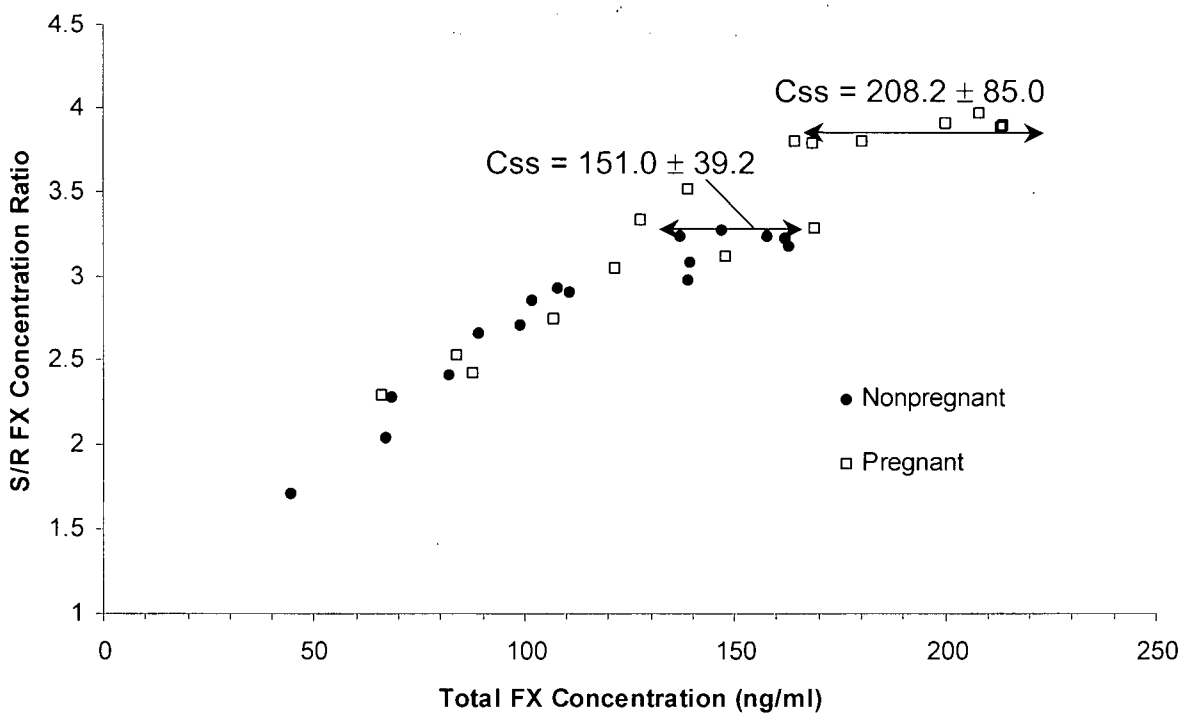


FIGURE 4.6 Plot of the S/R FX concentration ratio versus the corresponding total FX concentration data from the nonpregnant and pregnant studies, showing the plateau of S/R ratio at steady-state concentrations in each case.

variability in the pregnant animal data appeared to be responsible for the lack of statistical differences between the two groups.

4.2.2 Norfluoxetine

Representative plasma concentration versus time profiles of the R- and S-NFX enantiomers in a pregnant ewe are shown in Figure 4.1. A comparison of the average plasma concentration versus time profiles of total FX and NFX is depicted in Figure 4.2. Similar to the findings in nonpregnant experiment, no apparent differences were observed in the plasma concentration-time profiles between R- and S-NFX. As in the nonpregnant studies, the concentration-time profiles for the metabolite data also followed two-compartment model pharmacokinetics. Figures 4.7 and 4.8 compare the average plasma concentration-time profiles of total NFX and the two NFX enantiomers, respectively in pregnant and nonpregnant sheep. Unlike the situation in nonpregnant sheep, NFX continued to increase and did not reach steady-state by the end of the infusion period. The average concentration of total NFX before the termination of the drug infusion was 194.7 ± 79.3 ng/ml, which tended to be higher than the mean C_{ss} value of 143.2 ± 38.4 ng/mL in the nonpregnant sheep high dose experiment but the difference was not statistically significant (p = 0.19). Upon discontinuation of the FX infusion, NFX levels remained constant for up to 4 hours before starting to decline. Outwardly, the elimination of NFX appeared to be much slower than that of FX, with considerable amounts of the metabolite being measured in all samples collected 4 days post infusion.

The pharmacokinetic parameters determined for the NFX enantiomers from the pregnant experiments are presented in Table 4.2. Also summarized in this table are the

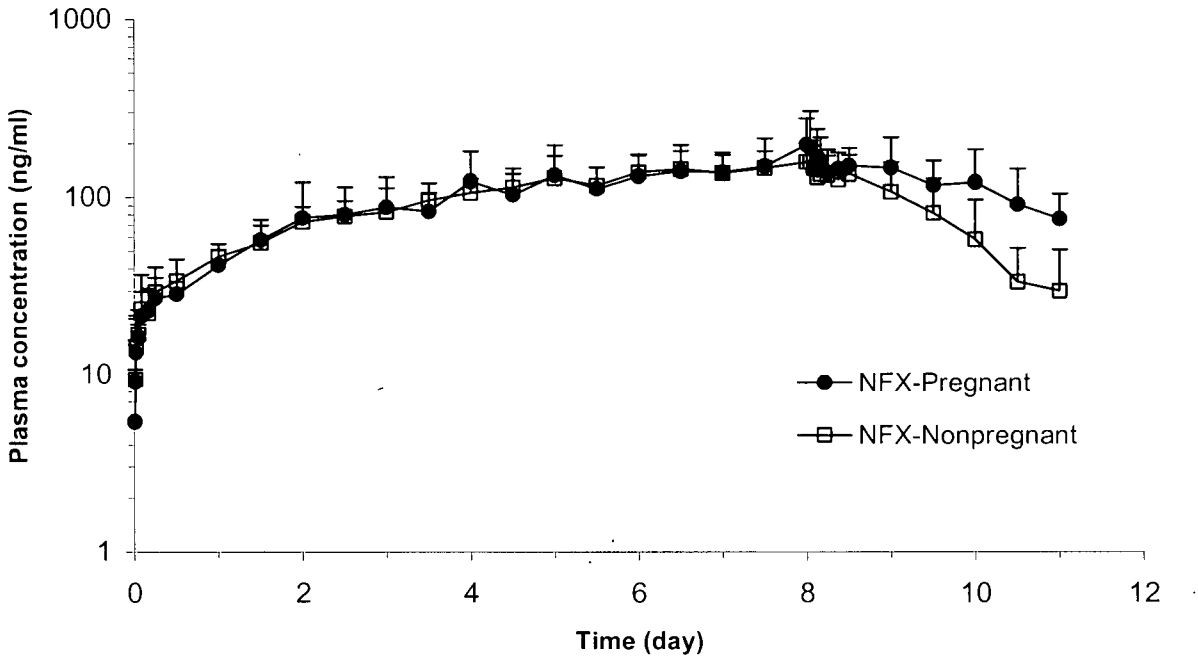


FIGURE 4.7 Comparison of the average plasma concentration versus time profiles of total NFX of pregnant and nonpregnant sheep during and following an 8-day *i.v.* infusion of racemic FX, showing longer elimination half-lives of NFX in pregnant sheep compared to nonpregnant sheep.

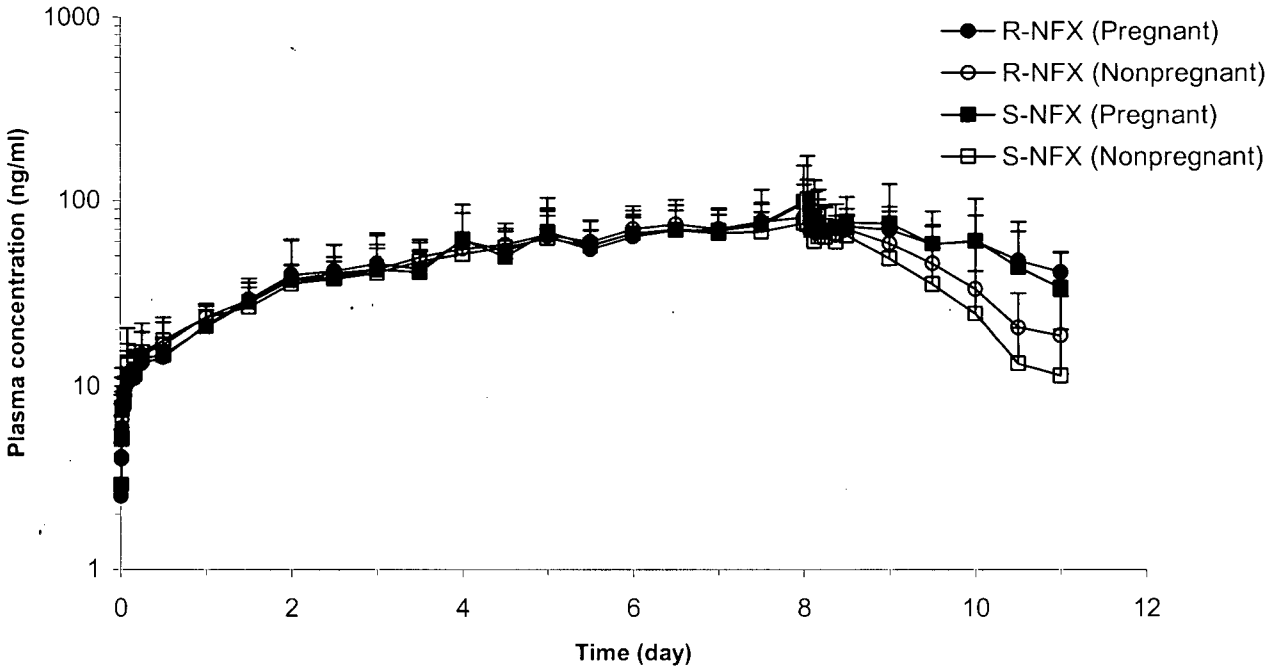


FIGURE 4.8 Comparison of the average plasma concentration versus time profiles of the NFX enantiomers in pregnant and nonpregnant sheep during and following an 8-day *i.v.* infusion of racemic FX.

TABLE 4.2 Comparison of pharmacokinetic parameters and S/R ratios of the NFX enantiomers in nonpregnant and pregnant sheep and the corresponding *p*-values.

Animal Number	$t_{1/2\beta}$ (h)		MRT (h)		AUC (μg*h/l)		NFX/FX AUC ratio		FX+NFX AUC (μg*h/l)		f_u	
	R-NFX	S-NFX	R-NFX	S-NFX	R-NFX	S-NFX	R-NFX	S-NFX	R-NFX	S-NFX	R-NFX	S-NFX
NE[†]	Refer to Table 3.3 for Individual Values											
Mean ± S.D.	26.0 ± 5.6	21.4 ± 4.2	124.8 ± 5.4	108.5 ± 3.6	13658 ± 4122	12265 ± 3169	2.26 ± 0.44	0.73 ± 0.26	19743 ± 5152	30321 ± 7206	2.02 ± 0.47	1.14 ± 0.35
S/R ratio	0.83 ± 0.10		0.87 ± 0.02		0.91 ± 0.11		0.32 ± 0.07		1.54 ± 0.11		0.57 ± 0.16	
Paired t-test R- vs. S-NFX *p*-values	<0.05		<0.0001		0.14		<0.0001		<0.0005		<0.005	
PE7103[†]	94.2	82.2	176.5	134.8	21137	16112	2.55	0.46	29414	51068	1.77	1.24
PE7132	73.3	71.2	145.1	115.4	15762	13535	2.19	0.42	22954	46019	2.68	2.05
PE5127	44.3	28.1	162.1	129.8	11717	12517	1.78	0.73	18314	29612	1.59	0.93
PE7250	63.6	40.3	168.3	135.6	25107	28742	2.44	0.59	35416	77295	2.14	1.29
PE4237	64.9	38.8	147.9	109.3	16270	11604	2.82	0.85	22047	25232	1.53	1.40
Mean ± S.D.	68.0 ± 18.0	52.1 ± 23.3	160.0 ± 13.3	125.0 ± 11.9	17999 ± 5192	16502 ± 7047	2.35 ± 0.39	0.61 ± 0.18	25629 ± 6775	45845 ± 20645	1.94 ± 0.48	1.38 ± 0.41
S/R Ratio	0.74 ± 0.17		0.78 ± 0.03		0.91 ± 0.19		0.27 ± 0.10		1.74 ± 0.40		0.71 ± 0.14	
R- vs. S-NFX *p*-value[‡]	<0.05		<0.001		0.42		<0.001		<0.05		<0.01	
PK Parameter Comparison NE vs. PE *p*-value[§]	<0.0005	<0.01	<0.0005	<0.01	0.16	0.22	0.74	0.41	0.14	0.12	0.80	0.32
S/R Ratio Comparison NE vs. PE *p*-value[§]	0.31		<0.001		1.00		0.31		0.28		0.14	

Note: Data are expressed as mean ± S.D.

[†] NE and PE refer to nonpregnant ewe and pregnant ewe, respectively.

[‡] Paired t-test, significance level $p < 0.05$.

[§] Two-sample t-test, significance level $p<0.05$.

statistical analyses for the comparison of the pharmacokinetic parameters and their corresponding S/R ratios between the pregnant and nonpregnant sheep. As in the nonpregnant ewes similar relationships between the S- and R-NFX enantiomers were observed in pregnant sheep. Significant differences were observed in $t_{1/2\beta}$, MRT, NFX/FX AUC metabolic ratio, combined FX+NFX AUC, and f_u between R-NFX and S-NFX. AUC did not differ significantly between R-NFX and S-NFX in both pregnant and nonpregnant sheep. A comparison of the individual enantiomers between the two study groups (i.e. $R\text{-}NFX_{(pregnant)}$ versus $R\text{-}NFX_{(nonpregnant)}$; $S\text{-}NFX_{(pregnant)}$ versus $S\text{-}NFX_{(nonpregnant)}$) revealed significant increases in $t_{1/2\beta}$ and MRT, and tendencies for increases in NFX AUC and combined FX+NFX AUC in pregnant sheep compared to the nonpregnant animals. The $t_{1/2\beta}$ of both R- and S-NFX were significantly prolonged in pregnant sheep compared to nonpregnant ewes (R-NFX: 68.0 ± 18.0 h versus 26.0 ± 5.6 h, $p<0.0005$; S-NFX: 52.1 ± 23.3 h versus 21.4 ± 4.2 h, $p<0.001$). Similarly, the MRTs of R- and S-NFX were significantly longer in pregnant compared to nonpregnant sheep (R-NFX: 160.0 ± 13.3 h versus 124.8 ± 5.4 h, $p<0.0005$; S-NFX: 125.0 ± 11.9 h versus 108.5 ± 3.6 h, $p<0.01$). No significant difference was found in the AUC of R- and S-NFX between pregnant and nonpregnant sheep, although the values tended to be higher in the pregnant animals (R-NFX: 17999 ± 5192 μg*h/l versus 13658 ± 4122 μg*h/l, $p = 0.16$; S-NFX: 16502 ± 7047 μg*h/l versus 12265 ± 3169 μg*h/l, $p = 0.22$). The NFX/FX AUC metabolic ratio did not differ significantly between the two groups (R-enantiomer: 2.35 ± 0.39 versus 2.26 ± 0.44, $p = 0.74$; S-enantiomer: 0.61 ± 0.18 versus 0.73 ± 0.26, $p = 0.41$). However, the combined AUC value of FX and NFX, which is a measurement of exposure to all pharmacologically active species, tended to be higher in pregnant compared to nonpregnant animals (R-NFX: 25629 ±

6775 μg*h/l versus 19743 ± 5152 μg*h/l, $p = 0.14$; S-NFX: 45845 ± 20645 μg*h/l versus 30321 ± 7206 μg*h/l, $p = 0.12$). Finally, no significant changes were observed in f_u of the NFX enantiomers between pregnant and nonpregnant animals (R-NFX: 1.94 ± 0.48 % versus 2.02 ± 0.47 %, $p = 0.80$; S-NFX: 1.38 ± 0.41 % versus 1.14 ± 0.35 %, $p = 0.32$). Comparison of the S/R ratios of NFX pharmacokinetic parameters between pregnant and nonpregnant animals only revealed a small (10%) but significant difference in stereoselectivity of the MRT values.

4.3 Discussion

As mentioned previously, data on the pharmacokinetics of FX and NFX in human pregnant subjects are extremely limited. Numerous clinical studies have been conducted to evaluate the safety of FX and NFX in human pregnancy but plasma drug concentrations were not monitored. The question of pregnancy-related physiological changes and their impact on drug disposition and pharmacotherapy has been neglected in the medical arena until recently, when Hostetter et al., 2000, reported that two thirds of 34 pregnant women taking fluoxetine, sertraline, or paroxetine, for the treatment of depression, required an increase in their daily dose of medication to maintain euthymia. A recent study by Heikkinen and co-workers, who longitudinally examined FX and NFX pharmacokinetics in pregnant & postpartum women, found an increase in FX concentration and a corresponding decrease in NFX concentration in the postnatal period (Heikkinen et al., 2003). Similar observations have been made in several pregnant subjects studied by our laboratory (Kim, 2000). These results strongly suggest that significant changes in the pharmacokinetics of these antidepressants must have occurred during pregnancy and highlight the need of more pharmacokinetic data in this population.

However, ethical and technical issues associated with *in utero* fetal blood sampling for detailed pharmacokinetic analysis presently precludes this. Animal experiment thus becomes an extremely valuable alternative to help answer some of these questions. Recently our lab has conducted a pharmacokinetic study of FX and NFX in pregnant sheep following a single *i.v.* bolus injection of racemic FX (Kim, 2000; Kim et al., 2004). In that study, five pregnant sheep at a gestational age of between 135-140 days and six nonpregnant ewes were given a 100 mg *i.v.* bolus dose of racemic FX and serial blood samples were collected to characterize the concentration-time profiles of FX and NFX and their respective enantiomers. It was reported that the apparent terminal half-lives of the FX enantiomers were significantly shorter in pregnant sheep (6.6 ± 2.5 h for R-FX and 6.7 ± 0.7 h for S-FX) than those in nonpregnant ewes (21.1 ± 6.0 h for R-FX and 14.8 ± 5.0 h for S-FX). Total body clearance values also appeared to be higher in pregnant sheep (3.1 ± 0.8 L/h/kg for R-FX and 1.9 ± 0.2 L/h/kg for S-FX) compared to those of the nonpregnant animals (1.97 ± 0.48 L/h/kg for R-FX and 1.44 ± 0.51 L/h/kg for S-FX). Similar findings of increased drug clearance have been reported for several compounds during human pregnancy. Citalopram, which is also a SSRI anti-depressant, has been shown to have lower trough steady-state plasma concentrations during pregnancy in humans. The mean desmethylcitalopram/citalopram and didesmethyl-citalopram/desmethylcitalopram metabolic ratios were significantly increased by 23% and 54%, respectively, during pregnancy compared to ratios determined 2 months after the delivery, suggesting increased metabolism during gestation (Heikkinen et al., 2002). During pregnancy, the oral clearance of metoprolol, a cardioselective β_1–adrenoceptor blocker, has also been reported to exceed that following pregnancy by a factor of 2 to 13, resulting in a lower oral bioavailability of the drug in pregnant women (Hogstedt et al.,

1985). In theory, the increased drug clearance reported by these studies could be related to one or more of the following changes during pregnancy: 1) protein binding, 2) renal elimination and 3) hepatic metabolism. As in our current studies, the free fractions of metoprolol were not significantly different in pre- and post-partum patients, suggesting that protein binding is unlikely to be the causal factor. FX, citalopram, and metoprolol are extensively metabolized by the liver with negligible amounts of unchanged drug being excreted renally. It is unlikely, then, that the increased clearance of these drugs during pregnancy is the result of increased glomerular filtration. Rather it would appear that the increase in drug clearance observed in pregnancy is the result of increased hepatic metabolism. A point of interest among these compounds is that they are metabolized predominantly by the same enzyme, cytochrome P450 2D6. Expression of the CYP2D6 enzyme is mainly under genetic control, and enzyme-inducing drugs have only a minor influence on its enzyme activity (Eichelbaum et al., 1986). However, the metabolic activity of CYP2D6 has been shown to increase in human pregnancy (Wadelius et al., 1997). The induction of CYP2D6 in pregnancy could explain the increase in clearance observed in the fore mentioned studies. In contrast to the observations from the previous single-dose FX studies (Kim, 2000), the present long-term FX infusion experiments did not show any change in systemic clearance between pregnant and nonpregnant animals. A possible explanation for this is that increased CYP2D6 activity in pregnancy may be offset by the inhibitory effect of FX on this enzyme upon long-term drug administration. In addition, there is no change in the NFX/FX AUC metabolic ratio between pregnant and nonpregnant animals, which lends further support to this argument. In the previous chapter it was demonstrated that FX was capable of inhibiting its own metabolism upon long-term administration based on the

differences in clearance between the single *i.v.* bolus versus steady-state data. In fact, our results for the infusion studies (Table 4.1) indicated a slight, but not statistically significant decrease, in clearance compared with the nonpregnant values. Wadelius et al., 1997, made a somewhat similar observation previously in human subjects. In their study, a single oral dose of dextromethorphan was given to seventeen female volunteers during and after pregnancy for CYP2D6 phenotyping. The metabolic ratio of dextromethorphan versus its metabolite dextrorphan, which served as a measurement of functional CYP2D6 activity, was determined during and after pregnancy. The study found that extensive metabolizers (EM) of CYP2D6 exhibited decreased dextromethorphan/dextrorphan metabolic ratios during pregnancy, suggesting the induction of CYP2D6 in pregnant women. Interestingly, an unexpected increase in dextromethorphan/dextrorphan metabolic ratio in poor metabolizers (PM) was observed during pregnancy, implying that metabolism of dextromethorphan was somehow compromised in this group of subjects. Due to the small number of subjects in the PM group (n=4), the authors did not perform any statistics or provide any explanations for this latter observation. It is possible that the experimental design of their study was a factor. Upon closer examination of their study, it was found that the determination of the dextromethorphan/dextrorphan metabolic ratio was based on a single blood sample collected 2 hours post oral dosing regardless of the subject's pregnancy and CYP2D6 metabolic status. The 2-hour time point was an approximation of the time to reach peak plasma concentration (C_{max}) of dextromethorphan and dextrorphan in CYP2D6 EMs. However, this experimental design contains three potential problems. First, the time to reach C_{max} (i.e. T_{max}) varies between EMs and PMs. Studies have shown that the T_{max} of dextromethorphan and its metabolites in PMs occurs at approximately 5 hours (range 4-6 h) following an oral dose,

which represents a delay of more than 200% compared to EMs (Schadel et al., 1995). The determination of the dextromethorphan/dextrorphan metabolic ratio at a time other than the actual C_{max} then, may lead to either an under- or over-estimation of the true metabolic ratio. Second, pregnancy is known to cause delays in gastric emptying. Studies with acetaminophen have shown that rate of absorption could be delayed by up to 50% in pregnant compared to nonpregnant women (Simpson et al., 1988; Levy et al., 1994). The change in gastric empting in pregnancy could also potentially be a confounding factor that affects the determination of dextromethorphan/dextrorphan metabolic ratio. Third, the use of C_{max} for the determination of metabolic ratio is prone to errors because C_{max} is a poor indicator of systemic exposure. Based on one compartment model pharmacokinetics with first order input, the C_{max} of a drug can be described as follows (Gibaldi and Perrier, 1982):

$$C_{max} = \frac{k_a FD}{Vd(k_a - k)}(e^{-kt_{max}} - e^{-k_a t_{max}}) \qquad (4.1)$$

Equation 4.1 shows that C_{max} is dependent on D, the orally administered dose; k, the first order elimination rate constant; k_a, the first order absorption rate constant; F, the fraction of the dose that is available to the systemic circulation and Vd, the volume of distribution. Among these parameters, k_a is subject to a high degree of intra-subject variability, especially during pregnancy. Area under the curve (AUC), which is the standard index for exposure, should be used instead for a more accurate determination of metabolic ratio. Unlike C_{max}, AUC is mainly dependent on systemic clearance and relatively unaffected by the rate of absorption (equation 4.2):

$$AUC = \frac{FD}{CL_{TB}} \qquad (4.2)$$

Metabolic ratios calculated based on AUC are relatively resistant to changes due to gastric emptying delays in drug absorption. However, estimation of the complete AUC requires an intensive blood sampling schedule that may not be suitable in human pregnant subjects. Under such circumstances, the single sample method is preferable but the time of sampling becomes very critical. According to statistical moment theory the concentration at the mean residence time (MRT) should be the best predictor of AUC (Zimmerman and Slattery, 1983). Alternatively, cumulative urine collection is a relatively non-invasive approach of sampling that can be used for the determination of the dextromethorphan/dextrorphan metabolic ratio. There are studies to support the use of urinary metabolic ratio as an index of functional CYP2D6 activity in human (Nagai et al., 1996; Capon et al., 1996; Streetman et al., 1999).

To determine whether the increased dextromethorphan/dextrorphan metabolic ratios in PM subjects reported by Wadelius et al., 1997, were truly due to the effect of pregnancy on drug metabolism rather than a flaw in experimental design, a simulation was performed. Concentration-time profiles for dextromethorphan and dextrorphan in nonpregnant and pregnant PMs were mimicked and the metabolic ratios calculated at different time points compared. According to literature data (Schadel et al., 1995; Capon et al., 1996), the pharmacokinetics of dextromethorphan and dextrorphan in PMs follow one compartment model pharmacokinetics therefore, their concentration-time relationships can be described by equations 4.3 and 4.4, respectively (Kochak and Rakhit, 1987):

$$C = \frac{k_a f_a (1 - E) D}{(k_a - k) Vd} (e^{-kt} - e^{-k_a t}) \qquad (4.3)$$

$$C_m = \left[\frac{k_a}{(k_a - k_m)}\right]\left[\frac{f_a ED}{Vd_m}\right]\left[e^{-k_m t} - e^{-k_a t}\right] + \left[\frac{k_a k_r f_a (1-E)D}{Vd_m}\right] \times$$
$$\left\{\left[\frac{1}{(k-k_a)(k_m-k_a)}\right]e^{-k_a t} + \left[\frac{1}{(k_a-k)(k_m-k)}\right]e^{-kt} + \left[\frac{1}{(k_a-k_m)(k-k_m)}\right]e^{-k_m t}\right\} \quad (4.4)$$

Where C_m is the concentration of the metabolite in plasma; k_m is the total elimination rate constant of the metabolite; f_a is the total fraction of the administered dose absorbed; k_a is the first order absorption rate constant; k is the overall first order elimination rate constant; E is the plasma hepatic extraction ratio; D is the dose of the parent drug; and Vd_m is the apparent volume of distribution of the metabolite. The simulated concentration-time profiles obtained using the pharmacokinetic parameter data obtained from the study by Schadel et al., 1995, are illustrated in Figure 4.9. Pharmacokinetic parameters derived from the simulated curves are summarized in Tables 4.3a and 4.3b. Three observations can be made from these simulations: 1) the metabolic ratio of dextromethorphan/dextrorphan is under-estimated by approximately 25% when it is calculated based on C_{max} as opposed to AUC; 2) the metabolic ratios determined at 2 h and 5 h (i.e. T_{max}) differ by 5% only; 3) the metabolic ratio determined at 2 h post dose remains unchanged when k_a is reduced by 50% to mimic the gastric emptying delay that occurs in pregnancy. Despite intra-subject variability in the dextromethorphan/dextrorphan metabolic ratio averaging 37% (Kashuba et al., 1998), it appears unlikely that the 63% increase in this ratio during pregnancy reported by Wadelius et al., 1997, was an artifact. As mentioned earlier, we observed a slight reduction in FX clearance in pregnant sheep from the long-term FX infusion experiments. In both scenarios, an apparent decrease in drug metabolism was observed and it appears that a common mechanism might be involved. Because of this, we are tempted to propose several hypotheses to explain these observations. First, we suspect that under conditions when

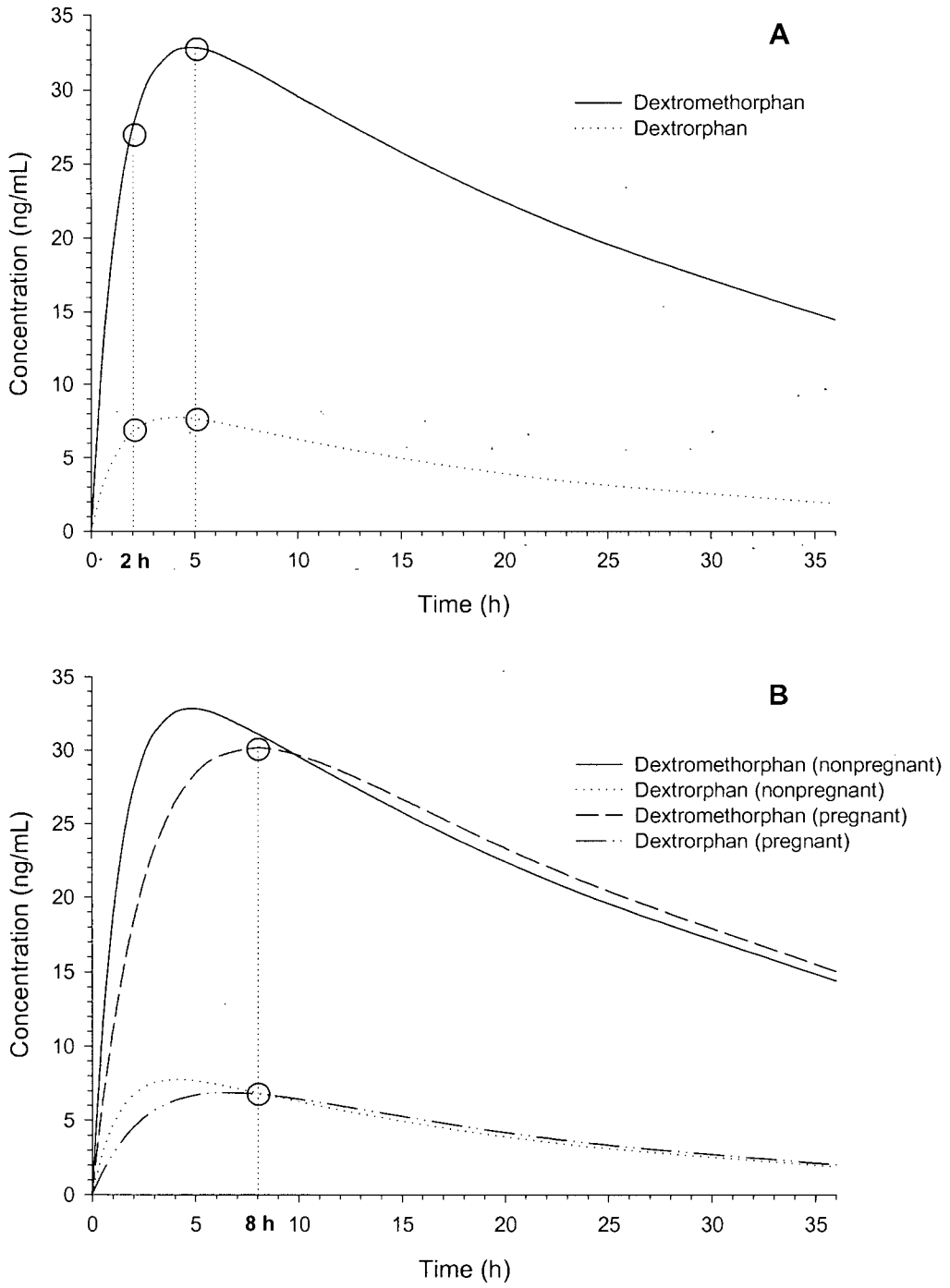


FIGURE 4.9 Simulations of the plasma concentration-time profiles for dextromethorphan and dextrorphan. Panel A illustrates the simulated profiles in poor metabolizer of CYP2D6 based on the literature data (Schadel et al.,1995). Metabolic ratios determined at 2 h and 5 h ($=T_{max}$) are 4.06 and 4.08, respectively. Panel B illustrates the comparative profiles between pregnant and nonpregnant subjects. C_{max} is delayed to 8 h when k_a is reduced by 50% to mimic the gastric delaying effect in pregnancy. Predicted values for the metabolic ratios at 2 h and 8 h during pregnancy is 4.05 and 4.45, respectively.

TABLE 4.3a Pharmacokinetic parameters derived from simulation of the dextromethorphan and dextrorphan plasma concentration-time profiles in human CYP2D6 poor metabolizers (PM) during and following pregnancy.

Pharmacokinetic Parameter	Schadel et al., 1995 CYP2D6 PM		Simulated Results Nonpregnant CYP2D6 PM		Simulated Results Pregnant CYP2D6 PM	
	DM	DX	DM	DX	DM	DX
C_{max} (ng/ml)[†]	32.52	7.71	32.81	7.75	30.15	6.86
T_{max} (h)	5	4	5	4	8	6
$T_{1/2\beta}$ (h)	25	NR	25.01	15.07	25.68	15.29
k (h^{-1})	0.03	NR	0.03	0.05	0.03	0.05
AUC (ng*h/ml)	1355	NR	1353	200	1373	201

Legends: DM = dextromethorphan; DX = dextrorphan; NR = not reported
[†] Units used in original report were nmol/ml

TABLE 4.3b Comparative dextromethorphan/dextrorphan (DM/DX) metabolic ratios determined at various individual time points as well as from AUC data.

Time Points (h)	DM/DX Metabolic Ratio based on Simulated Data	
	Nonpregnant	Pregnant
1	4.01	4.00
2	4.06	4.05
3	4.13	4.11
4	4.20	4.17
5	4.28	4.23
6	4.37	4.30
8	4.55	4.45
12	4.93	4.79
24	6.22	6.02
AUC	6.75	6.82

CYP2D6 activity is absent/reduced (e.g. in PMs) or inhibited (e.g. inhibition by FX), pregnancy paradoxically suppresses CYP2D6 activity (instead of inducing its activity as observed in EMs) or enhances the CYP2D6 inhibition imposed by the enzyme inhibitor. This hypothesis may explain the lowering of FX clearance observed in our pregnant sheep study after long term FX administration, but falls short of explaining the increased dextromethorphan/ dextrorphan metabolic ratio reported in human PMs. The pregnant PM subjects recruited in the study by Wadelius et al., 1997, possessed *CYP2D6*3* or *CYP2D6*4* mutant alleles, which are caused by frameshift mutations or altered splicing sites of the gene, respectively, resulting in completely non-functional enzymes (Rau et al., 2002). Other alleles such as **9, *10* and **41,* with alterations in amino acid sequence, are associated with a reduction of *in vivo* enzymatic activity (Raimundo et al., 2000) but none of these were detected in these particular pregnant individuals. Obviously, the increased dextromethorphan/dextrorphan metabolic ratio cannot be attributed to the suppression of CYP2D6 activity because there were simply no functional enzymes available for suppression in these pregnant subjects. The second part of this hypothesis postulates suppression of other metabolizing enzymes during pregnancy. In addition to the formation dextrorphan, the metabolism of dextromethorphan also involves a parallel pathway to form 3-methoxymorphinan (Figure 4.10). The formation of 3-methoxymorphinan is mediated by CYP3A4 enzyme (Jacqz-Aigrain et al., 1993; Yu and Haining, 2001). Similarly, the biotransformation of FX to NFX involves CYP2D6, CYP2C9 and CYP3A4 (Figure 1.2 in Chapter 1) although CYP2D6 is considered to be the principal metabolic enzyme for this demethylation pathway. It seems that pregnancy might have a negative effect on the activity of CYP3A4, resulting in the increased dextromethorphan/dextrorphan metabolic ratio in PMs

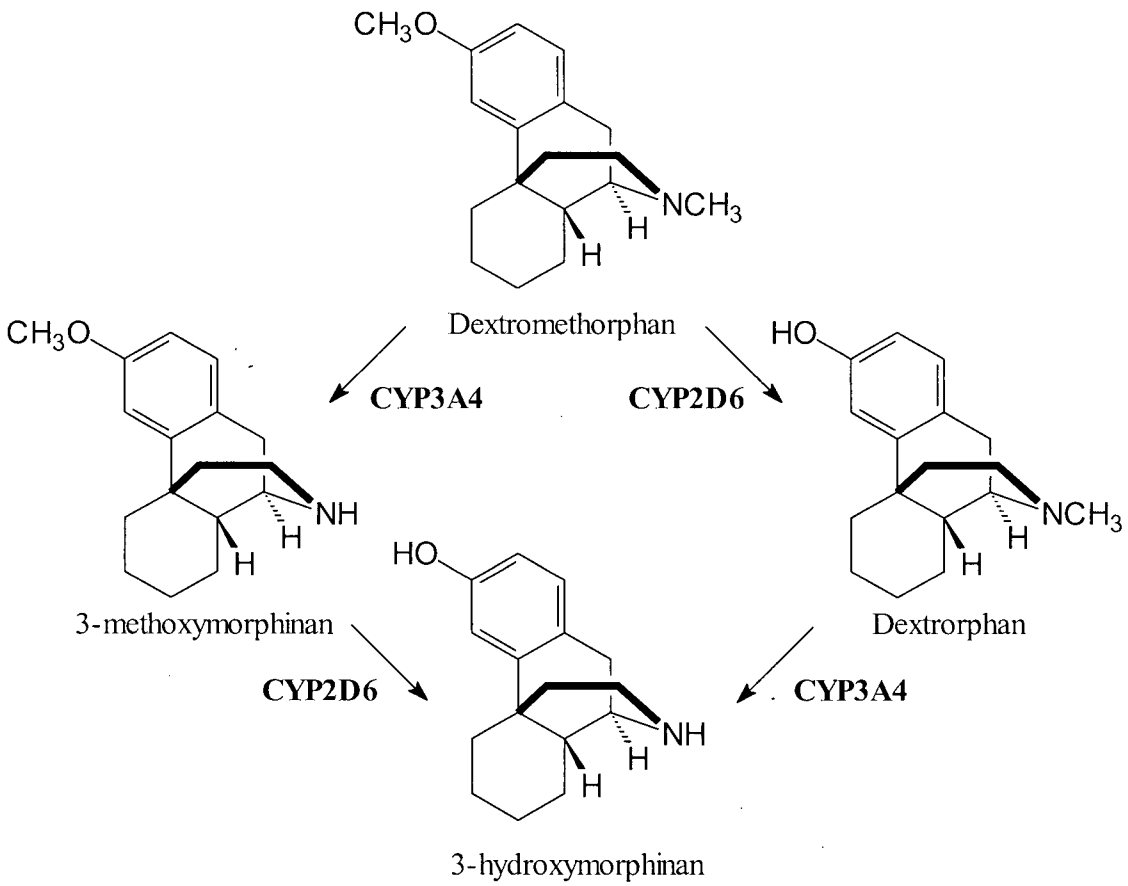


FIGURE 4.10 Metabolism of dextromethorphan by the CYP2D6 and CYP3A4 pathways to dextrorphan, 3-methoxymorphinan, and 3-hydroxymorphinan (adapted from Capon et al., 1996; Wadelius et al., 1997).

reported by Wadelius et al., 1997, as well as the decreased FX clearance observed in our long-term infusion experiments through an orthologous enzyme in sheep. The co-administration of oral contraceptives has resulted in reduced clearance of CYP3A4 substrates such as diazepam, alprazolam, and imipramine (Harris et al., 1995); hence the alterations in hormone levels that occur during pregnancy could have similar effects on CYP3A4. Wadelius et al., 1997, attempted to assess CYP3A4 activity by examining the dextro-methorphan/3-methoxymorphinan metabolic ratio but were unsuccessful because plasma 3-methoxymorphinan levels were below their assay limit of quantification. Instead, they calculated the dextrorphan/3-hydroxymorphinan metabolic ratio as an indirect estimate of CYP3A4 activity but showed no significant change in this ratio during pregnancy in all genotypes. The validity of using dextrorphan/3-hydroxymorphinan metabolic ratio to monitor CYP3A4 activity is, however, questionable and this may also be the case for the dextromethorphan/3-methoxymorphinan metabolic ratio, which, in theory, is supposed to be a better indicator of CYP3A4 activity. Several studies have shown that this latter ratio exhibits large intra-individual variability and the inability to discriminate moderate CYP3A4 inhibition suggesting that dextromethorphan is a suboptimal probe for accurately assessing CYP3A4 activity (Min et al., 1999; Kashuba et al., 1999). Midazolam, which is a specific CYP3A4 marker, is an effective probe for *in vivo* phenotyping for CYP3A4 activity using a single plasma concentration (Lin et al., 2001). It exhibits minimal transfer across placenta to the fetus and has been used clinically in pregnant women for nocturnal sedation prior to elective cesarean section (Kanto et al., 1983). On this basis, experiments that involve the administration of midazolam to women during and after pregnancy could potentially provide considerable information on the relationship between pregnancy and CYP3A4 activity.

Apart from modifications in drug metabolism, important distributional changes that occur in pregnancy may also have a significant impact on pharmacokinetics. The plasma free fraction of many drugs is altered during pregnancy (Reynolds and Knott, 1989; Loebstein et al., 1997; Little, 1999). Because FX is highly protein bound (>95%), it is anticipated that changes in plasma protein binding may lead to alteration in the unbound fraction and subsequently the pharmacological response. Albumin and α_1-acid glycoprotein are the two major plasma proteins for drug binding. In transgenic mice with elevated serum α_1-acid glycoprotein, there is a significant reduction in volume of distribution and elimination half-life of FX, suggesting that α_1-acid glycoprotein plays a larger role in its disposition (Holladay el al., 1998). In humans, maternal total plasma protein concentration falls particularly during the first trimester. The change is primarily due to a fall in the concentration of albumin while α_1-acid glycoprotein remains unchanged (Wood and Wood, 1981; Krauer et al., 1984). The unbound fractions of FX were previously determined in pregnant and nonpregnant sheep following a single *i.v.* bolus dose of FX (Kim et al., 2004). The results indicated that no change in the unbound fraction occurred during pregnancy in sheep. In the same study, the plasma unbound fraction of FX determined from women during pregnancy and postpartum also showed no changes in protein binding. In agreement with these previous reports, our long-term FX infusion study also revealed no significant difference in the free fraction and volume of distribution of FX between pregnant and nonpregnant animals. Although it is not known whether the concentration of α_1-acid glycoprotein is altered during pregnancy in sheep, the lack of a change in free faction of FX suggests that the concentration of α_1-acid glycoprotein remained unchanged. In general, unbound fraction normally correlates with the concentration of the binding protein so a

change in protein concentration usually affects the unbound fraction. However, this relationship is not always predictive. For example the free fraction of lidocaine, which is a basic drug that binds predominantly to α_1-acid glycoprotein (~ 65% bound), is greater in pregnant women at delivery compared to nonpregnant controls (48% versus 37%, respectively; Wood and Wood, 1981). The difference in unbound fractions could not be explained on the basis of the concentrations of plasma α_1-acid glycoprotein as they did not differ between the pregnant and nonpregnant groups. In the same study, the use of oral contraceptives was associated with an increased unbound lidocaine fraction of 37%. This suggests that endogenous sex hormones might act as binding inhibitors and cause the displacement of lidocaine from its protein binding sites leading to an increased free fraction during pregnancy. In addition, other factors such as changes in free fatty acids levels are also known to affect drug binding. The levels of free fatty acids increase by 2-fold during pregnancy (Nau et al., 1984), and this could significantly influence the amount of protein-bound drug. Propranolol is another example that shows a discordant correlation between drug binding and protein concentration in plasma. It is a basic drug that is highly bound to proteins in plasma (80-95%), and the major binding protein at therapeutic propranolol concentrations is α_1-acid glycoprotein. The plasma protein binding of propranolol is moderately decreased in pregnant patients (Wood and Wood, 1981), and at the time of delivery the unbound propranolol fraction was 20%. While this value is significantly different from the observed value of 15% in control patients, the plasma concentration of α_1-acid glycoprotein did not differ between the pregnant or control groups. However, it is important to be aware of the fact that a drug typically binds predominantly but not exclusively to a particular protein. Albumin exhibits low affinity but high capacity binding

for basic drugs like propranolol. The level of albumin falls during pregnancy and may then contribute, at least in part, to the change in free propranolol fraction. The negative correlation of the propranolol free fraction with albumin in fetal serum also appears to support this assumption (Krauer et al., 1986).

It is important to point out that the data presented for FX as well as those cited from the literature were derived from analyses of unrelated groups of pregnant and nonpregnant animals or human subjects. Although this may give an approximate picture, it must be realized that large interindividual differences make the comparison of disparate pregnant-nonpregnant groups subject to considerable error and that meaningful information can only be gained from a crossover study using the same animal or subject. Moreover, most studies comparing protein binding between pregnant and nonpregnant state (either in humans or animals) have been conducted during the late period of gestation. Haram et al., 1983, reported a statistically significant drop in α_1-acid glycoprotein concentration from 0.72 g/L at 8-11 weeks of pregnancy to 0.55 g/L at 16-27 weeks followed by a return to concentrations of 0.65 g/L, which are slightly lower than the 8-11 week values, by the end of the 37^{th} gestational week. The fluctuation of α_1-acid glycoprotein concentration throughout pregnancy strongly suggests that gestational age differences should also be taken into account when comparing pregnant and nonpregnant values.

While no apparent differences in the pharmacokinetics of FX were found between pregnant and nonpregnant animals, the disposition of NFX was subject to a few significant changes. Both $t_{1/2\beta}$ and MRT of NFX were significantly prolonged in the pregnant animals. Theoretically, alterations in clearance and/or volume of distribution of NFX could have contributed to these changes. A change in clearance, however, is unlikely based on the lack

of significant difference in NFX concentrations between pregnant and nonpregnant animals. Alternatively, an increase in volume of distribution is more likely to happen in pregnancy inasmuch as the fetus could act as a deeper tissue compartment for drug distribution. Upon discontinuation of drug infusion, anything that transferred across the placenta to the fetus will slowly diffuse back to the maternal circulation for elimination, assuming that the fetus lacks the capacity for drug elimination. If this redistribution process is slower than the maternal intrinsic rate of elimination, it will become a rate-limiting step for the overall drug elimination from the mother and manifests as a prolongation of $t_{1/2\beta}$ and MRT. Nevertheless, these changes in $t_{1/2\beta}$ and MRT did not result in a significant increase in AUC of NFX. The combined FX plus NFX AUC value, which is the sum of exposure of all pharmacological active species, appeared to be higher in pregnant compared to nonpregnant animals, but the difference was not large enough to reach statistical significance. Overall, our sheep data suggest that pregnancy does not pose a significant risk of increasing the exposure of the pharmacological active species to both the mother and the fetus. However, the human data reveal lower combined FX and NFX concentrations during pregnancy (Heikkinen et al., 2003). The same has been reported for citalopram, which exhibits low maternal trough plasma concentrations during pregnancy (Heikkinen et al., 2002). It is not clear if these low blood levels during pregnancy could lead to therapeutic failure, but clinicians should be aware of this possibility so that depression in pregnancy is not undertreated.

The effect of pregnancy with respect to the stereoselectivity of drug disposition is poorly understood. While gender differences may alter the stereoselective pharmacokinetics of drugs (Gilmore et al., 1992; Sasaki et al., 1993; Gupta et al., 1995; Hooper and Qing, 1990), it is anticipated that the unique physiological changes that occur in pregnancy could

influence the stereoselective disposition of enantiomers in their own right. Generally speaking, the mechanisms of stereoselective drug disposition mainly involve, but are not limited to, stereoselective protein binding, stereoselective metabolism, and stereoselective renal elimination. Enantiomers usually bind to the same class of plasma protein but the binding affinity may differ between enantiomers. In pregnancy, increased total plasma lipids and free fatty acids, altered concentrations of sex hormones and other endogenous substances may potentially influence drug binding. Displacement of drug from its plasma protein binding site(s) may occur when the concentration and affinity of the endogenous substance exceed those of the drug. Displacement may also have differential effects on enantiomers that exhibit different binding affinities to plasma protein, resulting in greater displacement for the enantiomer that has lower binding affinity thereby changing the stereoselectivity (or stereoselective index) of protein binding. For other racemic drugs that exhibit stereoselective pharmacokinetics because of stereoselective metabolism, the impact of pregnancy on certain metabolic enzymes has the potential to alter the metabolism of each enantiomer in a discriminating manner. An example of such interaction is the induction of CYP2D6 in pregnancy (Wadelius et al., 1997). In this example, the racemic drug is eliminated primarily by metabolism with one enantiomer being predominantly metabolized by CYP2D6 whereas a different enzyme exclusively metabolizes the other enantiomer. As a result the clearance of one enantiomer will be higher during pregnancy than that of its counterpart thus changing the AUC ratio of the enantiomers.

Compared to hepatic metabolism, fewer drugs have been reported to exhibit stereoselective renal elimination (i.e. active transport and/or renal metabolism) (Jamali et al., 1989; Tracy, 1995). Although glomerular filtration rate (GFR) increases by approximately

60-80% during pregnancy, it is believed this has no impact on stereoselective drug elimination as glomerular filtration is a passive process, which depends on the physiochemical property of the drug (e.g. molecular weight, pKa, partition coefficient, etc.), and for enantiomers these are identical. Other than GFR, renal secretion and reabsorption increase modestly by approximately 20% during pregnancy (Little, 1999). If we consider the situation where a racemic drug is mainly excreted by the kidney and exhibits stereoselective elimination and further that one enantiomer has a lower CL_{renal} due to preferential tubular reabsorption then, the clearance of that particular enantiomer will be expected to be reduced during pregnancy. This would occur as a result of more of it is being reabsorbed relative to its antipode. This phenomenon does not, however, appear to have been investigated.

The present study is the first to examine the effect of pregnancy on the stereoselective pharmacokinetics of FX and NFX. Our results showed a progressive increase of S/R ratio with respect to time (Figure 4.5). A positive correlation was also observed between S/R C_{ss} ratio and total FX concentration (Figure 4.6). The mean FX steady-state level in pregnant animals was slightly higher than that of nonpregnant animals resulting in a higher S/R ratio in the former group. The difference in S/R ratio between pregnant and nonpregnant animal is unlikely to be the result of CYP2D6 induction during pregnancy as we have proposed earlier in the discussion that any increase CYP2D6 activity was likely to be abolished by the CYP2D6 inhibitory effect of FX. When the S/R C_{ss} ratio was plotted against the CL_{TB} a negative correlation resulted ($p<0.01$, Spearman rank test; Figure 4.11). This finding suggests that reduced metabolism (probably through the inhibition of CYP2D6) plays a more important role in shifting the S/R ratios. For protein binding, no difference in the S/R f_u ratios of FX and NFX enantiomers was found between pregnant and nonpregnant sheep.

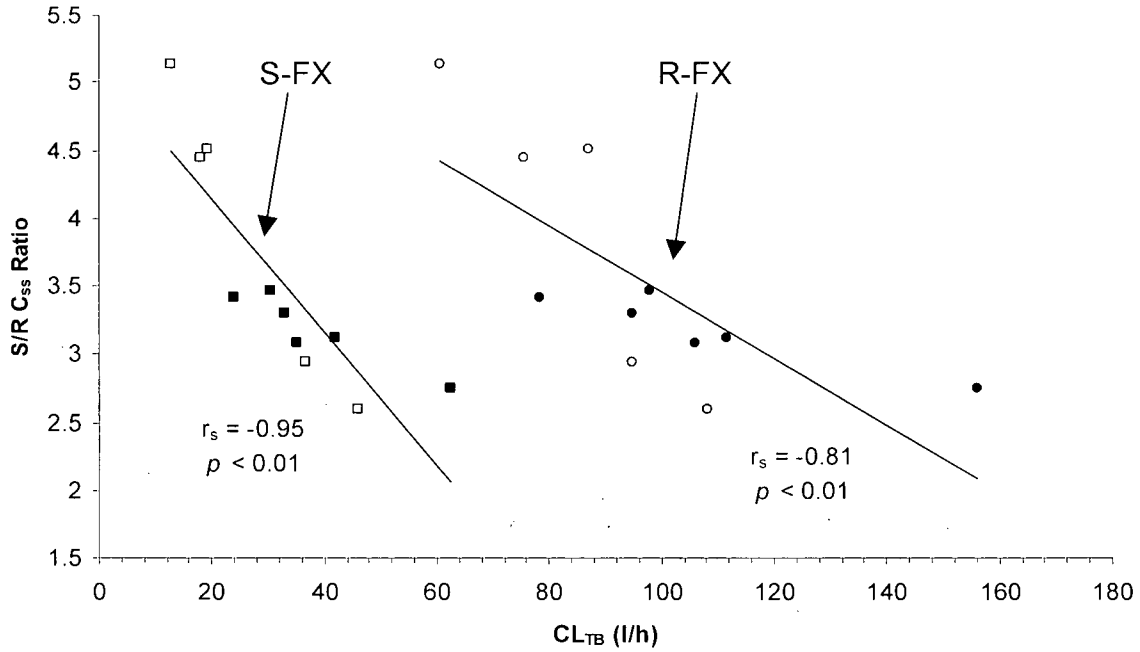


FIGURE 4.11 Plot of S/R FX C_{ss} ratio versus the corresponding total body clearance value (CL_{TB}) of FX enantiomers (data from the nonpregnant (closed symbol) and pregnant sheep (open symbol) experiments).

Similar findings were reported for other α_1-acid glycoprotein bound drugs such as propranolol and verapamil. Walle and colleagues reported an S/R unbound fraction ratio of 0.86 for propranolol in serum of healthy human subjects, which was similar to the value of 0.91 determined by Belpaire and colleagues in maternal serum (Walle et al., 1983; Belpaire et al., 1995). Similarly, S/R unbound fraction ratio of verapamil calculated based on the data reported by Gross and colleagues was 1.42 in the serum of human volunteers, which was similar to the value of 1.36 determined by Belpaire and colleagues in maternal serum (Gross et al., 1988; Belpaire et al., 1995). Based on the above findings, it appears that pregnancy has no significant impact on the S/R unbound fraction ratio for racemic drugs that bind predominantly to α_1-acid glycoprotein. The effect of pregnancy with respect to stereoselective renal elimination could not be explored in this study because maternal urine was not collected. Nevertheless, both FX and NFX were extensively metabolized and only minute amounts of the unchanged moieties (<1%) were excreted renally, so any changes occurring in this pathway during pregnancy should only affect their disposition to a very limited extent. However, for drugs that are predominantly eliminated by the kidney, pregnancy-related changes in renal function may be of great significance. The stereoselective pharmacokinetics of pindolol have been studied in pregnant women with hypertension in a recent report (Goncalves et al., 2002). Pindolol is marketed as a racemic mixture of the S-(−) and R-(+) enantiomers with the S-enantiomer being a more potent β-adrenoceptor blocking agent than the R-enantiomer. It is cleared approximately equally by both metabolism and renal excretion. Pindolol undergoes active secretion in the renal tubules with its CL_{renal} exceeding the glomerular filtration rate. Goncalves et al., 2002, reported that the renal elimination of pindolol is stereoselective in hypertensive pregnant women and

similar observations have also been reported in nonpregnant healthy (normotensive) subjects (Hsyu and Giacomini, 1985; Somogyi et al., 1992). The authors compared the pharmacokinetics of racemic pindolol between pregnant and nonpregnant subjects, but deliberately failed to examine any change in stereoselectivity during pregnancy, as apparently this was not within the scope of their study objectives. To facilitate the investigation of the effect of pregnancy on stereoselective renal elimination, we have combined the data from Goncalves et al., 2002, and Somogyi et al., 1992, for further analysis. S/R ratios of the reported pharmacokinetic parameters were calculated and the results are summarized in Table 4.4. The results indicate that the CL_{renal} of pindolol enantiomers and their corresponding S/R ratios were slightly lower in pregnant woman compared to nonpregnant subjects, suggesting that tubular reabsorption might have increased and that this process was more efficient for the S-enantiomer during pregnancy. Although these values have not been weight normalized, the reported ranges of body weight in the nonpregnant and pregnant groups were 52-78 kg and 68-97 kg, respectively (Somogyi et al., 1992; Goncalves et al., 2002), indicating that the difference in CL_{renal} is not due to a higher body weight in the nonpregnant subjects. Apart from renal clearance, we observed an interesting change in the direction of the S/R ratios for other parameters such as AUC and apparent total body clearance (CL/F). The S/R AUC ratio increased from 0.86 to 1.13 in nonpregnant and pregnant subjects, respectively, while the S/R CL/F ratio decreased from 1.17 in nonpregnant subjects to 0.89 in pregnant women. The authors commented that the increased CL/F in pregnant subjects was likely due to the induction of enzymes involved in the metabolism of pindolol. Further, our preliminary analysis shows a preferential increase in CL/F with respect to R-(+) pindolol compared to S-(–) pindolol, indicating a pregnancy-

TABLE 4.4 Comparative pharmacokinetic parameters of pindolol enantiomers in pregnant and nonpregnant human subjects.

Pharmacokinetic Parameter	Hypertensive Pregnant Women*			Nonpregnant Healthy Subjects**		
	S-(–)	R-(+)	S/R ratio	S-(–)	R-(+)	S/R ratio
AUC (ng*h/ml)	95.69	84.34	1.13	209	244	0.86
CL_{renal} (l/h)†	10.85	9.16	1.18	13.32	10.20	1.31
Ae (%)‡	35	27	1.31	35	29	1.21
CL/F (l/h)§	55.74	62.48	0.89	35.89	30.74	1.17
$CL_{non\text{-}renal}$/F (l/h)¶	44.89	53.32	0.84	22.57	20.54	1.10

* Mean values based on n = 8 following a 10 mg dose of racemic pindolol administered every 12 h for > 7 days (Goncalves et al., 2002)

** Mean values based on n = 8 following a single oral dose of 15 mg racemic pindolol (Somogyi et al., 1992)

† CL_{renal} was calculated by dividing the amount recovered in urine by AUC

‡ Ae, the amount excreted, was calculated as the amount of each enantiomers recovered in urine divided by the amount of each enantiomer administered

§ CL/F was calculated as amount of enantiomers administered divided by AUC

¶ $CL_{non\text{-}renal}$/F equals the difference between CL/F and CL_{renal}.

induced change in stereoselective pharmacokinetics. The finding of a higher non-renal clearance value ($CL_{non\text{-}renal}/F$) for R-(+) pindolol in pregnant subjects suggests the involvement of stereoselective metabolism, which was overlooked by other reports on pindolol pharmacokinetics.

4.4 Summary

In summary, this study has demonstrated that FX and NFX exhibit stereoselective pharmacokinetics in pregnant sheep. Increased clearance of FX was observed in the previous single *i.v.* bolus experiments in pregnant sheep but this effect was absent upon long-term FX infusion. The potent enzyme inhibitory effects of FX and NFX might have abolished any increased enzyme activity during pregnancy under steady-state conditions. The findings of decreased CL_{TB} of FX in pregnant sheep at steady-state together with the increased dextromethorphan/dextrorphan metabolic ratio in CYP2D6 PMs during pregnancy suggests a reduction of CYP3A4 activity during pregnancy. Protein binding of FX and NFX did not differ between pregnant and nonpregnant sheep. This finding is consistent with the lack of significant change in α_1-acid glycoprotein concentration during pregnancy. However, the relationship between unbound fraction and concentration of the binding protein does not always correlate, as exemplified by the alteration of binding of other α_1-acid glycoprotein bound drugs like lidocaine and propranolol during pregnancy. The theoretical basis of interactions between pregnancy and stereoselective drug disposition has been discussed. Although no remarkable change in the S/R ratio of FX was found in our experiments, a review of the literature for other racemic drugs shows that pregnancy does have the potential to alter stereoselective drug disposition. The clinical significance of such phenomena, however, remains to be elucidated.

CHAPTER 5

STUDY OF PLACENTAL TRANSFER OF FX AND NFX FOLLOWING MATERNAL INTRAVENOUS INFUSION OF RACEMIC FX TO STEADY-STATE

Preface

The placental transfer of FX and FX in humans has been reported, but detailed pharmacokinetic studies are lacking. Studies of placental drug transfer and fetal pharmacokinetics cannot be readily undertaken in humans, in whom observations have been largely confined to the comparison of single point drug concentrations in maternal and umbilical cord plasma at birth. Interpretation of such data is difficult because the single point fetal-to-maternal concentration ratio is highly dependent on the time of sampling relative to the time of maternal dosing. Moreover, such observations contribute little to the understanding of placental transfer, or of fetal drug distribution and elimination. The pregnant sheep preparation is not limited in this way, and the increased flexibility of experimental design allows much greater insight into the fetal handling of drugs.

The present study is an extension of the maternal infusion study in which fetal arterial blood, amniotic and fetal tracheal fluids were collected and analyzed for FX and NFX concentrations. The fetal exposure index (i.e. fetal-to-maternal AUC ratio) was calculated and components of fetal nonplacental drug elimination were explored.

5.1 Methods

5.1.1 Animal and Surgical Preparation

The experimental setup is the same as the pregnant sheep experiments in the previous chapter. Five pregnant ewes (Dorset and Suffolk breeds) at gestational ages ranging from 123 to 125 days and term ~145 days were used in this experiment. The study was 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. The surgical procedure was similar to that described previously in Chapter 3, Section 3.11. Addition details for the surgical procedures on the fetus have been described previously (Morrison et al., 2001). Briefly, a midline abdominal incision was made in the ewe after shaving, cleansing and disinfecting the abdomen, flank and groin areas with 10% povidone-iodine solution. The uterus was exposed and access to the fetus gained by an incision in an area free of placental cotyledons and major blood vessels. Polyvinyl catheters were implanted in the fetal femoral arteries and lateral tarsal veins for drug sampling and administration, respectively. Another two catheters were placed in the amniotic cavity and anchored to the skin on the neck and the abdomen of the fetus. A non-occlusive catheter was inserted into the trachea via a separate uterine incision. Electrodes of Teflon-coated stainless steel wire were implanted on the dura of the parietal cortex for the measurement of the electroencephalograph and on the zygomatic bone of each eye for electroocular recordings. A Transonic blood flow transducer was placed around the main uterine artery for measurement of uterine arterial blood flow. The hysterotomy and laparotomy incisions were then closed. All catheters and electrodes were tunneled subcutaneously and exited through a small incision on the side of the maternal flank. The catheters were stored in a plastic pouch

attached to the back of the ewe when not in use. After surgery, 500 mg of ampicillin was administered intramuscularly to the ewe for 3 days, and via the tarsal vein to the fetus on the day of surgery, and to the amniotic cavity for the duration of the preparation. The ewe was kept in a holding pen with other sheep and allowed free access to food and water. A minimum of 3 days were allowed for the ewes to recover before experimentation.

5.1.2 Experimental Protocol

The experimental protocol was the same as that used for the pregnant sheep experiments described in Chapter 4. Figure 3.1 in Chapter 3 is a schematic illustration of the general study protocol that was also used for the present experiment. Each animal received a 70 mg *i.v.* bolus loading dose over one minute followed by a continuous *i.v.* infusion of racemic FX via the maternal femoral vein at a rate of 6.92 mg/h. The drug infusion was continued for 8 days to achieve steady-state concentrations. Serial blood samples from the fetal femoral arterial (5 ml) were collected at 0, 5, 15, 30 min, 1, 2, 4, 6, 12, 24, 36, 48, 60, 72, 84, 96, 108, 120, 132, 144, 156, 168, 180 h, 192 h during the infusion, and at 5, 10, 20, 30, 45 min, 1, 2, 3, 4, 6, 9, 12, 24, 36, 48, 60, and 72 h post-infusion. Amniotic and fetal tracheal fluid samples (1 ml each) were also collected at 0, 12, 24, 36, 48, 60, 72, 84, 96, 108, 120, 132, 144, 156, 168, 180 h, 192 h during the infusion, and at 1, 2, 3, 4, 6, 9, 12, 24, 36, 48, 60, and 72 h post-infusion. All zero time samples were collected ~10 minutes prior to loading dose administration. During the experiment, the volume of fetal blood collected was replaced at intervals during the study by an equal volume of drug-free maternal blood.

All blood samples collected for drug and metabolite analysis were placed into K_3EDTA Vacutainer® tubes, gently mixed, and centrifuged at 2000g for 10 min to separate

the plasma. The plasma supernatant was transferred into a clean borosilicate test tube with polytetrafluoroethylene (PTFE)-lined cap. Amniotic and fetal tracheal fluid samples were transferred to borosilicate test tubes immediately upon collection. All samples were frozen at -20°C until the time of analysis.

At each blood collection, an aliquot of whole blood sample (~1 ml) was collected for blood gas, acid-base, and glucose/lactate analyses using the instrumentation outlined in Chapter 2, Section 2.2.2. In addition, the maternal and fetal arterial pressure and heart rate, uterine blood flow, fetal electrocorticographic activity (ECoG), rapid eye movements (REM), amniotic pressure, and fetal tracheal pressure [to monitor fetal breathing movements (FBM)] were continuously monitored with a Grass K2G polygraph (Astro-Med, West Warwick, RI) and digitally stored using a computerized data acquisition system (Chart® v4.2, AD Instruments, Grand Junction, CO). The values from the physiologic and behavioral state measurements have been reported separately (Morrison et al., 2001; Morrison et al., 2002; Morrison et al., 2004).

5.1.3 Pharmacokinetic Analysis

All pharmacokinetic parameters were calculated by standard pharmacokinetic procedures as described in Chapter 3, Section 3.1.4. In addition, the MRT of FX and NFX in the fetal circulation following maternal FX administration was calculated as the ratio of $AUMC_{fetal}$ to AUC_{fetal} minus the $MRT_{maternal}$ value (Kim 2000). The fetal-to-maternal exposure ratio (F/M ratio), which is also known as the index of fetal exposure, was calculated as AUC_{fetal} divided by $AUC_{maternal}$. The combined fetal-to-maternal exposure ratio (Combined F/M ratio), which is a measurement of the exposure to the pharmacologically

active species, was calculated as the sum of AUC_{fetal} for FX and NFX divided by the sum of the $AUC_{maternal}$ for FX and NFX.

5.1.4 Statistical Analysis

A paired t-test was used for statistical analysis of the pharmacokinetic parameters when comparing R- versus S-enantiomers. The Spearman rank correlation was used to test the correlation between two variables. The achievement of steady-state for plasma FX and NFX concentrations was evaluated by testing whether the slope of plasma concentration versus time curve was significantly different from the value of zero. The significance level used was $p<0.05$ in all cases. Unless otherwise specified, all data are reported as the mean ± standard deviation (S.D.).

5.2 Results

5.2.1 FX and NFX Levels in Fetal Arterial Plasma

The average plasma concentration versus time profiles of total FX and NFX are shown in Figure 5.1. There was rapid transfer of FX from the mother to the fetus across the placenta with FX detected in fetal plasma as early as 5 minutes following the initiation of maternal dosing. During the initial infusion period, there was a decline in the FX level in the fetus in the first 12 hours. After that, FX concentrations started to increase gradually over the next 5 days and reached steady state during day-6 to 8 of the infusion period. In contrast to FX, there was a delay in the appearance of NFX in fetal plasma. Plasma levels of NFX were below the limit of quantitation in the first 2 hours following the initiation of maternal dosing. After that, NFX began to increase slowly and continued to accumulate in the fetal circulation

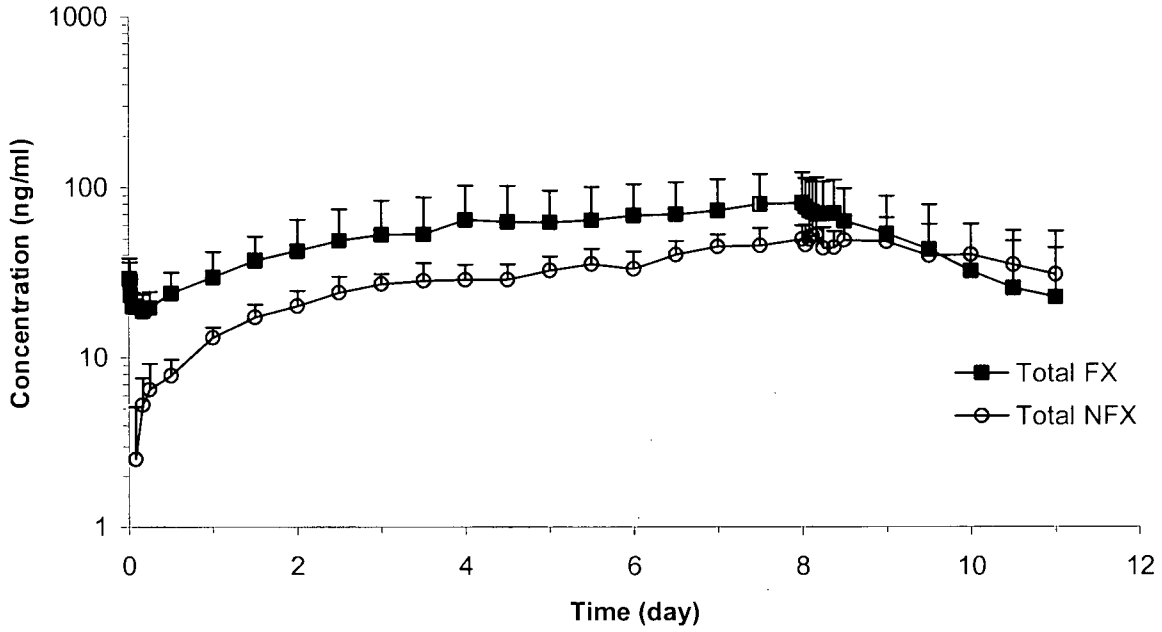


FIGURE 5.1 Average plasma concentration versus time profiles of total FX and NFX in fetal sheep (n=5) following an 8-day maternal *i.v.* infusion of racemic FX, showing concentration differences between FX and NFX from day-1 to 8 of infusion period.

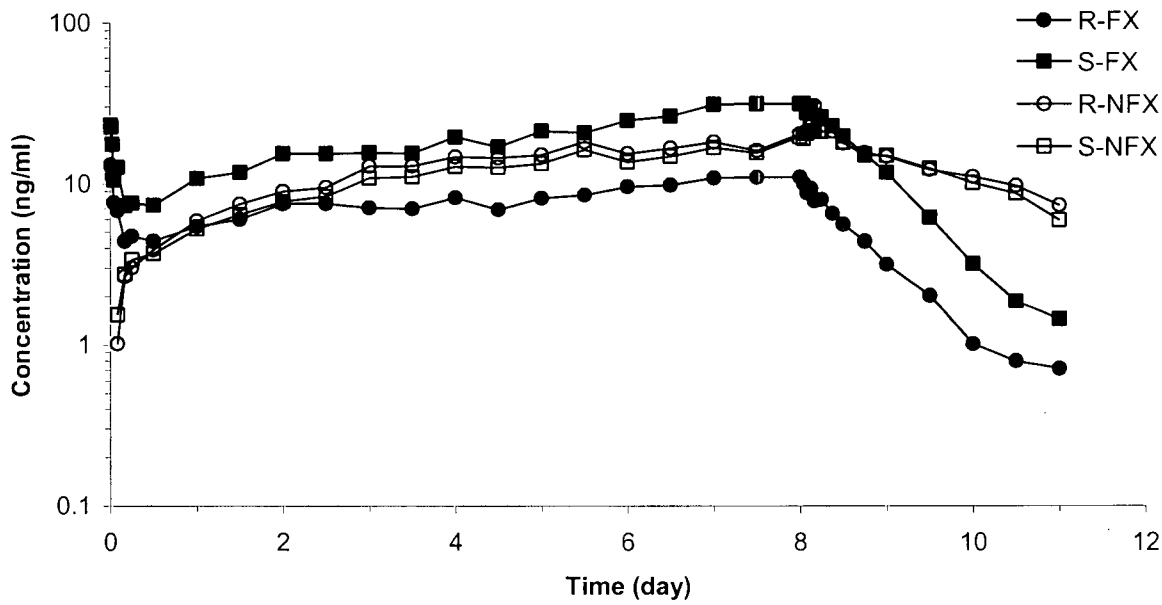


FIGURE 5.2 Representative plasma concentration versus time profiles of FX and NFX enantiomers in a fetus (E4237) following an 8-day maternal *i.v.* infusion of racemic FX, showing stereoselective disposition of FX enantiomers in the fetus.

over the entire infusion period without reaching steady-state. Upon termination of the maternal drug infusion, the concentration of FX persisted for approximately one hour and then began to decline whereas the concentration of NFX remained steady for up to 9 hours post-infusion before any signs of elimination occurred. Figure 5.2 depicts representative plasma concentration versus time profiles of FX and NFX enantiomers in a fetal lamb, showing stereoselective pharmacokinetics of FX enantiomers in the fetus. The stereoselective relationship between enantiomers of FX and NFX with respect to their steady-state concentrations in the fetus was similar to that in adult sheep (i.e. R-FX < S-FX and R-NFX ≅ S-NFX). The pharmacokinetic parameters for total FX, R-FX and S-FX are summarized in Table 5.1 while those for total NFX, R-NFX and S-NFX are summarized in Table 5.2. The elimination of FX appeared to be slower in the fetus compared to the mother (maternal $t_{1/2\beta}$ = 31.9 h for R-FX and 35.9 h for S-FX, maternal MRT = 40.2 h for R-FX and 57.8 for S-FX; Table 4.1 in Chapter 4). The elimination $t_{1/2\beta}$ for total FX, R-FX and S-FX was 35.8 ± 22.9 h, 35.0 ± 23.5 h, and 36.7 ± 23.3 h, respectively. MRTs for total FX, R-FX and S-FX were 100.2 ± 11.6 h, 103.6 ± 13.5 h, and 99.2 ± 12.1 h, respectively. No differences in $t_{1/2\beta}$ and MRT were observed between R-FX and S-FX. C_{ss} of R-FX was significantly lower than that of S-FX (14.7 ± 6.5 ng/ml vs. 61.4 ± 33.4 ng/ml, respectively; $p<0.05$) with a mean S/R ratio of 4.09 ± 0.85). Similarly, R-FX had a lower AUC compared to S-FX (3059 ± 1248 μg*h/l vs. 12497 ± 7107 μg*h/l, respectively; $p<0.05$), and the mean S/R AUC ratio was 3.83 ± 0.90. Figure 5.3 shows the comparative average plasma concentration versus time profiles of total FX in the mother and the fetus. The fetal concentration of total FX was consistently lower than the maternal concentration throughout the entire infusion period with an average F/M ratio of 0.40 ± 0.08. The combined F/M ratio

TABLE 5.1 Pharmacokinetic parameters of total, R- and S-FX in fetal lambs following an 8-day maternal infusion of racemic FX.

Animal Number	$t_{1/2\beta}$ (h)		MRT (h)		C_{ss} (ng/mL)		AUC (μg*h/l)		F/M AUC ratio†		Combined F/M AUC ratio‡	
	Total-FX		Total-FX		Total-FX		Total-FX		Total-FX		Total-FX+NFX	
PE7103†	58.5		111.9		110.9		23323		0.54		0.39	
PE7132	61.1		107.2		51.3		14795		0.37		0.41	
PE5127	13.2		91.5		51.3		8842		0.37		0.35	
PE7250	30.8		106.0		126.8		24702		0.42		0.38	
PE4237	15.4		84.6		40.4		6116		0.32		0.29	
Mean ± S.D.	35.8 ± 22.9		100.2 ± 11.6		76.1 ± 39.7		15556 ± 8348		0.40 ± 0.08		0.36 ± 0.05	
Animal Number	R-FX	S-FX	R-FX	S-FX	R-FX	S-FX	R-FX	S-FX	R-FX	S-FX	R-FX + R-NFX	S-FX + S-NFX
PE7103†	51.4	65.5	108.1	112.6	20.1	90.8	4356	18967	0.53	0.54	0.28	0.46
PE7132	66.9	55.2	119.4	104.5	8.7	42.6	2772	12023	0.39	0.37	0.41	0.42
PE5127	12.8	13.6	90.1	91.1	10.9	40.4	2050	6792	0.31	0.40	0.30	0.39
PE7250	28.5	33.1	111.7	104.8	23.1	103.7	4371	20331	0.42	0.42	0.34	0.39
PE4237	15.1	15.7	88.9	82.6	10.7	29.8	1746	4370	0.30	0.32	0.26	0.31
Mean ± S.D.	35.0 ± 23.5	36.7 ± 23.3	103.6 ± 13.5	99.2 ± 12.1	14.7 ± 6.5	61.4 ± 33.4	3059 ± 1248	12497 ± 7107	0.39 ± 0.09	0.41 ± 0.08	0.32 ± 0.06	0.39 ± 0.05
S/R Ratio	1.07 ± 0.17		0.96 ± 0.07		4.09 ± 0.85		3.83 ± 0.90		1.06 ± 0.13		1.26 ± 0.25	
Paired t-test R- vs. S-FX *p*-value	0.70		0.25		<0.05		<0.05		0.35		0.06	

Note: Average values are expressed as the mean ± standard deviation (S.D.)

† F/M AUC ratio = fetal-to-maternal area under the curve ratio.

‡ Calculated from the sum of the AUC for FX and NFX.

TABLE 5.2 Pharmacokinetic parameters of total, R- and S-NFX in fetal lambs following an 8-day maternal infusion of racemic FX.

Animal Number	$t_{1/2\beta}$ (h)		MRT (h)		AUC (μg*h/l)		F/M AUC ratio†		NFX/FX AUC ratio	
	Total-NFX		Total-NFX		Total-NFX		Total-NFX		Total-NFX	
E7103	42.8		71.6		8420		0.23		0.36	
E7132	88.5		129.3		13788		0.47		0.93	
E5127	34.0		69.7		8108		0.33		0.92	
E7250	54.9		117.4		17642		0.33		0.71	
E4237	47.0		85.8		7414		0.27		1.21	
Mean ± S.D.	53.4 ± 21.0		94.8 ± 27.2		11074 ± 4465		0.32 ± 0.09		0.83 ± 0.32	
Animal Number	R-NFX	S-NFX	R-NFX	S-NFX	R-NFX	S-NFX	R-NFX	S-NFX	R-NFX	S-NFX
E7103	42.0	43.6	79.7	67.3	3816	4604	0.18	0.29	0.88	0.24
E7132	91.1	85.8	125.3	124.3	6712	7076	0.43	0.52	2.42	0.59
E5127	41.1	26.8	80.1	63.3	3466	4642	0.30	0.37	1.69	0.68
E7250	46.7	63.1	103.3	125.2	7809	9833	0.31	0.34	1.79	0.48
E4237	50.8	43.1	84.0	85.1	3935	3479	0.24	0.30	2.25	0.80
Mean ± S.D.	54.4 ± 20.9	52.5 ± 22.6	94.5 ± 19.8	93.0 ± 30.1	5148 ± 1975	5927 ± 2548	0.29 ± 0.09	0.36 ± 0.09	1.81 ± 0.60	0.56 ± 0.21
S/R ratio	0.97 ± 0.26		0.97 ± 0.17		1.15 ± 0.18		1.28 ± 0.18		0.31 ± 0.07	
Paired t-test R- vs. S-NFX *p*-value	0.74		0.84		0.13		<0.01		<0.01	

Note: Average values are expressed as the mean ± standard deviation (S.D.)

† F/M AUC ratio = fetal to maternal area under the curve ratio.

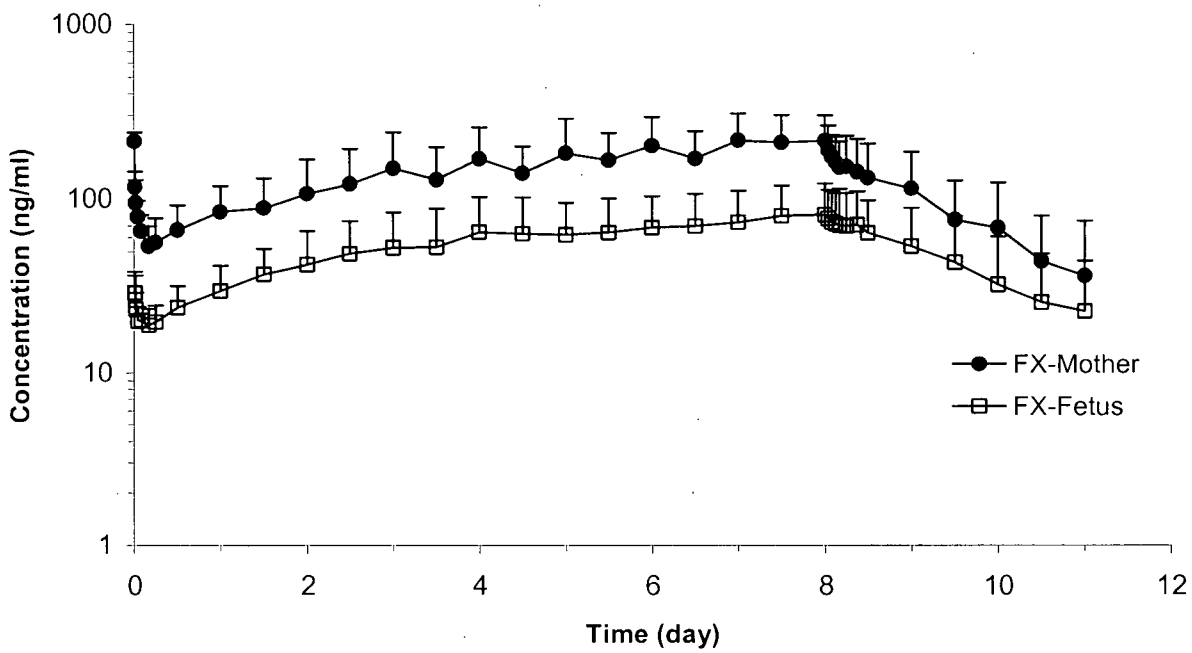


FIGURE 5.3 Average plasma concentration versus time profiles of total FX in the mother and fetus (n = 5) following an 8-day maternal *i.v.* infusion of racemic FX, with an average F/M AUC ratio of 0.40 ± 0.08.

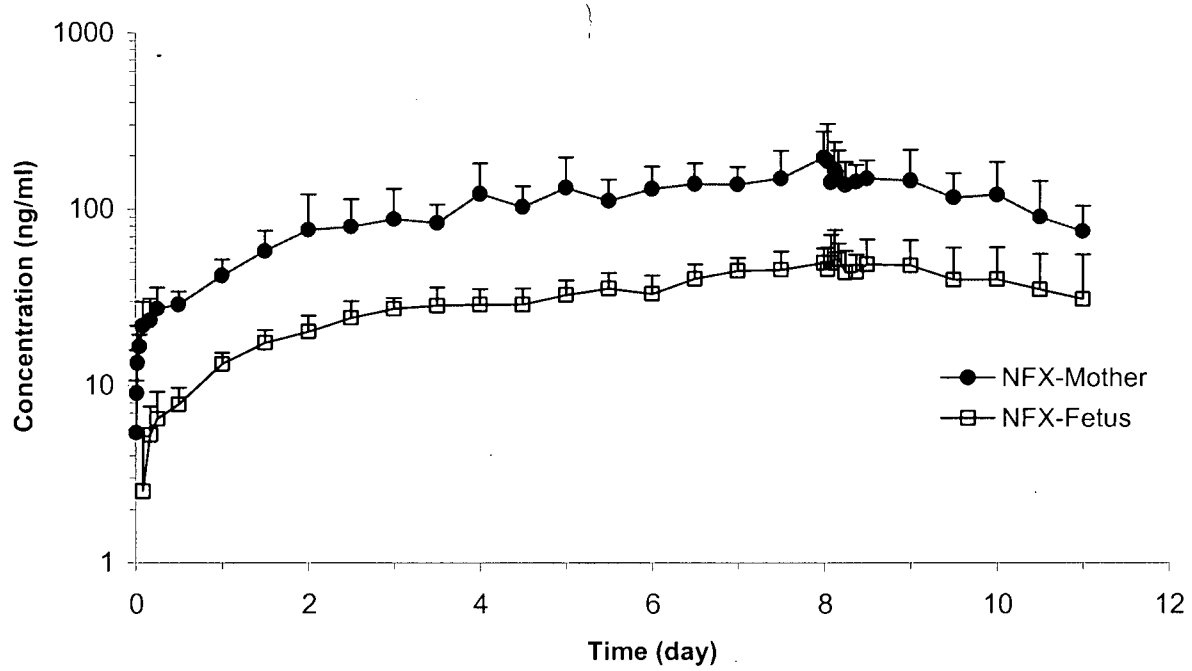


FIGURE 5.4 Average plasma concentration versus time profiles of total NFX in the mother and fetus (n = 5) following an 8-day maternal *i.v.* infusion of racemic FX, showing accumulation of NFX in the fetus with an average F/M AUC ratio of 0.33 ± 0.11.

had a similar value of 0.36 ± 0.05. Both the F/M ratio and combined F/M ratio did not differ significantly between R-FX and S-FX. For NFX, elimination seemed to be much slower in the fetus compared to the mother (see Table 4.2 in Chapter 4 for maternal data). Elimination $t_{1/2\beta}$'s for total NFX, R- NFX and S-NFX were 53.4 ± 21.0 h, 54.4 ± 20.9 h, and 52.5 ± 22.6 h, respectively. MRTs for total NFX, R-NFX and S-NFX were 94.8 ± 27.2 h, 94.5 ± 19.8 h, and 93.0 ± 30.1 h, respectively. MRT and $t_{1/2\beta}$ values for R- and S-NFX were not significantly different (p = 0.84). R- and S-NFX were equally distributed in the fetal plasma, with respective AUC values of 5148 ± 1975 μg*h/l and 5927 ± 2548 μg*h/l and an S/R ratio of 1.15 ± 0.18 (p = 0.13). Figure 5.4 shows the comparative average plasma concentration versus time profiles of total NFX in the mother and the fetus. Total NFX concentration was lower in the fetus compared to the mother with a mean F/M ratio of 0.32 ± 0.09. Interestingly, the F/M ratio for R-NFX was significantly lower than that of S-NFX (0.29 ±0.09 versus 0.36 ± 0.09; p<0.01) with an S/R ratio of 1.28 ± 0.18, suggesting a stereoselective transfer process in the maternal-fetal unit. Fetal exposure to total NFX was slightly lower compared to total FX, with a mean total NFX/FX AUC ratio of 0.83 ± 0.32. However, this ratio was significantly greater for R-NFX compared to S-NFX with respective values of 1.81 ± 0.60, and 0.56 ± 0.21 (p<0.01), resulting in a mean S/R ratio of 0.31 ± 0.07.

5.2.2 FX and NFX Levels in Amniotic and Fetal Tracheal Fluids

FX and NFX were also detected in amniotic (AMN) and fetal tracheal (TR) fluids. The pharmacokinetic parameters for both compounds in these fluids are summarized in Table 5.3. Figures 5.5 and 5.6 illustrate the average concentration-time profiles of total FX and NFX in amniotic fluid, fetal tracheal fluid and fetal arterial (FA) plasma, respectively.

TABLE 5.3 Pharmacokinetic parameters of FX and NFX in amniotic and fetal tracheal fluids following 8-day maternal infusion of racemic FX.

Animal Number	AMN $AUC_{0\text{-last}}$ (μg*h/l)			AMN/FA $AUC_{0\text{-last}}$ ratio			TR $AUC_{0\text{-last}}$ (μg*h/l)			TR/FA $AUC_{0\text{-last}}$ ratio		
	Total-FX	R-FX	S-FX	Total-FX	R-FX	S-FX	Total-FX	R-FX	S-FX	Total-FX	R-FX	S-FX
E7103	15271	5213	10058	0.80	1.38	0.66	23912	7539	16373	1.26	1.99	1.08
E7132	6489	2389	4100	0.56	1.12	0.44	16189	4784	11405	1.40	2.24	1.21
E5127	3073	1607	1466	0.35	0.78	0.22	6042	2617	3425	0.68	1.28	0.50
E7250	7918	2395	5523	0.34	0.57	0.29	23293	6298	16995	0.99	1.50	0.88
E4237	4002	1452	2550	0.66	0.84	0.59	6175	2290	3885	1.02	1.32	0.90
Mean ± S.D.	7351 ± 4830	2611 ± 1518	4739 ± 3347	0.54 ± 0.20	0.94 ± 0.31	0.44 ± 0.19	15122 ± 8770	4705 ± 2279	10417 ± 6544	1.07 ± 0.28	1.67 ± 0.43	0.91 ± 0.27
S/R ratio	-	1.72 ± 0.51		-	0.47 ± 0.16		-	2.05 ± 0.55		-	0.55 ± 0.10	
Paired t-test R- vs. S-FX *p*-value		0.07			<0.01			<0.05			<0.01	
Animal Number	Total-NFX	R-NFX	S-NFX	Total-NFX	R-NFX	S-NFX	Total-NFX	R-NFX	S-NFX	Total-NFX	R-NFX	S-NFX
E7103	5575	3484	2091	0.78	1.08	0.53	14075	8275	5800	1.96	2.57	1.46
E7132	4629	2619	2010	0.51	0.62	0.42	13798	8303	5495	1.53	1.97	1.15
E5127	2197	1168	1029	0.29	0.38	0.23	6465	3598	2867	0.86	1.18	0.65
E7250	5151	2742	2409	0.43	0.50	0.37	15146	8359	6787	1.26	1.51	1.04
E4237	4968	3099	1869	0.76	0.91	0.60	7356	4538	2817	1.13	1.34	0.91
Mean ± S.D.	4504 ± 1334	2623 ± 880	1882 ± 516	0.56 ± 0.21	0.70 ± 0.29	0.43 ± 0.14	11368 ± 4112	6615 ± 2348	4753 ± 1809	1.35 ± 0.42	1.71 ± 0.56	1.04 ± 0.30
S/R ratio	-	0.75 ± 0.14		-	0.64 ± 0.10		-	0.72 ± 0.08		-	0.61 ± 0.06	
Paired t-test R- vs. S-NFX *p*-value		<0.05			<0.05			<0.01			<0.01	

Note: Average values are expressed as mean ± standard deviation (S.D.)
Legend: AMN, amniotic fluid; TR, fetal tracheal fluid; FA, fetal arterial plasma; $AUC_{0\text{-last}}$, area under the curve from time zero to the last sampling time.

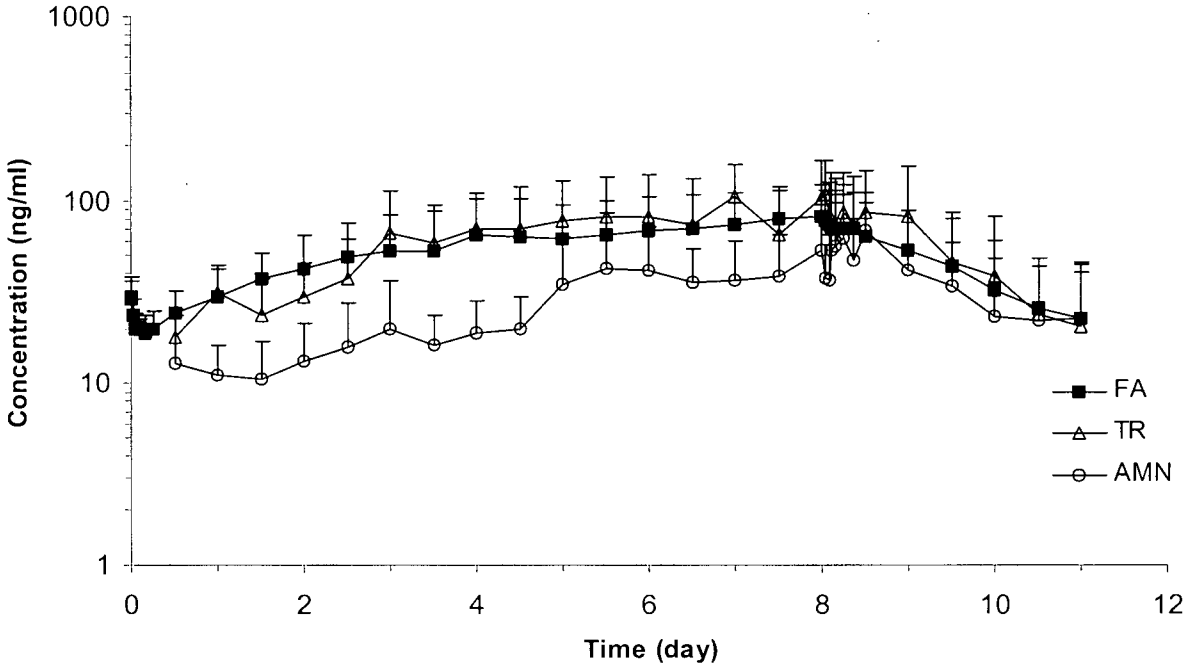


FIGURE 5.5 Average amniotic fluid (AMN), fetal tracheal fluid (TR) and fetal arterial plasma (FA) concentration versus time profiles of total FX in the fetus following an 8-day maternal *i.v.* infusion of racemic FX, showing comparable concentrations of FX in TR and FA but lower in AMN.

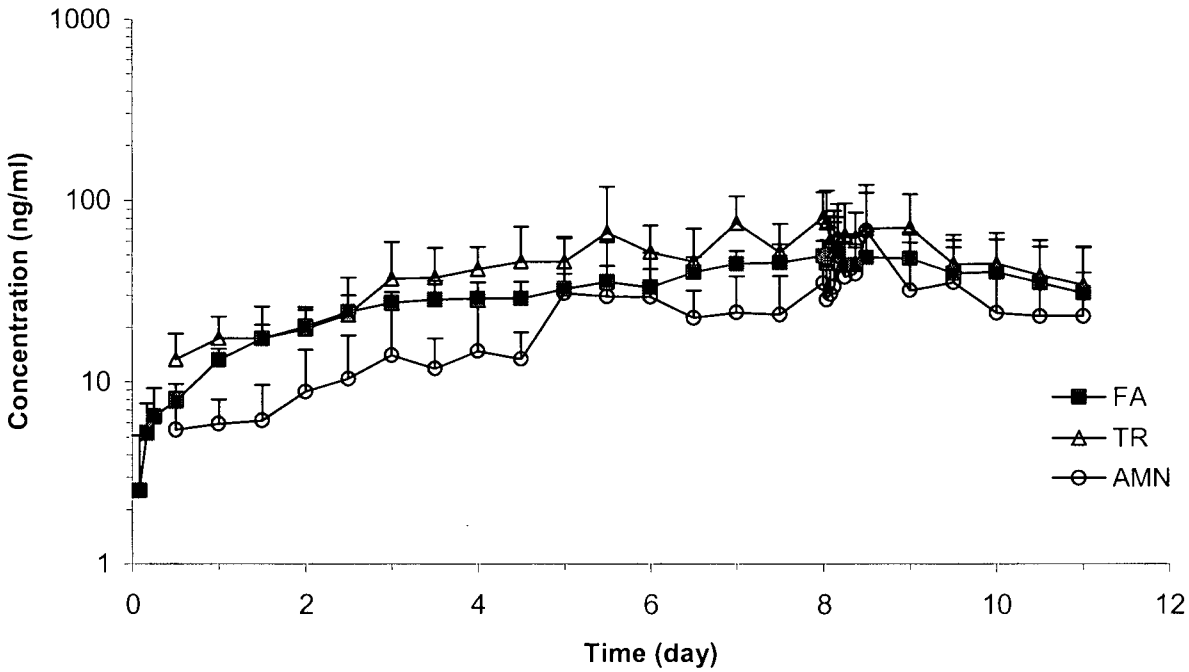


FIGURE 5.6 Average amniotic fluid (AMN), fetal tracheal fluid (TR) and fetal arterial plasma (FA) concentration versus time profiles of total NFX in the fetus following an 8-day maternal *i.v.* infusion of racemic FX, showing the degree of accumulation of NFX in the following order: TR ~ FA > AMN.

Throughout the infusion period, FX levels in amniotic fluid were lower than those in fetal plasma. The mean AUC in amniotic fluid for total FX was 7351 ± 4830 μg*h/l, which was about 50% of the AUC value in fetal plasma. The AUC of R-FX in amniotic fluid tended to be less than that of S-FX (2611 ± 1518 μg*h/l versus 4739 ± 3347 μg*h/l, S/R ratio = 1.72 ± 0.51) but the difference was not statistically significant (p = 0.07). The average amniotic fluid-to-fetal arterial plasma (AMN/FA) ratio for total FX was 0.54 ± 0.20. This ratio was significantly higher for R-FX compared to S-FX (0.94 ± 0.31 versus 0.44 ± 0.19; p<0.01), suggesting disproportional distribution of FX enantiomers in amniotic fluid. The extent of distribution of NFX in amniotic fluid was comparable to FX. The mean AUC in amniotic fluid for total NFX was 4504 ± 1334 μg*h/l, which was about half of that in fetal plasma. The AUC of R-NFX in amniotic fluid was significantly higher than that of S-NFX (2623 ± 880 μg*h/l versus 1882 ± 516 μg*h/l; p<0.05), with an average S/R ratio of 0.75 ± 0.14. This is an interesting finding because S/R ratios for the NFX enantiomers approximated unity in both maternal and fetal plasma. The average AMN/FA ratio for total NFX was 0.56 ± 0.21. Similar to FX, this ratio was significantly higher for R- compared to S-NFX (0.70 ± 0.29 versus 0.43 ± 0.14; p<0.05) with the S/R ratio averaging 0.64 ± 0.10. A representative amniotic fluid concentration versus time profile for the FX and NFX enantiomers is depicted in Figure 5.7.

Concentrations of FX in tracheal fluid were higher than those in amniotic fluid and comparable to fetal plasma. The mean AUC in fetal tracheal fluid for total FX was 15122 ± 8770 μg*h/l, which did not differ from that in fetal plasma. The AUC of R-FX in fetal tracheal fluid was significantly lower than that of S-FX (4705 ± 2279 μg*h/l versus 10417 ± 6544 μg*h/l; p<0.05), with an average S/R ratio of 2.05 ± 0.55. The average fetal tracheal

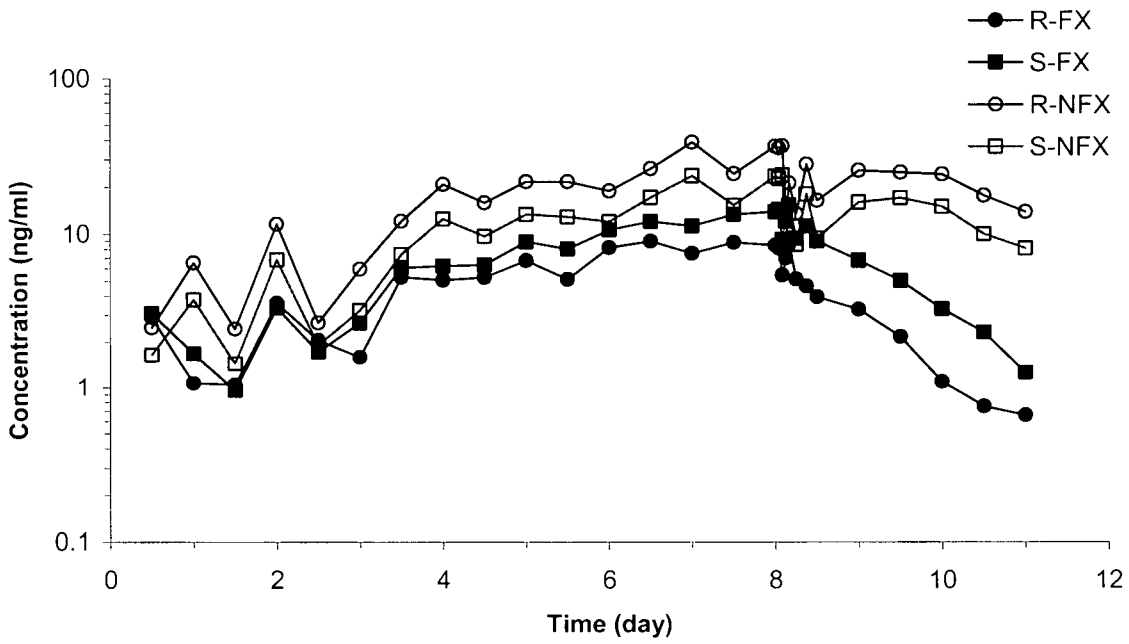


FIGURE 5.7 Representative amniotic fluid concentration versus time profiles of FX and NFX enantiomers in E4237 following an 8-day maternal *i.v.* infusion of racemic FX, showing stereoselective disposition of both FX and NFX enantiomers in the amniotic fluid.

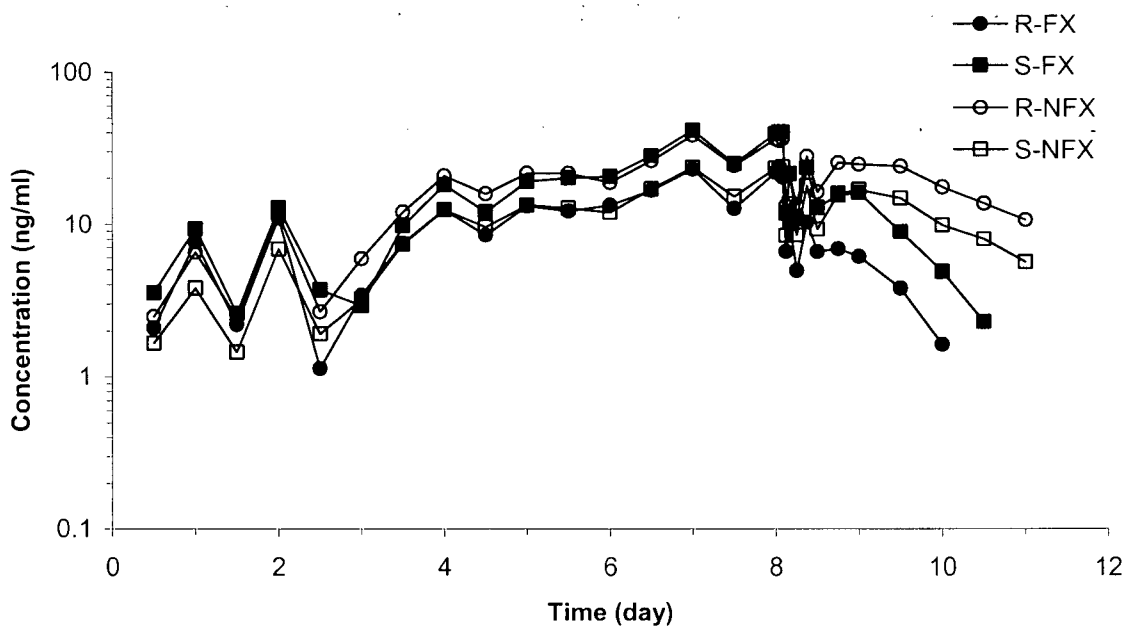


FIGURE 5.8 Representative fetal tracheal fluid concentration versus time profiles of FX and NFX enantiomers in E4237 following an 8-day maternal *i.v.* infusion of racemic FX, showing stereoselective disposition of both FX and NFX enantiomers in the fetal tracheal fluid.

fluid-to-fetal arterial plasma (TR/FA) ratio for total FX approximated unity (1.07 ± 0.28). Uneven distribution of the FX enantiomers in fetal tracheal fluid was observed, with the TR/FA ratio for R-FX being higher compared to S-FX (1.67 ± 0.43 versus 0.91 ± 0.27; $p<0.01$). The distribution of NFX in fetal tracheal fluid was comparable to FX, with mean AUC value for total NFX of 11368 ± 4112 μg*h/l. The AUC of R-NFX in fetal tracheal fluid was higher than that of S-NFX (6615 ± 2348 μg*h/l versus 4753 ± 1809 μg*h/l; $p<0.01$), with an average S/R ratio of 0.72 ± 0.08. This value was lower than the usual S/R ratios for the NFX enantiomers in either adult or fetal plasma. The average TR/FA ratio for total NFX was 1.35 ± 0.42. Similar to FX, this ratio was higher for R- compared to S-NFX (1.71 ± 0.56 versus 1.04 ± 0.30; $p<0.01$) with the S/R ratio averaging 0.61 ± 0.06. Representative fetal tracheal fluid concentration versus time profiles illustrating the stereoselective distribution of FX and NFX are shown in Figure 5.8.

5.3 Discussion

Kim, 2000, measured FX and NFX in cord (obtained from the umbilical cord at birth) and maternal venous plasma samples from 8 pregnant women who received a 10-30 mg oral dose of the drug daily during the course of pregnancy. The fetal FX concentration ranged from 7.9 to 94.3 ng/ml, with a mean of 45.4 ng/ml. These values are somewhat lower than those measured in fetal lambs in our current studies, which ranged from 40.4 to 110.9 ng/ml (mean = 76.1 ng/ml). On the other hand, fetal concentrations of NFX in human cord plasma ranged from 27.8 to 193.8 ng/ml (mean of 84.4 ng/ml), and were much higher than the concentrations measured in fetal sheep (range 29.4 to 92.4 ng/ml, mean = 49.5 ng/ml). When the concentrations of FX and NFX were added together, the combined concentration was

comparable between the human and sheep studies with mean values of 129.8 ng/ml and 125.6 ng/ml, respectively, indicating that they were exposed to the same levels of pharmacologically active moieties. This finding has significant implications in the interpretation of the physiological measurements that were collected in parallel with the current study (Morrison et al., 2001). In this report we demonstrated that maternal FX administration in pregnant sheep resulted in decreases in low voltage ECoG, REM and FBM in the fetal lambs. Postnatally, diminished weight gain following birth was also observed in these FX exposed lambs (Morrison et al., 2002). In two clinical studies, poor neonatal adaptation has been reported in human infants exposed to FX in the third trimester (Chambers et al., 1996; Cohen et al., 2000), and there is also a report of decreased facial and heart rate responses to painful stimuli in infants exposed prenatally to FX and other SSRIs (Oberlander et al., 2002). These postnatal effects could be due to alterations in fetal behavioral states, which are thought to be associated with normal brain development during the fetal and postnatal periods. Although the mechanisms underlying these effects elicited by FX and their relationships with drug concentrations remain to be elucidated, the comparable concentrations of the active moieties observed in the two species infers that similar fetal behavioral changes might occur in human fetuses exposed to FX.

The present study demonstrates rapid but limited maternal-fetal transfer of FX and NFX in pregnant sheep. Our F/M ratios for FX (0.40 ± 0.08) and NFX (0.32 ± 0.09) are slightly lower than the values determined previously from the maternal single *i.v.* bolus experiment (0.59 for FX and 0.63 for NFX) (Kim et al., 2004). In general, following a single dose of drug to the mother fetal exposure is substantially affected by placental permeability (Mihaly and Morgan, 1984). However, with multiple dosing or after steady-state infusion,

placental permeability will not determine fetal exposure, as the rate of placental drug transfer will not influence the final equilibrium achieved, but merely the time at which it is reached. The higher F/M AUC ratio obtained from the single *i.v.* bolus dose studies as opposed to steady-state infusion suggests that placental permeability does not seem to affect the transfer of FX and NFX. The transport of substances across the placenta may occur by simple diffusion, facilitated diffusion, active transport, and pinocytosis. For the transport of drugs, simple diffusion is usually the most important mechanism. This mode of transfer is largely dependent on the physicochemical properties of the drug. FX has a relatively low molecular weight and high lipid solubility, both of which favor placental transfer by this process. Elliott et al., 1994, reported a significant relationship by multiple regression between physicochemical properties (i.e. Log P, pKa, and MW) and the mean drug/antipyrine transport ratios obtained experimentally from an *in vitro* cotyledon human placenta model. However, in the *in vivo* situation the extent of placental transfer of drugs does not always appear to parallel their physicochemical properties as demonstrated by the data presented in Table 5.4. This table lists the physiochemical properties and F/M ratios of all therapeutic agents that have been studied in the pregnant sheep model as well as human data where available. The lipid solubility of the drug, as indicated by the octanol/water partition coefficient (Log P), is generally considered a good index of its ability to cross the placenta, i.e. the more lipid soluble the drug the more readily it should cross the placenta. However, as illustrated in Figure 5.9A, a plot of Log P versus F/M ratio based on the data from Table 5.4 does not reveal a significant correlation between lipophilicity and extent of transfer. For example, diphenhydramine and indomethacin have comparable log P values of 3.26 and 3.49, respectively, but there is a 20-fold difference in their F/M ratios (0.85 for diphenhydramine

TABLE 5.4 Literature data comparing the effect of physicochemical properties of various therapeutic agents on their degree of placental transfer.

Compound Name	Log P†	pKa (acid) †	pKa (base) †	Unionized fraction (%)†	Molecular Weight	F/M ratio (sheep)‡	F/M ratio (human) ‡	Reference
Acetaminophen	0.23	9.5	-	99.0	151.16	0.77	-	Wang et al., 1986
Aspirin (ASA)	1.22	3.5	-	0.0	180.16	0.40	-	Thiessen et al., 1984
Albendazole sulphoxide	1.91	9.7	5.2	98.9	281.33	0.46	-	Capece et al., 2002
Atosiban	-2.00	-	10.6	0.1	994.19	0.00	-	Greig et al., 1993
Atropine	1.53	-	9.9	0.4	289.37	1.00	-	Murad et al., 1981
Cimetidine	0.30	-	7.5	49.7	252.34	0.04	0.84	Mihaly et al., 1983; Howe et al., 1981
Cisapride	3.94	-	9.6	0.8	465.94	0.71	-	Veereman-Wauters et al., 1991
Cocaine	2.42	-	8.6	7.3	303.35	0.13	-	DeVane et al., 1991
Dermorphin analog (DALDA)	-2.00	9.9	13.7	0.0	611.73	0.00	-	Szeto et al., 1998
Diphenhydramine	3.26	-	8.8	4.7	255.35	0.85	-	Yoo et al., 1986
Ethanol	-0.07	-	-	100.0	46.07	1.00	-	Brien et al., 1985; Cumming et al., 1984
Fluoxetine	**4.84**	**-**	**10.6**	**0.1**	**309.33**	**0.40**	**0.65-1**	Kim et al., 2004; Heikkinen et al., 2003
Glycopyrrolate	3.95	-	-	0.0	318.43	0.13	-	Murad et al., 1981
Hexoprenaline	0.76	10.0	8.6	7.3	420.50	0.00	-	Lipshitz et al., 1981
Indomethacin	3.49	4.6	-	0.1	357.79	0.04	0.45	Harris et al., 1981; Lampela et al., 1999
Ketanserin	2.14	7.0	7.5	12.0	395.43	0.18	-	Schneider et al., 1996
Labetalol	2.24	8.3	9.2	1.7	328.40	0.14	0.50	Yeleswaram et al., 1992; Rogers et al., 1990
Lidocaine	3.06	-	7.9	28.2	234.34	0.76	0.50	Biehl et al., 1978; Banzai et al., 1995
Meperidine	2.18	-	8.4	11.1	247.33	0.30	0.83	Szeto et al., 1978; Rothberg et al., 1978
Methadone	4.47	-	8.6	7.3	309.44	0.14	0.14	Szeto et al., 1981; Doberczak et al., 1993
Methamphetamine	2.15	-	9.7	0.6	149.23	0.79	-	Burchfield et al., 1991
Metoclopramide	2.27	-	9.3	1.5	299.80	0.74	0.63	Riggs et al., 1990; Arvela et al., 1983
Midazolam	4.28	-	6.2	95.2	325.77	0.15	0.56	Vree et al., 1984; Kanto et al., 1983
Morphine	0.65	10.0	8.1	19.8	285.34	0.13	0.73	Szeto et al., 1982a; Gerdin et al., 1990
Nifedipine	1.12	-	4.0	100.0	346.33	0.59	0.76	Nugent et al., 1991; Pirhonen et al., 1990
Norfluoxetine	**4.42**	**-**	**10.6**	**0.1**	**295.30**	**0.32**	**0.72-1**	Kim et al., 2004; Heikkinen et al., 2003
Omeprazole	2.57	8.6	4.0	92.7	345.42	0.18	-	Ching et al., 1986
Propranolol	3.04	-	9.6	0.8	259.34	0.09	0.25	Mihaly et al., 1982b; Erkkola et al., 1982
Quinidne	2.29	-	8.6	7.3	324.42	0.10	0.82	Czuba et al., 1991; Hill & Malkasian, 1979
Quinine	2.29	-	8.6	7.3	324.42	0.15	0.32	Czuba et al., 1991; Phillips et al., 1986
Ranitidine	0.13	-	8.6	7.3	314.40	0.03	0.67	Mihaly et al., 1982a; Liu et al., 1985
Ridogrel	3.04	4.9	5.5	0.3	366.33	0.25	-	Schneider et al., 1999
Ritodrine	1.93	10.3	8.5	9.0	287.35	0.04	0.26	Fujimoto et al., 1984; Fujimoto et al., 1986
Salicylic acid	2.04	3.0	-	0.0	138.12	0.40	1.64	Thiessen et al., 1984; Levy et al., 1975
Sulphamethoxazole	0.86	5.6	1.8	1.3	253.28	1.00	-	Vree et al., 1983
Valproic acid	2.29	4.9	-	0.3	144.21	0.70	1.70	Kumar et al., 2000a; Nau et al., 1981
Verapamil	4.93	-	9.1	2.4	454.60	0.40	-	Murad et al., 1985

† log P, pKa, and unionized fraction (at physiological pH) were calculated by ADME Boxes, version 1.0, Pharma Algorithms, Toronto, ON, Canada.

‡ F/M ratios are calculated based on either C_{ss} or AUC; C_{max} is used in some single dose studies.

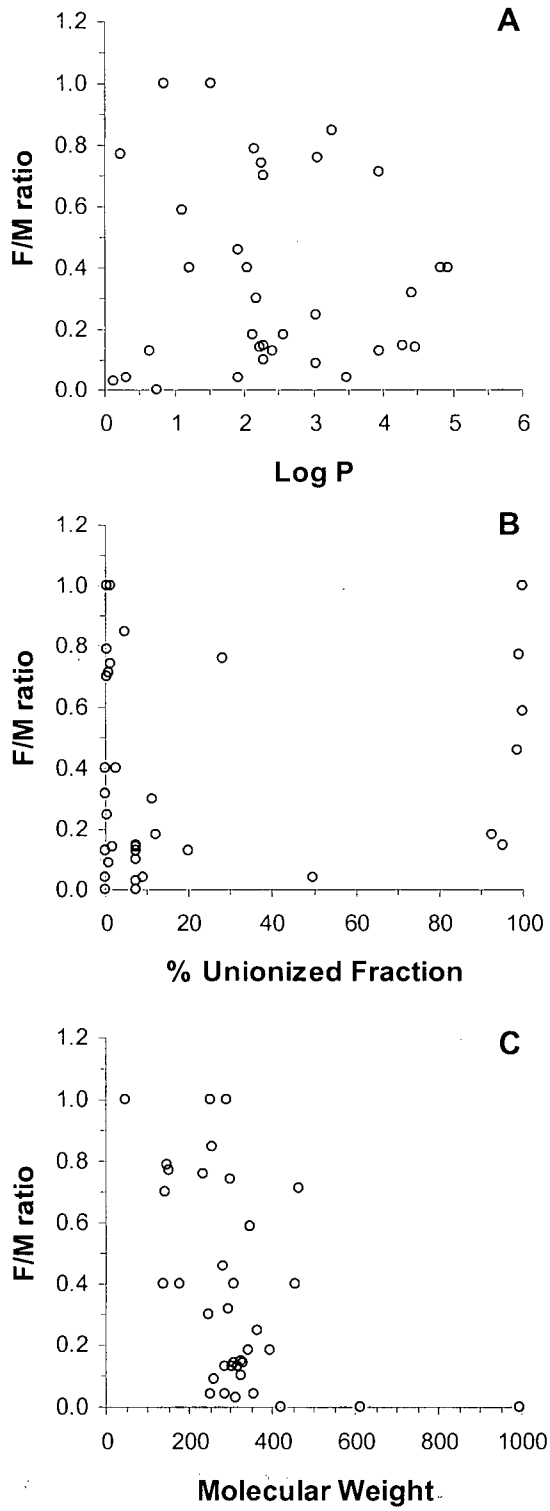
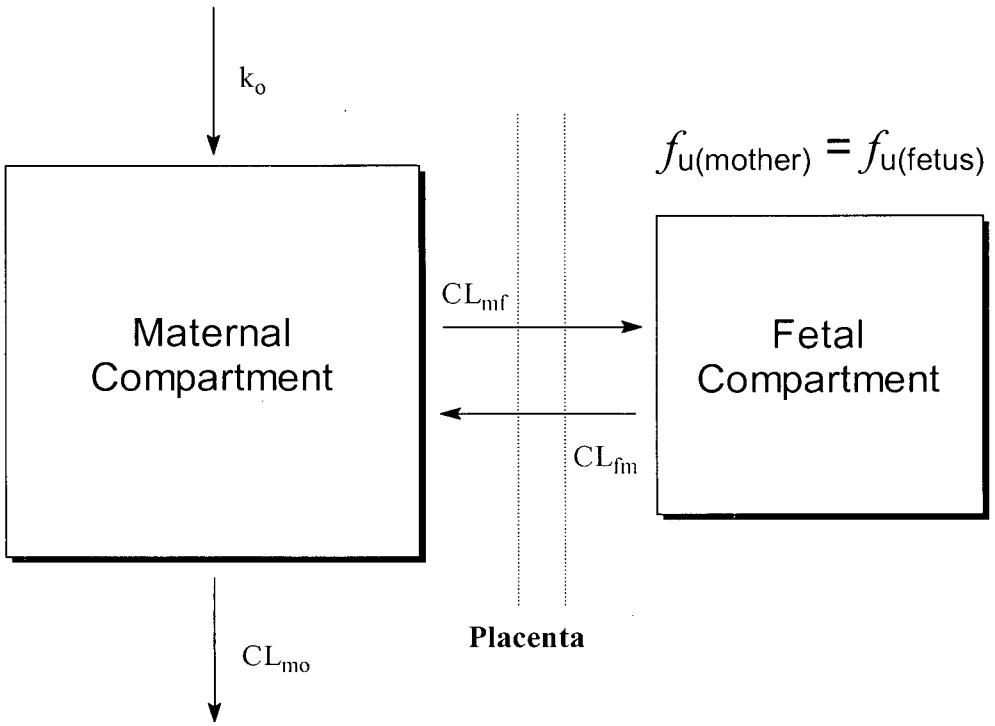


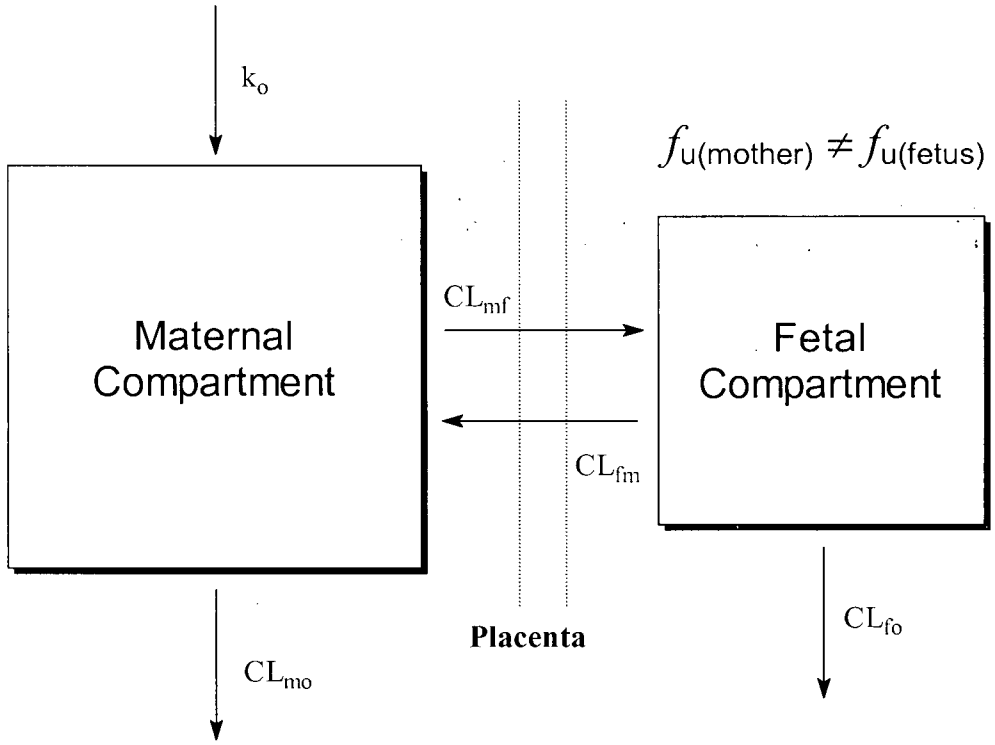
FIGURE 5.9 Placental transfer of therapeutic agents in pregnant sheep in relation to their physicochemical properties. Fetal-maternal exposure ratios (F/M ratio) were determined following maternal drug administration (see Table 5.4 for individual values). Plot A: log P vs. F/M ratio; Plot B: unionized fraction vs. F/M ratio; Plot C: molecular weight vs. F/M ratio. Only compounds with molecular weight of 100-600 are included in plots A and B.

and 0.04 for indomethacin) in sheep. The degree to which the drug is ionized, as determined by the pKa of the ionizable group, may limit the extent of drug transfer across placenta. Thus neutral species are expected to cross the placenta more efficiently while ionized species are less efficient for transfer. Biehl et al., 1978 have shown that with fetal acidemia, the F/M ratio of lidocaine, which has a pKa of 7.9 increased significantly from 0.76 to 1.21 because more drug was ionized and trapped in the fetal plasma; a mechanism known as "ion-trapping." Since fetal blood pH (~7.35) is slightly lower than maternal blood pH (~7.45), ion trapping can occur even under normal physiological conditions and contribute to the F/M gradient particularly for drugs that possess pKa values close to the physiological pH. However, many other drugs which have pKa values outside the range of normal physiological pH are either completely ionized or unionized in the plasma therefore ion trapping becomes less relevant in those situations. Figure 5.9B depicts the relationship between the percentage ionization of drug at physiological pH versus F/M ratio, showing that no apparent correlation exists between the two parameters. For example, FX and albendazole sulphoxide have unionized fraction of 0.1% and 98.9 % at physiological pH, respectively, but their F/M ratios do not differ significantly (0.40 versus 0.46, respectively). The sheep placenta has a distinctive molecular cut off at which it becomes a significant barrier to transfer for substances having a molecular weight (MW) > 600 Da (Faber and Thornburg, 1983; Rurak et al., 1991). Molecules with a MW less than 600 Da appear to cross the placenta freely but no correlation is observed between molecular size and the extent of transfer, i.e. a low MW ≠ a higher F/M ratio (Figure 5.9C). For example, FX and methadone have almost the same MW (~309 Da) but the F/M ratio of FX is 3-fold higher than that of methadone (0.40 vs. 0.14). Overall, these data have shown that the *in vivo* F/M

ratio is not merely a reflection of drug transfer across the lipid bilayer membrane of the placenta. Instead, it is a complicated physiological process within the maternal-fetal unit, which cannot simply be explained or predetermined by the physicochemical characteristics of the drug alone. For most drugs following maternal administration, fetal plasma concentrations are found to be lower than the maternal plasma concentrations at steady-state. Hill & Abramson, 1988, reported that in humans the degree of maternal and fetal protein binding is the most important factor in determining the F/M drug ratio at steady-state. When differential plasma protein binding of FX and NFX in the mother (see Chapter 4 for maternal f_u values) and the fetus (see Chapter 6 for fetal f_u values) is taken into consideration, the F/M ratio of unbound drug increases to 0.71 for FX and 1.0 for NFX. It appears that differential protein binding is sufficient to explain the maternal-fetal concentration differences for NFX but not for FX. Szeto et al., 1982b, use a two compartment pharmacokinetic model (Figure 5.10) to propose that the difference in fetal and maternal unbound steady-state concentrations could be due partly to fetal drug elimination, which is commonly denoted as fetal nonplacental clearance (CL_{fo}). Common routes of fetal drug elimination include: 1) fetal drug metabolism, 2) fetal renal excretion, 3) excretion in amniotic fluids, and 4) excretion in fetal tracheal fluid (Rurak et al., 1991). Preliminary experiments involving incubation of FX in ovine fetal hepatic subcellular fractions did not show any formation NFX from FX following a 30-min incubation (Kim et al., 2004). Moreover, direct *i.v.* bolus injection of FX to the ovine fetuses resulted in no detectable level of NFX in the fetal plasma (Kim et al., 2004). These findings suggest the absence of fetal metabolic capability to convert FX to NFX. In our infusion experiments, the presence of NFX in the fetal circulation and other fluids is probably due to the placental transfer of the preformed metabolite from the maternal



Case 1. $C_{ss(mother)} = C_{ss(fetus)}$ in the absence of fetal nonplacental clearance. Protein binding is assumed to be the same between the mother and the fetus.



Case 2. $C_{ss(mother)} > C_{ss(fetus)}$ in the presence of fetal nonplacental clearance (CL_{fo}) and difference in the protein binding between the mother and the fetus.

FIGURE 5.10 Compartmental model of the maternal-fetal unit with continuous drug input into the maternal compartment (Szeto et al., 1982b).

circulation. In adult sheep, hepatic metabolism is the major elimination route for FX whereas renal excretion accounts for less than 1% of the total body clearance. While the fetal liver is generally less developed for drug metabolism, studies have shown that the fetal kidney has the capacity for efficient renal tubular secretion of meperidine and cimetidine (Szeto et al., 1979; Mihaly et al., 1983). Thus, a compensatory mechanism might take place in the fetal kidney whereby renal excretion becomes the predominant factor for FX elimination rather than the liver, however further experiments are necessary to assess this possibility. Significant excretion of drug into the amniotic fluid could not account for the large gradient because FX and NFX concentrations were low in amniotic fluid, with the AMN/FA ratio averaging 0.54 and 0.56 for total FX and total NFX, respectively (Table 5.3). This is in agreement with findings in previous maternal *i.v.* bolus experiments where insignificant accumulation of FX and NFX in amniotic fluid was observed (Kim et al., 2004). A lack of accumulation in amniotic fluid has also been reported for several acidic drugs in sheep, such as valproic acid (Gordon et al., 1995), diphenylmethoxyacetic acid (DPMA), a diphenhydramine metabolite (Tonn et al., 1995), and indomethacin (Krishna et al., 2002). In contrast, most amine drugs including metoclopramide (Riggs et al., 1987), ritodrine (Wright et al., 1991), labetalol (Yeleswaram et al., 1992), and diphenhydramine (Kumar et al., 1997) have been found to accumulate substantially in amniotic fluid with concentrations approximately 4-15 times higher than those in fetal plasma. In spite of being amines, FX and NFX do not appear to follow these same general distribution characteristics in amniotic fluid. As fetal urine is a major source of amniotic fluid, low drug concentrations in amniotic fluid imply that fetal renal excretion of FX and NFX might also be low. Intra-membranous pathways are also important in fluid and solute exchange between amniotic fluid and fetal

blood (Gilbert & Brace 1989). However, the low amniotic concentrations of FX and NFX suggest that this pathway is of minor importance. Protein binding may also be a factor in determining the distribution of drugs in amniotic fluid. The protein binding of FX and NFX in pooled fetal plasma following single *i.v.* bolus administration to the fetus ranged from 90-94% (Kim et al., 2004). Indomethacin and DPMA are also both highly protein bound in fetal plasma (~98%) and exhibit low concentrations in amniotic fluid (Anderson et al., 1980a; Kumar et al., 1999a).

FX and NFX were detected in fetal tracheal fluid at levels similar to those in fetal plasma. This is in contrast to the results for most amine drugs such as diphenhydramine, metoclopramide, labetalol and ritodrine, which are present in high concentrations in tracheal fluid (ranging 2- to 15-fold higher than fetal plasma) following either maternal or fetal drug administration (Riggs et al., 1987; Wright et al., 1991). The reason for the difference between FX and the other basic lipophilic drugs studied is unclear. There is extensive accumulation of FX and other basic lipophilic psychotropic drugs in the rat lung via lysosomal trapping and phospholipid binding (Daniel and Wöjcikowski, 1997). The former process involves diffusion of the non-ionized form of the drug into the acidic interior of lysosome where protonation of the drug occurs, preventing efflux from the lysosome. As FX is more lipophilic than the other basic drugs that have been studied, it may be that lysosomal trapping is more important for this compound resulting in limited efflux and accumulation in lung fluid. However, to determine if this might be a factor, measurement of FX in subcellular components of the lung in comparison to other drugs is necessary.

The possibility of placental drug metabolism in addition to fetal drug elimination should be considered. Several drug biotransformation processes have been identified in

animal and human placentas, but the overall role of the placenta in xenobiotic metabolism still remains unclear (Juchau, 1980; Pasanen, 1999). As such, the relative importance of placental compared to fetal elimination of FX requires further investigation.

Active transport of FX from fetus to mother could theoretically be responsible for the unbound drug gradient from mother to fetus. Specific transporters are found in placenta that facilitates the transfer of nutrients and other endogenous substances at the maternal-fetal interface (Ganapathy et al., 2000). Specificity of these transporters in the placenta is not strictly restricted to endogenous substances. Xenobiotics that bear significant structural similarity to these substrates have the potential to be recognized by the transporters expressed in the placenta. Thus placental transporters could play a crucial role in the distribution of drugs across the maternal-fetal interface. Numerous transporters have been identified in placental tissues with P-glycoprotein (P-gp) the most extensively studied in terms of drug disposition (von Moltke and Greenblatt, 2000; Lin and Yamazaki, 2003). P-glycoprotein-mediated transport occurs unidirectionally facilitating the efflux, and not the influx of various substances. This drug efflux transporter appears to have evolved as a protective mechanism in animals against the potential toxic effects of environmental toxins and other xenobiotics. Animal studies with P-gp-knockout mice have provided strong supporting evidence for an important role of placental P-gp in protecting the fetus from potentially harmful and therapeutic agents (Lankas et al., 1998; Smit et al., 1999). Intravenous administration of the P-gp substrates digoxin, saquinavir, and taxol to pregnant animals led to a 7-fold greater transfer of these drugs across the placenta into the fetus in P-gp knockout mice than in wild-type type mice. An *in vitro* placental transfer study of glyburide, which is a P-gp substrate, showed that the drug only minimally crossed the human placenta (Elliott et al., 1991). P-gp

was proposed to be the underlying mechanism as neither its pKa, solubility nor molecular weight precludes it from being able to cross the placenta. A similar observation was made for midazolam, which is also a P-gp substrate and exhibits a low degree of placental transfer in humans (Tolle-Sander et al., 2003; Kanto et al., 1983). Recent studies regarding the interactions between antidepressants and P-gp showed that FX and NFX are moderate P-gp inhibitors, suggesting the presence of substrate-recognition between P-gp and FX (Weiss et al., 2003). However, conflicting results were observed in a blood-brain-barrier uptake study where the brain-to-tissue distribution ratios of FX and its metabolites were indistinguishable between P-gp knockout mice and wild-type mice (Uhr et al., 2000). The expression of P-gp in the sheep placenta has not yet been confirmed, therefore it is difficult to attribute any of our findings to P-gp at present. It is clear that further studies are indicated to examine the role of P-gp in the placental transfer of FX.

Our F/M ratios in sheep for FX and NFX were much lower than the cord blood/maternal venous plasma concentration ratio reported in humans with the latter approximating unity (Kim, 2000). This could be due to one or more of the following reasons. First, the epitheliochorial sheep placenta differs from the hemochorial human placenta in having a lower permeability to hydrophilic polar substances (Faber and Thornberg, 1983). Second, the human data are based on a single point determination of the fetal-to-maternal drug concentration ratio, which changes constantly with respect to the time of dosing in non-steady-state conditions (Anderson et al., 1980b). Third, the extensive maternal and fetal hemodynamic changes that occur during and immediately after delivery may affect the disposition of the drug in the mother and/or the fetus (Hamshaw-Thomas et al., 1984) and hence extrapolation of the concentration ratio at delivery to the antepartum

intrauterine conditions may be erroneous. The last two points are exemplified in a recent study by Heikkinen et al., 2003, who reported a different newborn-to-maternal plasma concentration ratio for FX and NFX. They reported a mean F/M ratio of 0.65 for FX and 0.72 for NFX, which are about 30-40% lower compared to those reported previously by Kim (2000). Discrepancies in these results indicate that the single point determination of fetal-to-maternal drug concentration ratios in humans or animal species is subject to high variability. In addition, from a pharmacokinetic point of view the extent of fetal exposure to a drug is more accurately reflected by the F/M AUC ratio than by a comparison of fetal and maternal plasma drug concentrations at any time, t, after drug administration. Although care should be exercised in extrapolating results from animals to the clinical situation, our data indicate that the fetus could be exposed to upwards of 70-100% of the concentrations of the pharmacologically active form (i.e. unbound drug) of FX and NFX present in the maternal plasma.

The placental transfer of several compounds has been studied in both sheep and humans (Table 5.4), but no one has attempted to explore the correlation between the two species in this regard. On this basis, we decided to investigate the possibility of extrapolating the F/M ratio determined in sheep to that in humans. A total of nineteen compounds for which placental transfer in both sheep and human has been studied were used to test for a correlation. In our initial trial no correlation was found as illustrated in Figure 5.11, plot A. Attempts were made to identify outliers from the original data set based on the following two criteria: 1) human-to-sheep F/M ratio ≤ 1 (based on the presumption that the sheep placenta cannot be equally or more permeable to drugs compared to the human placenta); 2) human-to-sheep F/M ratio $> Q3 + (Q3-Q1) \times 1.5$, where Q1 indicates the bottom quartile and Q3 the top quartile of the human-to-sheep F/M ratio (Zar, 1996; Ogawa et al., 2003). Q1, Q3, and

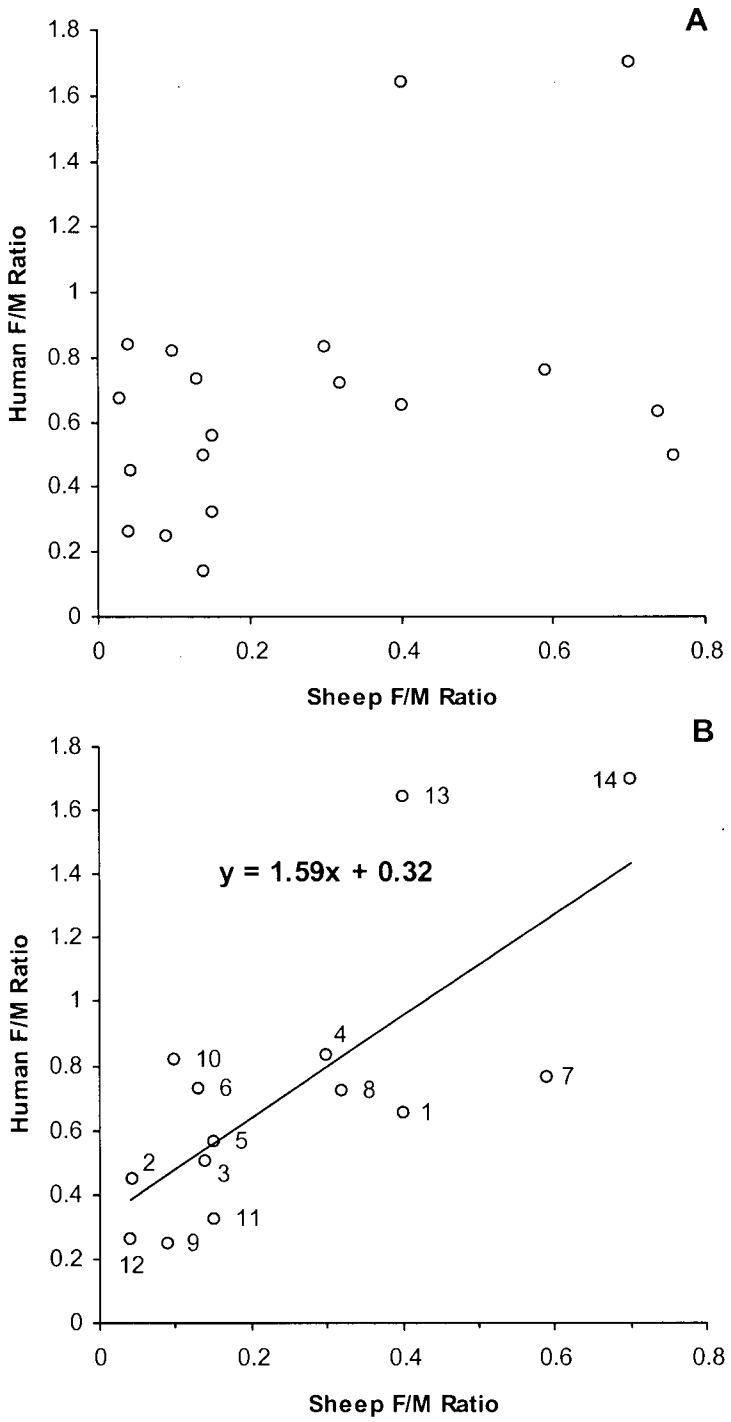


FIGURE 5.11 Relationship of F/M ratios between sheep and human. In Plot A, no correlation is observed when all 19 compounds are included for analysis (Table 5.4). In Plot B, significant linear correlation was observed between sheep and human values with 14 compounds when outliers are excluded ($p<0.01$, Spearman rank correlation). Compounds that fit the correlation include (1) fluoxetine, (2) indomethacin, (3) labetalol, (4) meperidine, (5) midazolam, (6) morphine, (7) nifedipine, (8) norfluoxetine, (9) propranolol, (10) quinidine, (11) quinine, (12) ritodrine, (13) salicylic acid, and (14) valproic acid.

the upper outlier limit are determined as 1.6, 6.5, and 13.8, respectively. Using these criteria, methadone, lidocaine, metoclopramide, cimetidine, and ranitidine were identified as outliers. Upon exclusion of these outliers, a second analysis was performed and the result revealed a significant linear relationship for the F/M ratio between sheep and humans as illustrated in Figure 5.11B (r_s = 0.69; $p< 0.01$, Spearman rank correlation). When subject to linear regression analysis, the data set (outliers excluded) yielded the following regression equation: $y = 1.59x + 0.32$ ($r^2 = 0.54$; $p< 0.01$ when H_o: $\beta=0$, but not statistically significant when H_o: $\beta=1$*). This regression equation tends to predict a higher F/M ratio in human with respect to the value in sheep, which is not illogical considering the placental structure in the two species. However, factors that influenced the correlation remain to be explored. Variability in human studies could be a causal factor accounting for some of the deviations. Empirically, drugs that follow the correlation might share similar physicochemical and ADME (absorption, distribution, metabolism and elimination) properties but this hypothesis requires further testing.

Extensive pharmacokinetic analysis of the fetal FX concentrations was not attempted in our current study because the bidirectional placental flux of FX as well as fetal nonplacental clearance could not be calculated from the available data. The significantly longer apparent elimination $t_{1/2\beta}$ of FX and NFX in the fetus when compared to the ewe could be due to a small fetal-to-maternal transplacental clearance of the drug. It may be also be related to the extensive binding of FX to and consequent slow release from proteins and/or tissues in the fetus. Reduced drug elimination capacity in the fetus or recirculation of FX from the amniotic fluid compartment back to the fetus could also be an additional factor. In a

*H_o = null hypothesis, β = slope of the regression line

clinical case study, Heikkinen et al., 2003, reported that FX and NFX levels in newborns at 4 days post-delivery were about 60-80% of the levels at birth. The mothers of these infants had received oral dose of FX for at least 2 weeks before delivery. In the present study we observed that FX and NFX were excreted in amniotic and tracheal fluids and declined at a rate similar to that in fetal plasma (Figures 5.5 and 5.6). This indicates the rate-limiting step of drug elimination in fetal plasma could reside in these fluid compartments. Similar observations have been made with labetalol and metoclopramide in pregnant sheep following maternal administration (Riggs et al., 1988; Yeleswaram et al., 1992). The elimination $t_{1/2}$ of labetalol was comparable among fetal plasma, amniotic fluid and fetal tracheal fluid, but longer compared to maternal plasma (Yeleswaram et al., 1992). For metoclopramide the rate of elimination was substantially slower in the fetus compared to the mother with the drug accumulating and persisting longer in the amniotic and tracheal fluids (Riggs et al., 1988).

Stereoselectivity in placental drug transfer is poorly characterized, partly due to the limited number of chiral compounds that have been studied in this regard. In the present study, stereoselectivity in the F/M ratio was observed. The F/M ratio for R-NFX was significantly lower than that for S-NFX (0.29 vs. 0.36) whereas no difference was observed between R-FX and S-FX (0.39 vs. 0.41). The 25% higher F/M AUC ratio for S-NFX compared to R-NFX indicating that NFX distributed between the mother and the fetus in a stereoselective manner. A clinically important consideration for this difference in F/M AUC ratio lies in the fact that S-NFX is 20-times more active than R-NFX in terms of serotonin reuptake inhibition, and hence the fetus is actually exposed to more pharmacological active species than one would normally predict based on racemic data. A similar finding was observed for albendazole sulphoxide (ABZSO), which is a racemic mixture used as an

anthelmintic drug in veterinary medicine. Studies with pregnant sheep showed that the AUC ratio between mother and fetus for (+)-ABZSO and (−)-ABZSO was 2.5 and 1.4, respectively (Capece et al., 2002). In the maternal plasma, the AUC ratio between (+)-ABZSO and (−)-ABZSO was 2.8 whereas in the fetal plasma the ratio decreased to 1.6. In our experiments we also observed a difference in the S/R AUC ratio of NFX enantiomers between the mother (0.91, Table 4.2) and the fetus (1.15, Table 5.2). As discussed earlier, differential protein binding between enantiomers could affect the concentration gradient between the mother and the fetus. When S/R AUC ratio is corrected for the free drug, the ratio becomes 0.65 for the mother and 0.91 for the fetus, which results in a corresponding F/M ratio of 0.94 for R-NFX and 1.32 for S-NFX. These results suggest that protein binding alone is not sufficient to explain the stereoselective difference in F/M ratios. Other mechanisms must, therefore, be in effect to cause the difference in disposition of NFX enantiomers between the mother and the fetus. Drug levels in the fetus are affected by both placental and fetal nonplacental clearances. These clearance processes could occur in a stereoselective manner. We observed that the excretion of NFX in amniotic and fetal tracheal fluid showed significant preference for R-NFX compared with S-NFX ($p<0.05$). This is in contrast to what we observed in adult plasma where the S/R NFX AUC ratio approximated unity. The higher excretion of R-NFX into these fluids corresponds with a lower fetal plasma level of R-NFX, resulting in a smaller F/M AUC ratio for R-NFX compared to S-NFX. The mechanism of stereoselectivity behind these excretion processes is unclear. Drug entry into amniotic fluid is mainly through renal excretion, therefore stereoselective renal excretion could be involved, but further studies are necessary to examine this possibility. The difference in F/M ratio between enantiomers might also suggest an enantioselective transport through the placental membranes. As we

have discussed earlier, P-gp is expressed in placental tissues and plays an important role in controlling drug transfer across placenta. The 3-dimensional interaction between protein and substrate allows for the possibility that one enantiomer will be a better substrate than the other. As such, there may be a difference in efflux between enantiomers. Little regard has been paid to stereoselective placental transfer as most drugs cross the placenta by simple diffusion. Since enantiomers have the same physicochemical properties the degree of placental transfer should be the same. However, more research is now emerging to indicate that the role of drug transporters in this process is more important than originally perceived (Ganapathy et al., 2000; Young et al., 2003). It is clear then, that further studies examining the stereoselective aspects of drug transporters and their relationships with placental drug transfer and fetal drug disposition should be emphasized.

5.4 Summary

In summary, we have found moderate placental transfer of FX and NFX in pregnant sheep. Physicochemical properties of the drug alone cannot provide a satisfactory explanation for the concentration gradient between the maternal and fetal circulations. The protein binding corrected F/M AUC ratio for FX has a value of less than unity, suggesting the presence of fetal nonplacental FX elimination. FX and NFX were excreted into amniotic and fetal tracheal fluids with levels not exceeding those in fetal plasma. There are possibilities for fetal renal excretion, active placental transport, and placental and fetal metabolism, although previous *in vivo* and *in vitro* experiments suggest that the later two elimination mechanisms are probably of minor significance. The extent of placental transfer in sheep is less than that in humans probably due to the permeability characteristics of the sheep placenta. An empirical correlation for F/M ratios between sheep and human was

derived from several compounds that were studied for their placental transfer in both species, however this relationship requires further testing. Stereoselectivity in the F/M ratio was observed for NFX. Factors causing the difference in S/R ratio between the mother and the fetus could involve stereoselective fetal drug elimination (e.g. renal excretion) and stereoselective carrier mediated transfer (e.g. P-glycoprotein).

CHAPTER 6

DISPOSITION OF FX AND NFX IN FETAL LAMBS UNDER STEADY-STATE CONDITIONS

Preface

In a previous study conducted in our lab with direct *i.v.* bolus injection of FX to fetal lambs, no NFX was found in fetal plasma (Kim et al., 2004). This finding suggests that the fetus may be incapable of eliminating FX by metabolism. In human newborns from mothers who received FX during pregnancy, the rate of elimination of FX was found to be much slower compared to the adult, suggesting that fetal elimination of FX is impaired during the perinatal period (Heikkinen et al., 2003). In the present study, racemic FX was administered to the fetus by infusion to allow further investigation of the fetal disposition of FX and NFX under steady-state conditions. Moreover, the stereoselective pharmacokinetics of FX and NFX and the underlying mechanisms were examined in the fetus. Fetal urine was collected to determine the role of renal elimination in the fetal nonplacental clearance of FX and NFX. Comparative analyses of pharmacokinetic parameters in fetal, maternal and nonpregnant adult sheep were performed to assess the ontogeny of FX and NFX pharmacokinetics. Finally, computations of maternal and fetal placental and nonplacental clearances using the method described by Szeto et al., 1982b, which utilizes data from both maternal and fetal infusion experiments, were attempted to estimate the contribution of fetal nonplacental clearance to the overall clearance of the drug in the maternal-fetal unit.

6.1 Methods

6.1.1 Animal and Surgical Preparation

Five pregnant ewes (Dorset and Suffolk breeds) at gestational age ranging from 119 to 126 days and term ~145 days were used in these experiments. The study was approved by University of British Columbia Animal Care Committee and the procedures performed on sheep conformed to the guidelines of the Canadian Council on Animal Care. The surgical procedure was similar to that described previously in Chapter 5, Section 5.11 except that brain electrodes and blood flow transducers were not implanted. In addition, the fetal bladder was cannulated through a suprapubic incision to allow fetal urine collection. After surgery, ampicillin of 500 mg was administered intramuscularly for 3 days to the ewe, and via the tarsal vein to the fetus at the time of surgery and daily into the amniotic fluid for the duration of the preparation. A minimum of 3 days were allowed for the ewes to recover before experimentation.

6.1.2 Experimental Protocol

A preliminary experiment was performed in one pregnant sheep to determine the appropriate infusion rate to achieve the desired steady-state concentration. A step-wise fetal *i.v.* infusion experiment at two different rates (k_o) was performed (k_o = 2.1 mg/h for step 1 and 4.2 mg/h for step 2) for the purpose of achieving a target FX concentration of 50-100 ng/ml. Each infusion step lasted for 4 days. Figure 6.1A is a schematic illustration of the experimental protocol used for this preliminary 2-step fetal infusion study. Serial blood samples were collected from both the fetus and the mother over the entire course of the infusion. Cumulative fetal urine samples were collected every 12 hours until the end of the

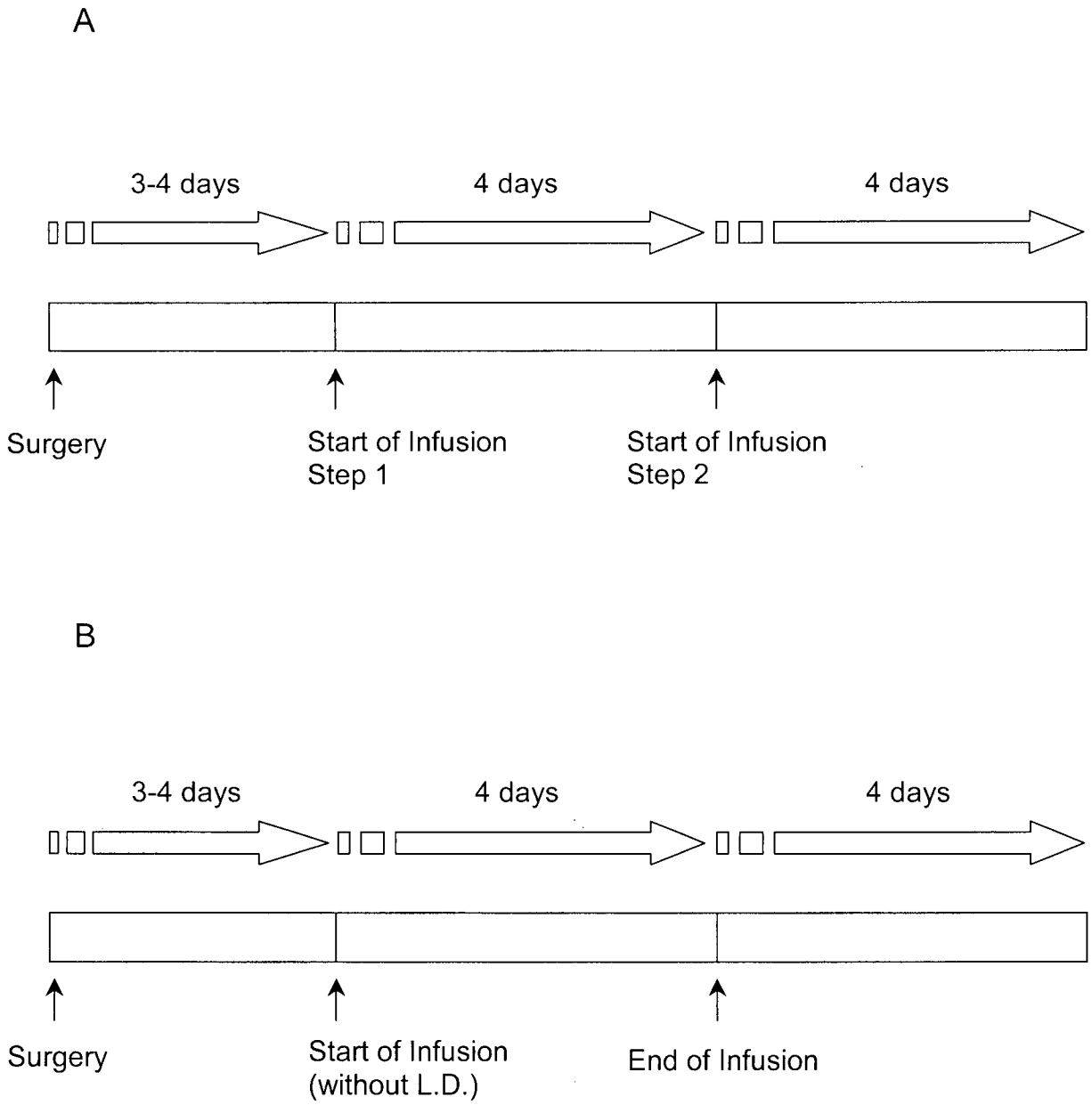


FIGURE 6.1 Schematic diagram of the experimental protocol used for (A) the preliminary 2-step fetal infusion study, where infusion rate at step 2 is double the rate of step 1; (B) the 4-day fetal infusion study (L.D. = loading dose) followed by a 4-day post-infusion period.

infusion. Figure 6.1B is a schematic illustration of the final experimental protocol selected for the fetal infusion studies based on the targeted FX plasma concentration. Each animal received a continuous *i.v.* infusion of racemic FX HCl via the fetal tarsal vein for 4 days to achieve steady-state concentrations. Serial blood samples from the maternal and fetal femoral arteries (5 ml) were collected at 0, 15, 30, 45 min, 1, 2, 4, 6, 12, 24, 36, 48, 60, 72, 75, 78, 81, 84, 96 h during the infusion, and at 1, 2, 3, 4, 6, 9, 12, 18, 24, 27, 30, 36, 48, 51, 60, 72, 75, 84, and 96 h post-infusion. The zero time samples were collected within 10 minutes prior to starting the drug infusion. The volume of fetal blood collected was replaced by an equal volume of drug-free maternal blood at intervals during the study. Fetal urine samples were collected immediately before dosing, then at 4 h intervals from 73 to 77 hours during the infusion, and from 1 to 5, 7 to 11, 25 to 29, 49 to 53, 73 to 77 hours after the discontinuation of the infusion.

All blood samples collected for drug and metabolite analysis were placed into K_3EDTA Vacutainer® tubes, gently mixed, and centrifuged at 2000g for 10 min to separate the plasma. The plasma supernatant was transferred into clean borosilicate test tubes with polytetrafluoroethylene (PTFE)-lined caps. The volume of fetal urine collected at each time interval was recorded and a 10 ml aliquot was taken and transferred into a clean borosilicate test tube for subsequent drug and metabolite analysis. The remaining urine was infused into the amniotic cavity. All samples were stored at -20°C until the time of analysis.

6.1.3 Physiological Recording and Monitoring Procedures

An aliquot of whole blood sample (~1 ml) was collected from the fetus pre-dose and at 15, 30, 45 min, 1, 2, 4, 6, 12, 24, 48, 72, and 96 h during the infusion (the same sampling

schedule repeated at the initiation of the second step of the preliminary 2-step infusion experiment), and at 24-hour intervals during the post-infusion period for blood gas, acid-base, electrolytes and glucose/lactate analyses. The equipment used for these measurements has been previously described in Chapter 2, Section 2.2.2.

6.1.4 Determination of FX and NFX Plasma Protein Binding

Plasma protein binding of FX and NFX was measured *ex vivo* using the equilibrium dialysis procedure as described in Chapter 3, Section 3.1.3, except that the dialysis of fetal plasma was performed at a temperature of 39.5°C, the physiological temperature of fetal lamb. Plasma unbound fraction (f_u) was calculated as described in Chapter 3, Section 3.1.3, Equation 3.1.

6.1.5 Pharmacokinetic Analysis

All pharmacokinetic parameters were calculated using standard pharmacokinetic procedures as described in Chapter 3, Section 3.1.4. The fetal-to-maternal exposure ratio (F/M AUC ratio) was calculated the same way as described in Chapter 5, Section 5.1.4. Fetal renal clearance (CL_{renal}) was determined from the slope of the plot of cumulative drug excreted in the urine from t_n to t_{n+1} versus AUC between the same collection intervals. Fetal weight at the time of experiment was estimated using the following equation for fetal ovine growth described by Gresham et al., 1972:

$$\log \text{intrauterine weight (g)} = \log \text{birth weight (g)} - 0.0153*(\Delta t)$$

where Δt is the time difference, in days, between the day of delivery and the day of experiment.

6.1.6 Statistical Analysis

A paired t-test was used for statistical analysis of the pharmacokinetic parameters when comparing R- versus S-enantiomers. Comparative analyses of the pharmacokinetic parameters obtained from the fetal, maternal, and nonpregnant adult sheep were performed using one-way ANOVA and post hoc Tukey's tests. The achievement of steady-state for plasma FX and NFX concentrations was evaluated by testing whether the slope of plasma concentration versus time curve was significantly different from the value of zero. The significance level used was $p<0.05$ in all cases. All pharmacokinetic data are reported as the mean ± standard deviation (S.D.). Fetal blood gas data were analyzed using repeated measures ANOVA followed by post hoc Tukey's tests to determine the effect of time on changes in measurements from pre-infusion values. All physiological data are presented as mean ± standard error (S.E.).

6.2 Results

Mean fetal birth weight averaged 2945 ± 340 g and the mean time between the day of delivery and the day of the experiment 15.5 ± 1 days, therefore the estimated average intrauterine weight at the time of experiments was 1707 ± 219 g. Figure 6.2 shows the fetal plasma concentration versus time profiles of total FX and NFX from the preliminary 2-step infusion experiment. FX reached steady-state at approximately 1-2 days after the initiation of each infusion step. The mean C_{ss} of total FX during the step 1 and step 2 infusions were 40.2 ± 5.4 ng/ml and 101.7 ± 2.3 ng/ml, respectively. The lack of proportionality between infusion rate and C_{ss} (i.e. 2 times k_o resulted in 2.5 times increase in C_{ss}) could be related to the nonlinear profile of FX pharmacokinetics discussed earlier in Chapter 3. In contrast to

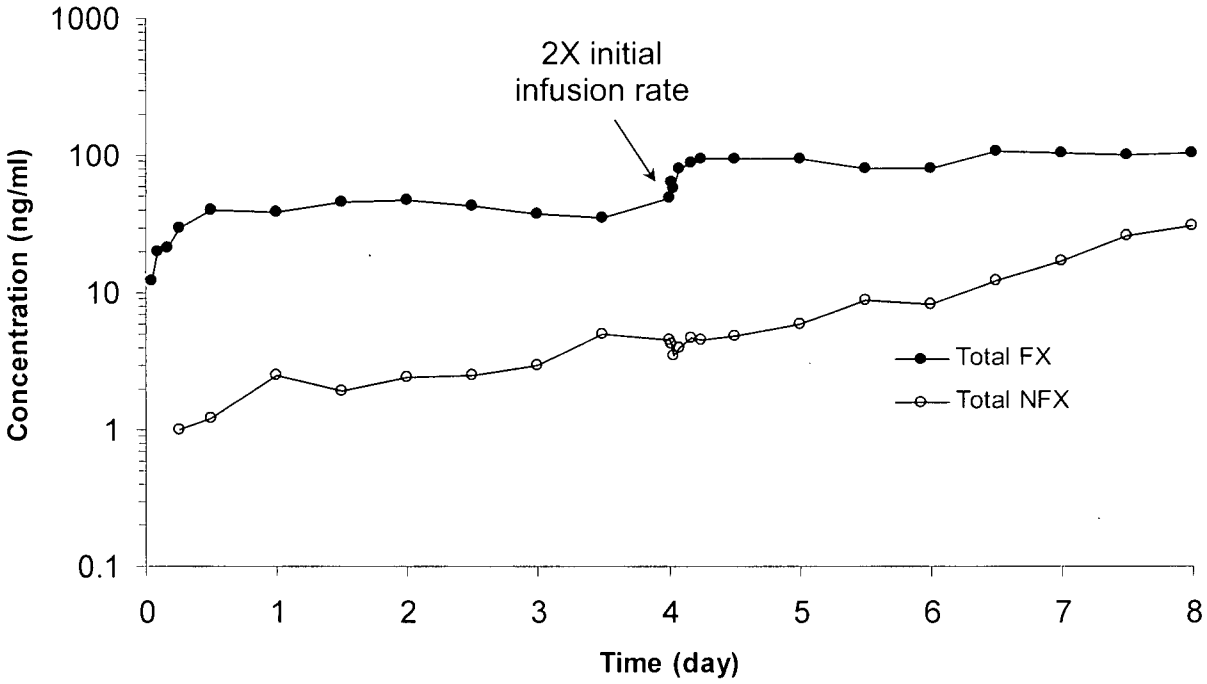


FIGURE 6.2 Plasma concentration versus time profiles of total (racemic) FX and NFX in the fetus (E8105) after step-wise fetal *i.v.* infusion of racemic FX at two different rates, showing steady-state concentrations of FX after each infusion rate and continuous accumulation of NFX during the entire infusion period.

the parent drug, NFX did not reach steady-state and continued to accumulate over the entire duration of the infusion. Concentrations of total NFX at the end of each infusion step were 4.6 ng/ml and 30.1 ng/ml, respectively. From this preliminary experiment, an infusion rate of 2.1 mg/h targeting a C_{ss} of total FX of 50-100 ng/ml was selected for all subsequent infusion experiments following the protocol outlined in Figure 6.1b. Figure 6.3 depicts the mean plasma concentration versus time profiles of total FX and NFX in fetal lambs from these 4-day fetal infusion experiments. Steady-state was achieved between day 2-4 and the average C_{ss} of total FX was 81.2 ± 8.6 ng/ml (n = 4). NFX was below the assay limit of detection in the fetal plasma during the first 6 hours of the infusion. After that, NFX slowly accumulated in the fetal circulation over the entire infusion period. The concentration of total NFX at the end of infusion averaged 18.1 ± 3.7 ng/ml.

6.2.1 Stereoselective Disposition of FX and NFX in the Fetus

Figure 6.4 depicts representative plasma concentration versus time profiles for the FX enantiomers in a fetus, showing a consistent concentration difference between the FX enantiomers. Fetal pharmacokinetic parameter data for the FX and NFX enantiomers and their corresponding S/R ratios are summarized in Table 6.1. The stereoselective pharmacokinetics of FX enantiomers observed in the fetus is consistent with the findings in the adult sheep. With the exception of elimination $t_{1/2\beta}$ and MRT, significant differences were found in most parameters (e.g. AUC, C_{ss}, Vd_{ss}, and CL_{TB}) between R-FX and S-FX. The $t_{1/2\beta}$ of R-FX was not significantly different from that of S-FX (48.9 ± 12.2 h versus 43.4 ± 6.4 h, S/R ratio = 0.93 ± 0.24; p = 0.46). Likewise, the MRT of R-FX was comparable to that of S-FX (25.4 ± 10.5 h versus 20.7 ± 5.4 h, S/R ratio = 0.82 ± 0.15, p = 0.11). The AUC

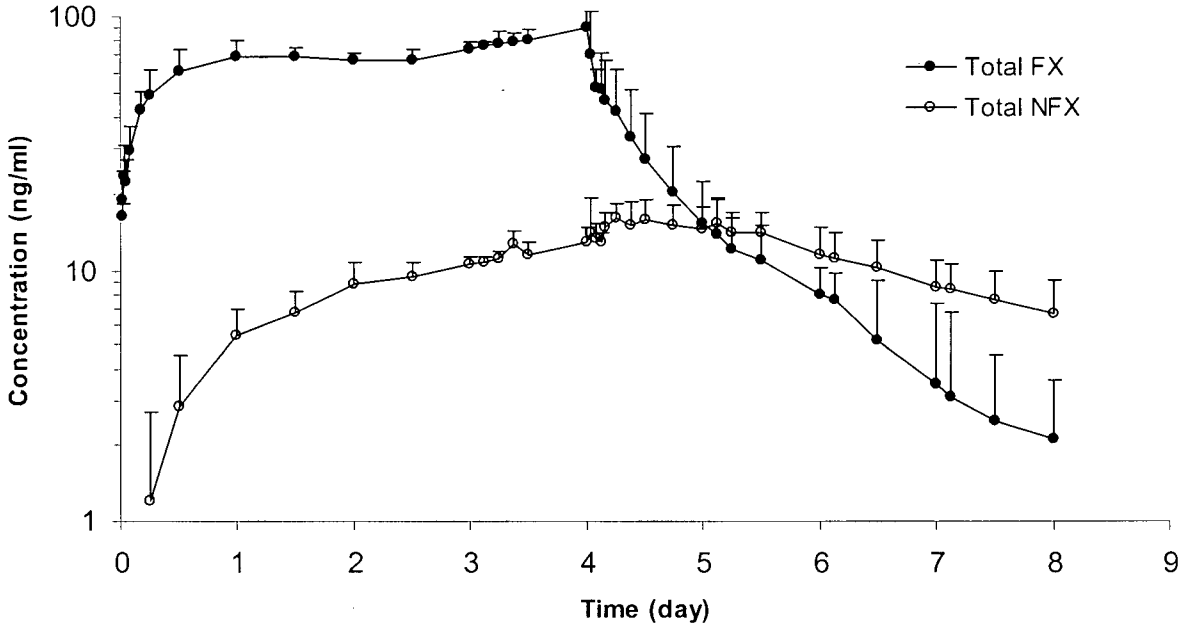


FIGURE 6.3 Comparative average plasma concentration versus time profiles of total (i.e. R plus S enantiomers) FX and NFX in the fetuses (n=4) after a 4-day fetal *i.v.* infusion of racemic FX, illustrating the concentration differences between parent drug and metabolite.

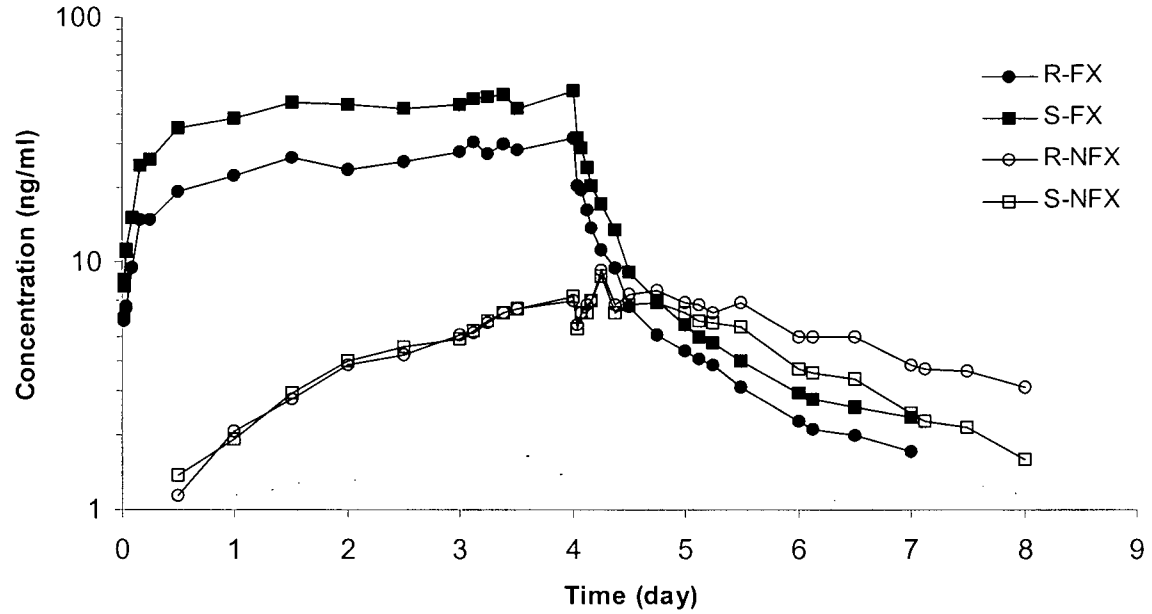


FIGURE 6.4 Representative plasma concentration versus time profiles of FX and NFX enantiomers in a fetus (E9140) after a 4-day fetal *i.v.* infusion of racemic FX, showing stereoselective steady-state concentrations of the FX enantiomers and a low level of NFX accumulation in the fetal plasma.

TABLE 6.1 Pharmacokinetic parameters for the FX and NFX enantiomers in fetal lambs after a 4-day fetal infusion of racemic FX.

Fluoxetine	$t_{1/2\,\beta}$ (h)		MRT (h)		AUC (μg*h/l)		C_{ss} (ng/ml)		Vd_{ss} (l/kg)		CL_{tb} (l/h/kg)		F/M AUC ratio†		f_u (%)		CL_{renal} (ml/h/kg)	
Animal #	R-FX	S-FX	R-FX	S-FX	R-FX	S-FX	R-FX	S-FX	R-FX	S-FX	R-FX	S-FX	R-FX	S-FX	R-FX	S-FX	R-FX	S-FX
E5134	46.1	52.7	29.1	26.3	3960	5761	42.0	49.6	411.8	255.9	14.17	9.74	4.34	3.70	4.03	1.82	124.6	86.3
E9140	34.0	38.1	17.7	15.8	2773	4519	29.5	46.4	430.1	234.9	24.29	14.91	3.14	2.97	5.39	1.33	10.7	6.1
E9156	63.3	42.2	27.2	16.3	3199	4944	27.9	44.6	549.8	213.8	20.21	13.08	3.19	2.70	4.46	1.06	28.4	15.9
E9168	52.3	40.5	27.5	24.5	3043	4529	34.4	50.2	461.9	276.7	16.80	11.29	1.97	1.50	5.30	0.89	44.7	26.7
Mean ± S.D.	48.9 ± 12.2	43.4 ± 6.4	25.4 ± 10.5	20.7 ± 5.4	3244 ± 509	4938 ± 583	33.4 ± 6.3	47.7 ± 2.7	463.4 ± 61.2	245.3 ± 21.1	18.87 ± 4.38	12.25 ± 2.23	3.16 ± 0.97	2.72 ± 0.91	4.79 ± 0.66	1.27 ± 0.40	52.1 ± 50.3	33.8 ± 36.1
S/R ratio	0.93 ± 0.24		0.82 ± 0.15		1.53 ± 0.08		1.45 ± 0.19		0.54 ± 0.10		0.66 ± 0.03		0.85 ± 0.08		0.28 ± 0.12		0.61 ± 0.06	
R- vs. S-FX *p*-value‡	0.46		0.11		<0.01		<0.01		<0.01		<0.01		<0.05		<0.01		0.08	
Norfluoxetine	$t_{1/2\,\beta}$ (h)		MRT (h)		AUC (μg*h/l)		F/M AUC ratio		NFX/FX AUC ratio†		f_u (%)		CL_{renal} (ml/h/kg)	
Animal #	R-NFX	S-NFX	R-NFX	S-NFX	R-NFX	S-NFX	R-NFX	S-NFX	R-NFX	S-NFX	R-NFX	S-NFX	R-NFX	S-NFX
E5134	67.5	48.4	129.9	104.1	1410	1138	0.74	0.70	0.36	0.20	5.43	5.47	49.4	55.1
E9140	63.7	38.5	133.7	100.2	1157	857	0.46	0.42	0.42	0.19	7.05	4.68	39.0	19.5
E9156	51.9	50.8	110.4	113.9	1014	913	0.59	0.68	0.32	0.18	6.36	5.61	17.6	19.9
E9168	67.7	49.4	142.0	115.9	1673	1494	0.70	0.68	0.55	0.33	6.79	4.60	15.3	5.6
Mean ± S.D.	62.7 ± 7.4	46.8 ± 5.6	129.0 ± 13.4	108.5 ± 7.6	1313 ± 291	1100 ± 289	0.62 ± 0.13	0.62 ± 0.13	0.41 ± 0.10	0.23 ± 0.07	6.41 ± 0.71	5.09 ± 0.52	30.3 ± 16.6	25.1 ± 21.1
S/R Ratio	0.76 ± 0.16		0.85 ± 0.12		0.84 ± 0.08		1.00 ± 0.10		0.55 ± 0.07		0.81 ± 0.17		0.78 ± 0.40	
R- vs. S-NFX *p*-value‡	0.06		0.09		<0.05		0.94		<0.01		<0.05		0.43	

Note: Average values are expressed as the mean ± standard deviation (S.D.)

† F/M AUC ratio = fetal to maternal area under the curve ratio.

‡ Paired t-test, significance level $p = 0.05$.

of R-FX was significantly lower than that of S-FX (3244 ± 509 μg*h/l versus 4938 ± 583 μg*h/l, S/R ratio = 1.53 ± 0.08; $p<0.01$). Similarly, C_{ss} was significantly lower for R-FX compared to S-FX (33.4 ± 6.3 ng/ml versus 47.7 ± 2.7 ng/ml, S/R ratio = 1.45 ± 0.19; $p<0.01$). R-FX had a higher Vd_{ss} compared to S-FX (463.4 ± 61.2 l/kg versus 245.3 ± 21.1 l/kg, S/R ratio = 0.54 ± 0.10; $p<0.01$). CL_{TB} of R-FX was significantly higher than that of S-FX (18.87 ± 4.38 l/h/kg versus 12.25 ± 2.23 l/h/kg, S/R ratio = 0.66 ± 0.03; $p<0.01$). The comparative average plasma concentration versus time profiles of total FX between the mother and fetus are depicted in Figure 6.5. The F/M AUC ratio for total (racemic) FX averaged 2.87 ± 0.93. This represents a fetal-to-maternal gradient of similar magnitude, but with the order of the enantiomers opposite that found in the maternal infusion experiments. The F/M AUC ratio of R-FX was significantly higher than that of S-FX (3.16 ± 0.97 versus 2.72 ± 0.91, S/R ratio = 0.85 ± 0.08; $p<0.05$). Stereoselective protein binding was observed in the fetal plasma, with the f_u of R-FX being significantly higher than that of S-FX (4.79 ± 0.66 versus 1.27 ± 0.40, S/R ratio = 0.28 ± 0.12; $p<0.01$).

Excretion of FX was observed in fetal urine as illustrated in Figure 6.6, which depicts the time course of cumulative amount excreted in fetal urine from the 2-step infusion experiment. The renal elimination of FX in the fetus followed a linear process with the elimination rate increasing proportionally with infusion rate. Figure 6.7 illustrates the graphical method of determining CL_{renal} from the slope of the plot of the amount of the FX enantiomers excreted in the urine versus AUC during the urine collection time interval t_n–t_{n+1} in a 4-day fetal infusion experiment. The straight line obtained from the slope of graph confirms the linearity of the fetal renal elimination process. Fetal CL_{renal} averaged 52.1 ± 50.3 ml/h/kg and 33.8 ± 36.1 ml/h/kg for R-FX and S-FX, respectively (Table 6.1). The

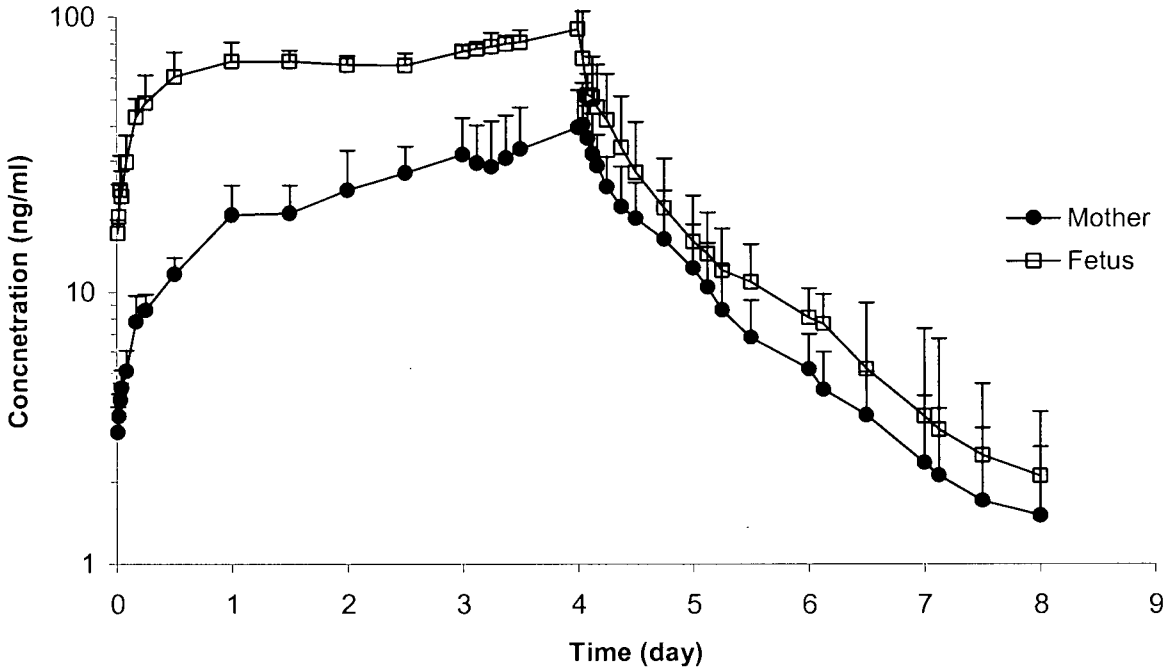


FIGURE 6.5 Average plasma concentration versus time profiles of total FX (i.e. R plus S enantiomers) in the mother and fetus (n=4) after a 4-day fetal *i.v.* infusion of racemic FX, with F/M AUC ratio averaging 2.87 ± 0.93.

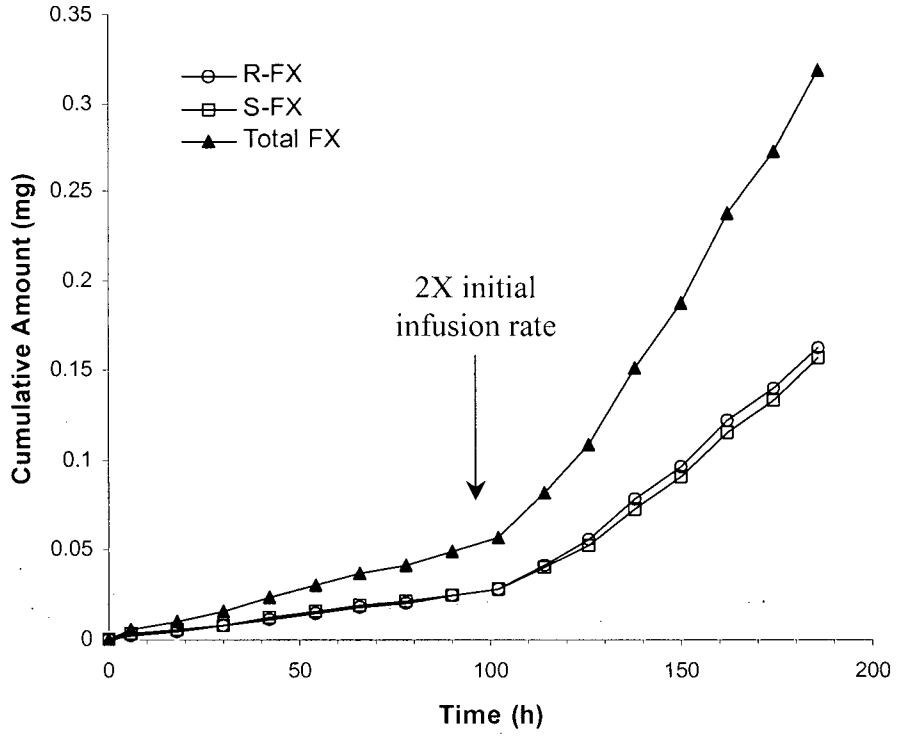


FIGURE 6.6 Plot of the time course of the cumulative amount of FX excreted in urine of a fetus (E8105) from the 2-step racemic FX fetal *i.v.* infusion experiment, demonstrating a linear relationship between renal elimination rate and dose.

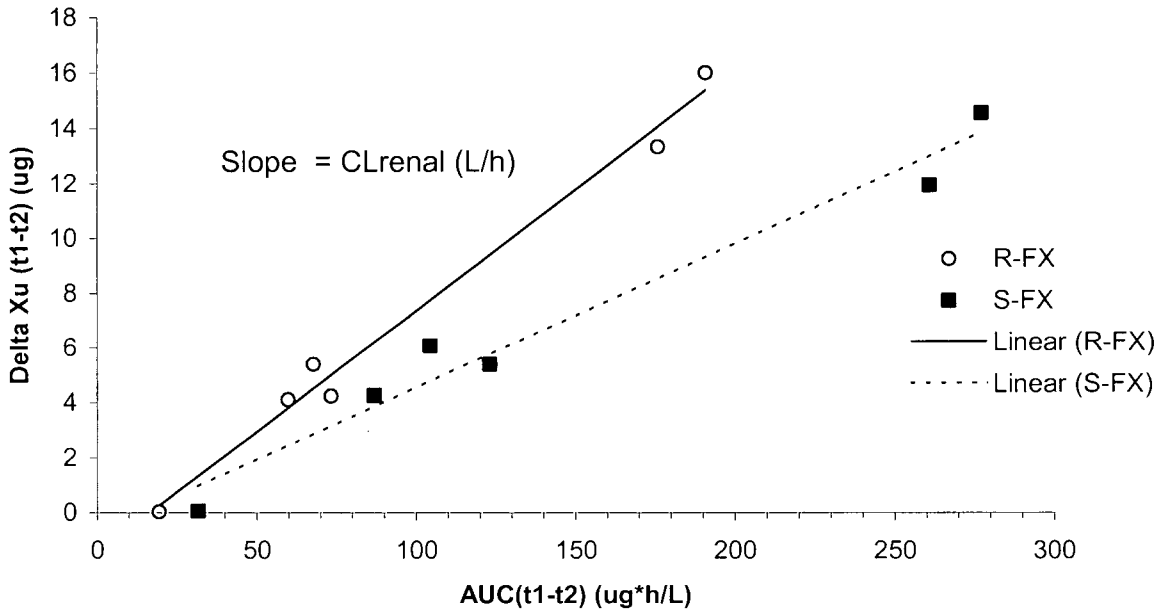


FIGURE 6.7 Representative plot of the amount of FX enantiomers excreted in fetal urine versus AUC during the urine collection time intervals t_n–t_{n+1} for a 4-day FX fetal *i.v.* infusion experiment (E9168).

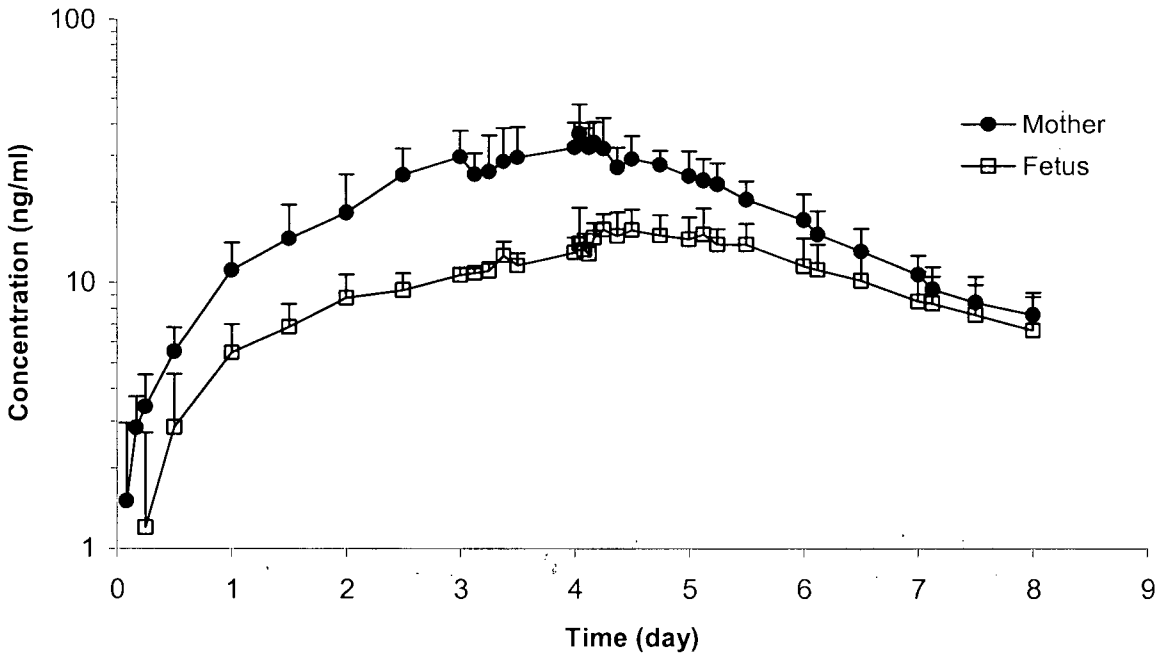


FIGURE 6.8 Average plasma concentration versus time profiles of total NFX (i.e. R plus S enantiomers) in the mother and fetus after a 4-day fetal *i.v.* infusion of racemic FX, showing higher accumulation of NFX in maternal plasma compared to fetal plasma (F/M AUC ratio = 0.62 ± 0.13; n=4).

CL_{renal} of R-FX tended to be higher than that of (S/R ratio = 0.61 ± 0.06) but the difference was not statistically significant (p=0.08) due to high variability among the four animals. Overall, CL_{renal} accounts for less than 1% of CL_{TB}, indicating that renal excretion of FX is a minor route of elimination in the fetus.

Unlike the parent drug, no apparent difference was observed in the plasma concentration-time profiles of the NFX enantiomers as illustrated in Figure 6.4. Both the $t_{1/2\beta}$ and MRT of R-NFX tended to be longer compared to S-NFX but their differences were not statistically significant ($t_{1/2\beta}$: 62.7 ± 7.4 h versus 46.8 ± 5.6 h, p=0.06; MRT: 129.0 ± 13.4 h versus 108.5 ± 7.6 h, p=0.09) (Table 6.1). The AUC of R-NFX was significantly higher than that of S-NFX (1313 ± 291 μg*h/l versus 1100 ± 289 μg*h/l, S/R ratio = 0.84 ± 0.08; p<0.05). The comparative plasma concentration versus time profiles of total NFX between the mother and the fetus are shown in Figure 6.8. Both R-NFX and S-NFX have the same average F/M AUC ratios of 0.62 ± 0.13, resulting in an S/R ratio approximating unity. The NFX/FX AUC ratio was significantly higher for the R-enantiomer compared to the S-enantiomer (0.41 ± 0.10 versus 0.23 ± 0.07, S/R ratio = 0.55 ± 0.07; p<0.01). Similar to FX, stereoselective protein binding of NFX was found in the fetal plasma, with the f_u being significantly higher for R-NFX compared to S-NFX (6.41 ± 0.71 % versus 5.09 ± 0.52 %, S/R ratio = 0.81 ± 0.17; p<0.05). NFX was excreted in fetal urine but to a lesser extent compared to the parent drug. CL_{renal} values for the NFX enantiomers were calculated in a manner analogous to that of FX. As for the parent drug, there was no significant difference in CL_{renal} between R-NFX and S-NFX (30.3 ± 16.6 ml/h/kg versus 25.1 ± 21.1 ml/h/kg, S/R ratio = 0.78 ± 0.40; p = 0.43).

6.2.2 Comparative Pharmacokinetics of FX and NFX in Adult, Maternal, and Fetal Sheep

Comparisons of the FX and NFX enantiomer pharmacokinetic parameters values were made between sheep at different developmental periods to examine the effects of any associated changes in physiological and biochemical processes on drug disposition. The results of the ANOVA analyses on the pharmacokinetic data from fetal sheep (from the current chapter), maternal sheep (from chapter 4) and nonpregnant adult sheep (from chapter 3) are summarized in Table 6.2 for FX and Table 6.3 for NFX. The $t_{1/2\beta}$ of R-FX and S-FX in fetal lambs were similar to those in the maternal sheep, but significantly longer compared to nonpregnant adult sheep ($p<0.01$). On the other hand, the MRT of R-and S-FX were comparable between the fetal lambs and nonpregnant adult sheep. The MRT for FX in maternal sheep was significantly larger than in the fetus, but not different from the that in the nonpregnant ewes. Weight normalized Vd_{ss} of the FX enantiomers was significantly higher in fetal lambs, with values that were approximately 10-fold larger compared to maternal and nonpregnant adult sheep ($p<0.01$). Similarly, the weight normalized CL_{TB}'s of R- and S-FX in the fetal lambs were much higher than the values in maternal and nonpregnant adult sheep ($p<0.01$). Protein binding of R-FX was lower in the fetal plasma with f_u being significantly higher in the fetal lambs compared to both maternal and nonpregnant adult sheep ($p<0.01$). For S-FX, however, this difference was only observed between the fetus and nonpregnant ewes ($p<0.05$). The higher f_u is consistent with the observation of low α_1-acid glycoprotein concentration in fetal plasma compared to adult plasma (Wood and Wood, 1981; Hill and Abramson, 1988). In spite of its low importance relative to the overall systemic clearance, CL_{renal} in the fetus appeared to be much higher compared to adult sheep, but due to the high

TABLE 6.2 Comparison of FX enantiomers pharmacokinetic parameters values in nonpregnant adult, pregnant and fetal sheep.

FX Pharmacokinetic Parameter	Enantiomer	Dosing Group†	Mean ± S.D.	*p*-value	Dosing Group Comparison‡		
					Nonpregnant Adult	Mother	Fetus
$t_{1/2\beta}$ (h)	R	Nonpregnant	18.4 ± 7.7	<0.01	-	NS	**
		Mother	31.9 ± 14.6		NS	-	NS
		Fetus	48.9 ± 12.2		**	NS	-
	S	Nonpregnant	14.8 ± 3.3	<0.01	-	**	**
		Mother	35.9 ± 18.3		**	-	NS
		Fetus	43.4 ± 6.4		**	NS	-
MRT (h)	R	Nonpregnant	24.6 ± 7.0	0.15	-	NS	NS
		Mother	40.2 ± 21.0		NS	-	NS
		Fetus	25.4 ± 10.5		NS	NS	-
	S	Nonpregnant	34.9 ± 8.6	<0.05	-	NS	NS
		Mother	57.8 ± 24.3		NS	-	*
		Fetus	20.7 ± 5.4		NS	*	-
Vd_{ss} (l/kg)	R	Nonpregnant	44.9 ± 11.3	<0.01	-	NS	**
		Mother	48.2 ± 25.3		NS	-	**
		Fetus	463.4 ± 61.2		**	**	-
	S	Nonpregnant	22.7 ± 8.3	<0.01	-	NS	**
		Mother	20.2 ± 10.9		NS	-	**
		Fetus	245.3 ± 21.1		**	**	-
CL_{TB} (l/h/kg)	R	Nonpregnant	1.91 ± 0.61	<0.01	-	NS	**
		Mother	1.20 ± 0.32		NS	-	**
		Fetus	18.87 ± 4.38		**	**	-
	S	Nonpregnant	0.68 ± 0.28	<0.01	-	NS	**
		Mother	0.38 ± 0.22		NS	-	**
		Fetus	12.25 ± 2.23		**	**	-
f_u (%)	R	Nonpregnant	2.42 ± 0.82	<0.01	-	NS	**
		Mother	2.71 ± 0.48		NS	-	**
		Fetus	4.79 ± 0.66		**	**	-
	S	Nonpregnant	0.71 ± 0.23	<0.05	-	NS	*
		Mother	0.83 ± 0.26		NS	-	NS
		Fetus	1.27 ± 0.40		*	NS	-
CL_{renal} (ml/h/kg)	R	Nonpregnant	0.97 ± 0.40	0.15	-	-	NS
		Mother	n/a		-	-	-
		Fetus	52.1 ± 50.3		NS	-	-
	S	Nonpregnant	0.66 ± 0.38	0.18	-	-	NS
		Mother	n/a		-	-	-
		Fetus	33.8 ± 36.1		NS	-	-

† Nonpregnant and maternal sheep data are from the 8-day FX *i.v.* infusion experiments (see chapters 3 and 4).

‡ From one-way ANOVA with post hoc Tukey comparison (* $p \leq 0.05$, ** $p \leq 0.01$, NS = not significant).

n/a – data not available as urine was not collected from the pregnant ewes.

TABLE 6.3 Comparison of NFX enantiomers pharmacokinetic parameter values in nonpregnant adult, pregnant and fetal sheep.

NFX Pharmacokinetic Parameter	Enantiomer	Dosing Group†	Mean ± S.D.	*p*-value	Dosing Group Comparison‡		
					Nonpregnant Adult	Mother	Fetus
$t_{1/2\beta}$ (h)	R	Nonpregnant	26.0 ± 5.6	<0.01	-	**	**
		Mother	68.0 ± 18.0		**	-	NS
		Fetus	62.7 ± 7.4		**	NS	-
	S	Nonpregnant	21.4 ± 4.2	<0.01	-	**	**
		Mother	52.1 ± 23.3		**	-	NS
		Fetus	46.8 ± 5.6		**	NS	-
MRT (h)	R	Nonpregnant	124.8 ± 5.4	<0.01	-	**	NS
		Mother	160.0 ± 13.3		**	-	**
		Fetus	129.0 ± 13.4		NS	**	-
	S	Nonpregnant	108.5 ± 3.6	<0.05	-	**	NS
		Mother	125.0 ± 11.9		**	-	**
		Fetus	108.5 ± 7.6		NS	**	-
NFX/FX AUC ratio	R	Nonpregnant	2.26 ± 0.44	<0.01	-	NS	**
		Mother	2.35 ± 0.39		NS	-	**
		Fetus	0.41 ± 0.10		**	**	-
	S	Nonpregnant	0.73 ± 0.26	<0.01	-	NS	**
		Mother	0.61 ± 0.18		NS	-	**
		Fetus	0.23 ± 0.07		**	**	-
f_u (%)	R	Nonpregnant	2.02 ± 0.47	<0.01	-	NS	**
		Mother	1.94 ± 0.48		NS	-	**
		Fetus	6.41 ± 0.71		**	**	-
	S	Nonpregnant	1.14 ± 0.35	<0.01	-	NS	**
		Mother	1.38 ± 0.41		NS	-	**
		Fetus	5.09 ± 0.52		**	**	-
CL_{renal} (ml/h/kg)	R	Nonpregnant	1.46 ± 1.03	<0.05	-	-	*
		Mother	n/a		-	-	-
		Fetus	30.3 ± 16.6		*	-	-
	S	Nonpregnant	1.07 ± 0.57	0.11	-	-	NS
		Mother	n/a		-	-	-
		Fetus	25.1 ± 21.1		NS	-	-

† Nonpregnant and pregnant sheep data are from the 8-day FX *i.v.* infusion experiments (see chapters 3 and 4).

‡ From one-way ANOVA with post hoc Tukey comparison (* $p \leq 0.05$, ** $p \leq 0.01$, NS = not significant).

n/a – data not available as urine was not collected from the pregnant ewes.

variability among animals, statistically significant differences were not achieved. The $t_{1/2\beta}$ of the NFX enantiomers in the fetuses were similar to those in maternal sheep, but higher compared to the nonpregnant adults ($p<0.01$). In contrast, the MRTs of the NFX enantiomers in the fetal lambs were similar to the values obtained in the nonpregnant adult sheep, but higher compared to maternal sheep ($p<0.05$). NFX/FX AUC ratios were significantly lower in the fetal lambs compared to both maternal and nonpregnant adult sheep ($p<0.01$). These ratios were less than 20% and 40% of the adult values for R-NFX and S-NFX, respectively, indicating decreased metabolic efficiency in the fetuses. Similar to FX, the protein binding of NFX was significantly lower in the fetal plasma. Overall, the f_u of both R- and S-NFX in the fetal lambs were about 3 to 4-fold higher than the respective values in maternal and nonpregnant adult sheep. Urinary excretion of NFX was higher in the fetuses, which had higher CL_{renal} compared to the adult sheep although only the comparison for R-NFX was statistically significant ($p<0.05$).

6.2.3 Fetal Blood Gas Results

Mean fetal arterial blood gas and pH values during the first 24 h of drug infusion (pooled data from Day 1 of Step #1 of the preliminary 2-step infusion experiment and Day 1 of the 4-day infusion studies) are illustrated in Figures 6.9 and 6.10. Data from the maternal FX *i.v.* infusion experiments are also included for comparison (from Morrison et al., 2002). Compared with the pre-infusion values, fetal pH, P_{O_2}, and oxygen saturation tended to decrease, whereas P_{CO_2} tended to increase after fetal drug infusion, but none of these changes were statistically significant. Moreover, the changes were less than those occurring during the maternal FX infusion experiments (Morrison et al., 2002). Mean fetal P_{O_2}

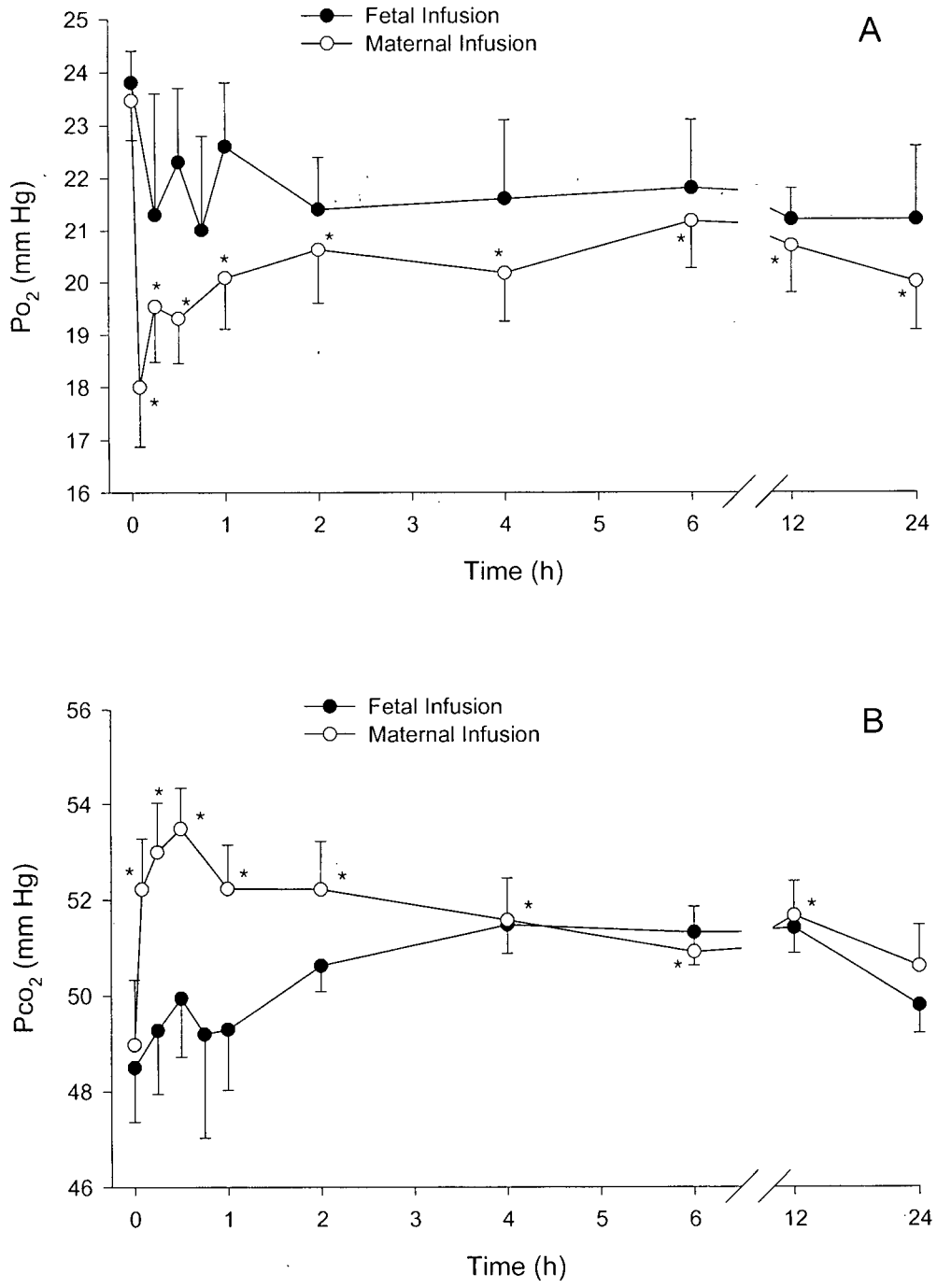


FIGURE 6.9 Mean fetal arterial PO_2 (A) and PCO_2 (B) during the first 24 h of fetal FX infusion (closed symbols, n = 5) or maternal FX infusion (open symbols, n = 13). # and * denote significant differences ($p<0.05$) from pre-infusion day in fetal and maternal infusion experiments, respectively.

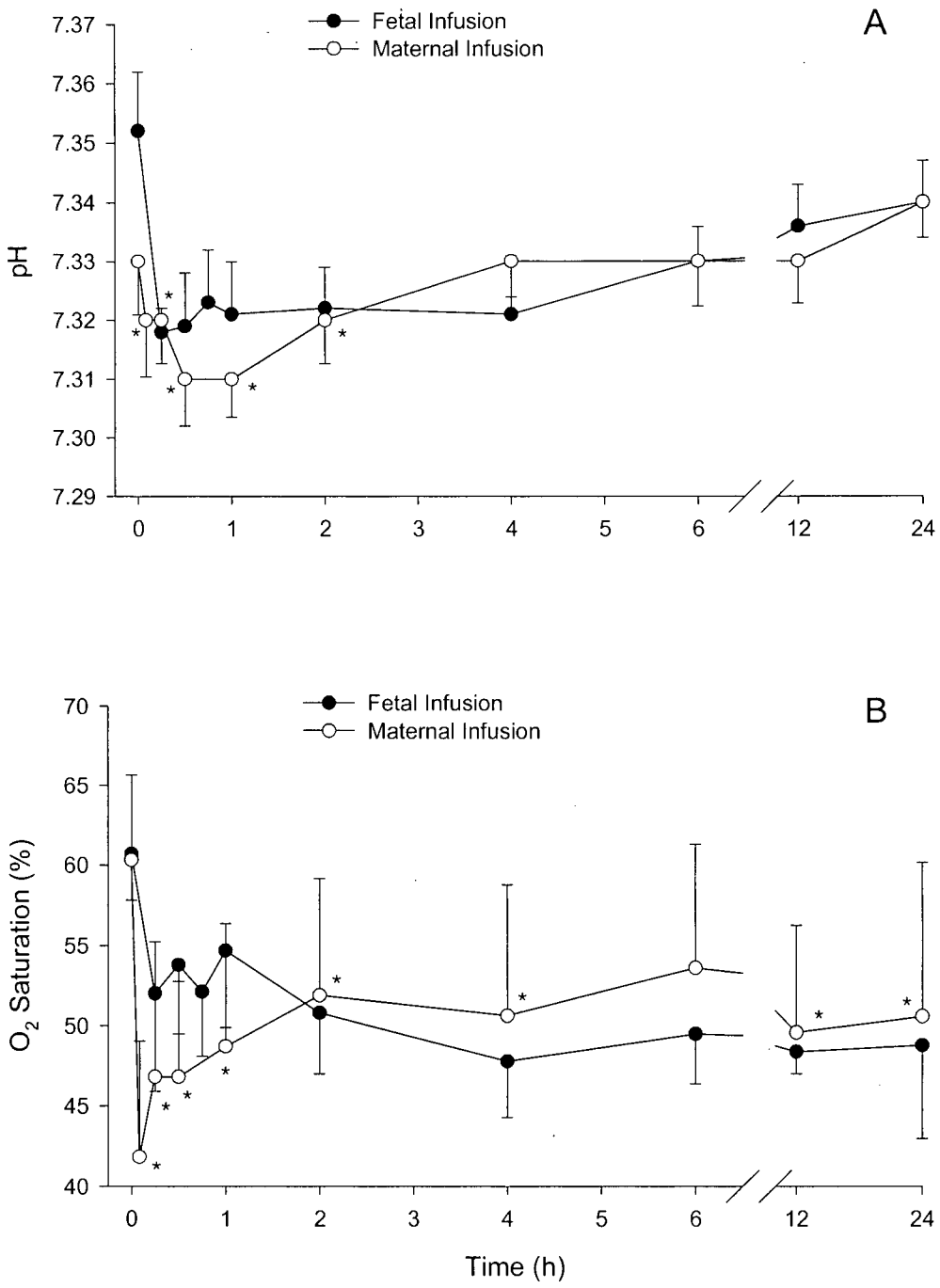


FIGURE 6.10 Mean fetal arterial pH (A) and oxygen saturation (B) during the first 24 h of fetal FX infusion (closed symbols, n = 5) or maternal FX infusion (open symbols, n = 13). # and * denote significant differences ($p<0.05$) from pre-infusion day in fetal and maternal infusion experiments, respectively.

dropped from 23.8 ± 0.6 mm Hg at pre-infusion to a minimum of 21.0 ± 1.8 mm Hg at 0.75 h after infusion, and remained steady over the next 24 h period. A similar decrease in O_2 saturation was observed, with an initial decrease from 60.7 ± 2.9 to 47.8 ± 3.5 % at 4 h post-infusion. Mean fetal P_{CO_2} increased gradually and reached a maximum of 51.5 ± 0.6 mm Hg at 4 h post-infusion. Mean fetal blood pH decreased slightly from 7.352 ± 0.010 to 7.319 ± 0.009 at 30 min after infusion, but returned to pre-infusion levels after 24 h. The pH decrease appeared to be associated with a similar decrease in base excess, which was 1.2 ± 0.3 mEq/L before FX administration and -0.3 ± 0.4 mEq/L at 30 min. Figure 6.11 illustrates the changes in mean fetal lactate and glucose concentration during the first 24 hours. Both lactate and glucose gradually increased and reached the highest concentrations of 1.47 ± 0.38 mM and 1.07 ± 0.14 mM, respectively, at 6 h post-infusion ($p<0.05$), then returned to pre-infusion levels after 24 h. A similar pattern of changes in fetal blood gas status was observed during the first 24 hours of Step #2 of the preliminary 2-step infusion experiment. The daily blood gas status monitored at 0700 h throughout the infusion and post-infusion period are summarized in Table 6.4. No significant changes in any fetal blood gas parameters were observed.

6.3 Discussion

In general the present experiments demonstrated that the FX enantiomers exhibit significant stereoselective disposition in the fetus following fetal drug infusion to steady-state. Overall, the stereoselective relationships between R-FX and S-FX are similar to those previously observed in adult sheep, but the magnitude of the S/R ratios was less. For example, S/R ratios for AUC and C_{ss} averaged 1.5 in the fetal infusion experiments

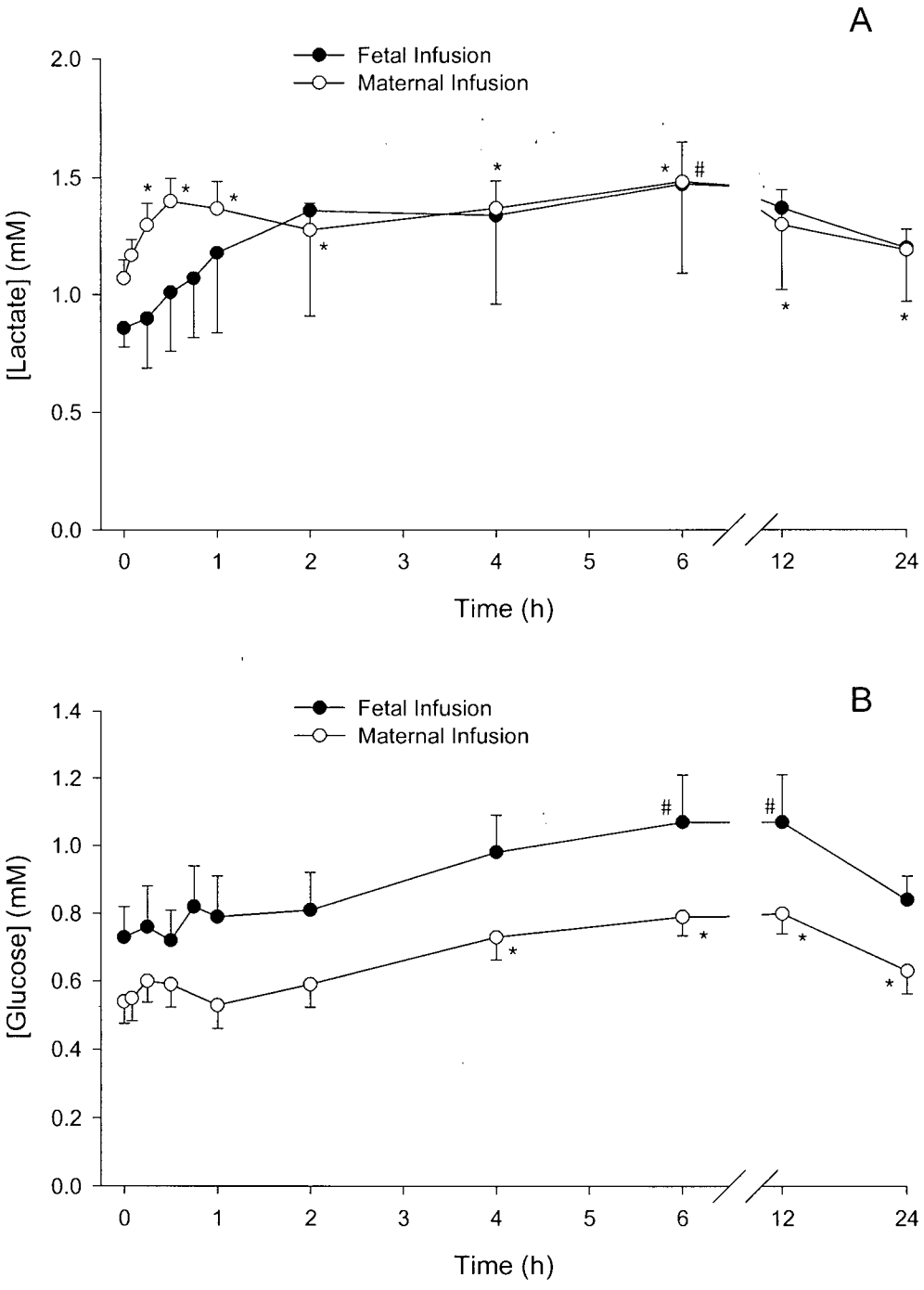


FIGURE 6.11 Mean fetal arterial lactate (A) and glucose (B) concentrations during the first 24 h of fetal FX infusion (closed symbols, n = 5) or maternal FX infusion (open symbols, n = 13). # and * denote significant differences ($p<0.05$) from pre-infusion day in fetal and maternal infusion experiments, respectively.

TABLE 6.4 Mean fetal arterial blood gas status at 0700 h on each day of a 4-day fetal FX *i.v.* infusion and post-infusion period.

		Control[a]	Inf 1[a]	Inf 2[a]	Inf 3[a]	Inf 4[a]	PI 1[b]	PI 2[b]	PI 3[b]	PI 4[b]	PI 5[b]
P_{O_2} (mm Hg)	Mean	24.8	23.8	21.2	22.2	22.0	23.0	24.8	24.8	22.8	22.8
	S.E.	0.8	0.6	1.4	0.6	1.0	1.6	1.1	1.8	2.1	2.0
O_2 Sat. (%)	Mean	62.4	60.7	48.8	56.4	52.2	51.0	50.0	46.6	47.7	45.8
	S.E.	4.3	2.9	5.8	5.1	5.4	3.8	1.0	1.9	1.1	1.7
P_{CO_2} (mm Hg)	Mean	48.4	48.5	49.8	49.4	49.6	48.5	49.2	48.9	47.6	49.5
	S.E.	1.2	1.2	0.6	0.6	1.1	0.4	1.1	1.6	0.4	0.7
pH	Mean	7.347	7.352	7.340	7.343	7.318	7.318	7.306	7.308	7.324	7.330
	S.E.	0.011	0.010	0.007	0.010	0.010	0.027	0.016	0.033	0.023	0.009
BE (mEq/L)	Mean	1.3	1.2	1.4	1.5	0.1	-0.9	-1.1	-1.0	-0.4	0.8
	S.E.	0.5	0.3	0.3	0.5	0.6	1.7	1.4	2.6	1.5	0.8
Hb (g/dL)	Mean	10.26	9.78	9.82	9.50	9.04	9.20	9.15	9.13	9.43	9.85
	S.E.	0.52	0.72	0.53	0.59	0.57	0.32	0.63	0.64	0.29	0.31
Lactate (mM)	Mean	0.87	0.86	1.20	0.92	0.92	0.86	0.96	0.90	1.00	0.98
	S.E.	0.11	0.08	0.23	0.06	0.16	0.13	0.18	0.14	0.13	0.17
Glucose (mM)	Mean	0.77	0.73	0.84	0.71	0.69	0.63	0.70	0.65	0.60	0.60
	S.E.	0.08	0.09	0.07	0.06	0.10	0.08	0.03	0.05	0.10	0.09

Note that Control and Infusion Day 1 are pre-infusion values.

Inf, infusion day; PI, post-infusion day; O_2 Sat., oxygen saturation; BE, base excess; Hb, Hemoglobin.

* Significant difference from pre-infusion day ($p < 0.05$); [a] n=5 from Control to Inf 4; [b] n=4 from PI 1 to PI 5.

compared to 3 to 4 in the maternal infusion experiments. In chapter 3, it was shown that stereoselective protein binding and metabolism were key factors in determining the stereoselective disposition of FX in adult sheep. Although the extent of protein binding was lower in fetal plasma, stereoselectivity was preserved with the S/R ratio averaging 0.28, which is comparable to the adult value (0.30). Stereoselective metabolic formation of NFX is primarily due to the difference in intrinsic clearance between FX enantiomers. Previous *in vitro* metabolism studies using fetal ovine hepatic microsomes did not show any formation of NFX from FX (Kim et al., 2004). Thus, it is believed that the fetal liver does not contribute significantly to the observed difference in fetal enantiomer concentrations. Instead, the apparent difference in concentration between R- and S-FX in the fetus at equilibrium is more likely to be a byproduct of stereoselective metabolism from the maternal side, with subsequently placental transfer. This is supported by the finding of comparable S/R AUC ratios in the mother (1.80) and the fetus (1.53) in the present study. Moreover, the lower S/R ratios for C_{ss} and AUC correspond with the lower FX concentrations in maternal plasma (mean total FX C_{ss} of 33.4 ng/ml; Figure 6.5). These results also parallel the positive correlation observed between FX concentration and S/R ratio (Figure 4.6) in the adult sheep experiments. Figure 6.12 is a modification of Figure 4.6, from which the maternal FX concentrations and S/R ratios from the present fetal infusion experiment were included to further illustrate the presence of a positive correlation between FX concentration and S/R ratio.

A previous study with direct *i.v.* bolus injection of FX to fetal lambs resulted in no measurable level of NFX in fetal plasma (Kim et al., 2004). In contrast, NFX was detected in fetal plasma and urine during the current fetal steady-state FX infusions, with the mean

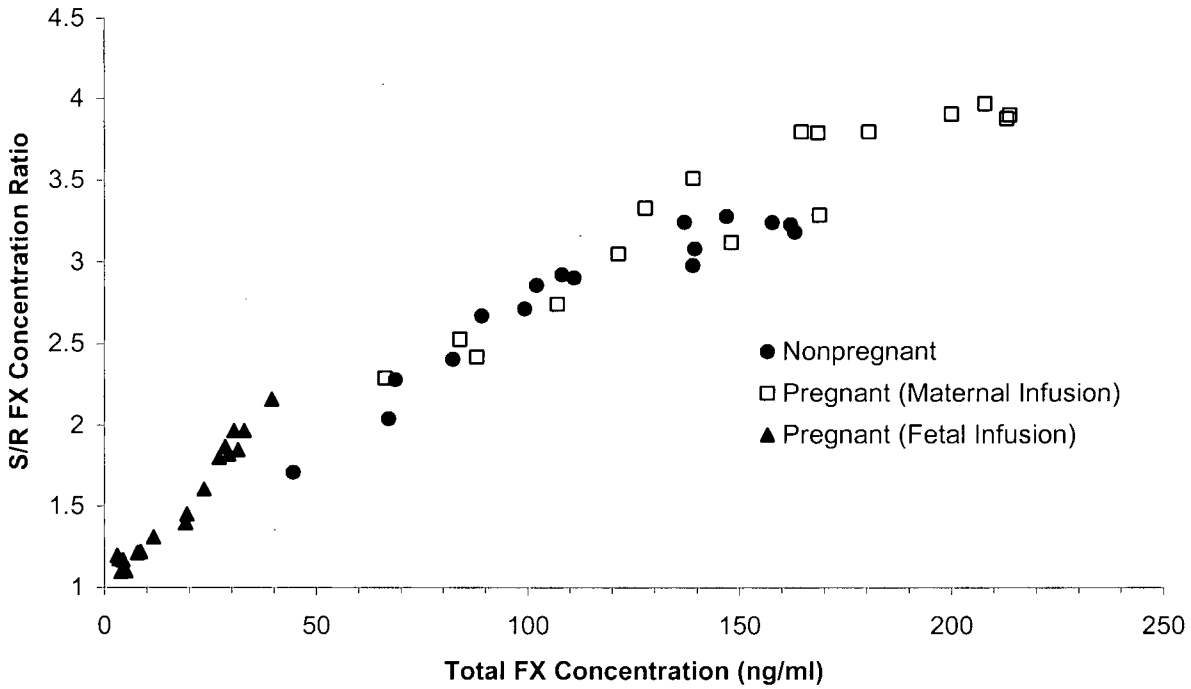


FIGURE 6.12 Plot of the maternal S/R FX concentration ratio versus the corresponding total FX concentration, a compilation of all data from the nonpregnant and pregnant (following either maternal or fetal *i.v.* FX infusion) sheep experiments.

AUC for total NFX accounting for 30% of the parent drug. The presence of drug metabolites in the fetal circulation may be the result of maternal-to-fetal placental transfer or biotransformation by the fetus itself. As previously discussed, the presence of NFX in fetal plasma during the maternal infusions appears to be the result of metabolite formation in the ewe followed by placental transfer to the fetus. Since the fetal lambs in the present studies were not surgically prepared to allow us to determine the existence of a concentration gradient across the fetal liver for NFX, we are not able to provide direct evidence for the fetus to biotransform FX to NFX. Nevertheless, the low NFX F/M ratios along with the delay in the appearance of NFX in the fetal circulation indirectly suggest a deficiency for the fetal liver to biotransform FX to NFX. Since the majority of this biotransformation occurs through CYP2D6 in humans (see Chapter 5, discussion), these results suggest the lack of orthologous enzyme activity in the fetal lamb. In human fetal liver, CYP2D6 mRNA has been detected however, CYP2D6 protein content was negligible (Treluyer et al., 1991; Hakkola et al., 1994). Using dextrorphan formation, via dextromethorphan *O*-demethylation (see Figure 4.10 in chapter 4), as a probe for CYP2D6 activity Treluyer et al., 1991, showed that human fetal liver samples accounted for only 1% of the adult values. A substantial metabolic contribution by the fetal liver to the FX-to-NFX pathway is therefore improbable.

Besides the difference in drug biotransformation, significant alterations in other pharmacokinetic properties of FX and NFX were observed between the fetus and the adult. The Vd_{ss} of FX in the fetus was 10-fold greater than that in the nonpregnant adult or the mother. In addition to the inherent high tissue distribution characteristics of FX, it is likely that the large volumes of amniotic fluid surrounding the fetus provide additional extravascular fluid space for FX fetal distribution. Another possibility is that the maternal

compartment, which acts as a sink, also contributes additional space for FX distribution from the fetus. Moreover, a remarkable difference in the weight normalized CL_{TB} of FX was observed between the fetus and the adult. Our maternal and fetal infusion studies in the pregnant ewes and their fetal lambs indicate that FX is transferred across the sheep placenta from both sides of the membrane. Placental transfer of FX from the fetus to the mother serves as the dominant removal process for FX from the fetus. Further experiments in neonatal lambs may confirm if the high CL_{TB} in the fetus will be lost at birth and the newborn must then eliminate FX using its own immature metabolic pathways.

Protein binding of both FX and NFX was significantly lower (higher f_u) in the fetus compared to the adult. This is in accordance with the low level of α_1-acid glycoprotein, which is the major binding protein for FX and NFX, in (human) fetal plasma (Wood and Wood, 1981). This implies that the fetus is exposed to more unbound drug (i.e. the pharmacological active moiety) if there are equivalent total drug concentrations between the maternal and fetal circulations. Therefore the fetus may be at a higher risk of experiencing toxicity if drug levels exceed the therapeutic window. Our experiments also showed that the CL_{renal} of FX and NFX was much higher than the corresponding values in adult sheep (Tables 6.2 and 6.3). However, the contribution of CL_{renal} to the overall elimination of FX was negligible and accounted for less than 1% of CL_{TB} in all animals. Comparable CL_{renal} values have also been observed in fetal lambs for the amine drug diphenhydramine (range 7.8 to 60 ml/h/kg; Kumar et al., 1997) and like FX and NFX they were also higher than in the adult. The average CL_{renal} values of two other amine drugs cimetidine and meperidine in fetal lambs are however, considerably higher (117 ml/h/kg and 186 ml/h/kg, respectively) than those of FX and NFX (Mihaly et al., 1983; Szeto et al., 1979). For both these drugs,

however, metabolism is negligible and renal excretion is the primary route of elimination. These findings for amine drugs are in contrast to acidic compounds such as valproic acid (Kumar et al., 2000b), diphenylmethoxyacetic acid (Kumar et al., 1999a) and acetaminophen (Wang et al., 1986) all of which exhibit very low CL_{renal} in fetuses. Robillard et al., 1977, reported that the renal clearance of inulin in fetal sheep ranged from 23.4 to 114.6 ml/h/kg. The CL_{renal} of FX and NFX in our fetal lamb experiments were highly variable, with some animals having values that exceed those reported in the literature for inulin clearance. This suggests that the fetal kidney may be able to eliminate FX and NFX by tubular secretion, which may partly explain the reason why R-FX was preferentially concentrated in fetal urine in higher amounts compared to S-FX.

Overall, we have observed that the fetus has 1) limited capability to metabolically biotransform FX to NFX, 2) negligible renal excretion of FX in urine, and 3) insignificant distribution of FX in amniotic fluid and fetal tracheal fluid (based on the results from Chapter 5). Unfortunately, the findings we have gathered so far are still far from explaining the fetal-to-maternal concentration gradient observed in the maternal infusion experiments (0.4 for total drug [bound plus unbound drug] and 0.7 for unbound drug). Apparently, other routes of elimination probably exist in the fetus. To further access the significance of fetal drug elimination by nonplacental routes, we attempted to calculate the placental and nonplacental clearances of FX in the ewe and the fetus from the maternal and fetal steady-state arterial plasma FX concentrations according to the two-compartment model described by Szeto et al., 1982b (Figure 6.13). This model is based on separate steady-state maternal and fetal drug administration and assumes bidirectional placental transfer and drug elimination from both maternal and fetal compartments. The model is defined by four clearance parameters,

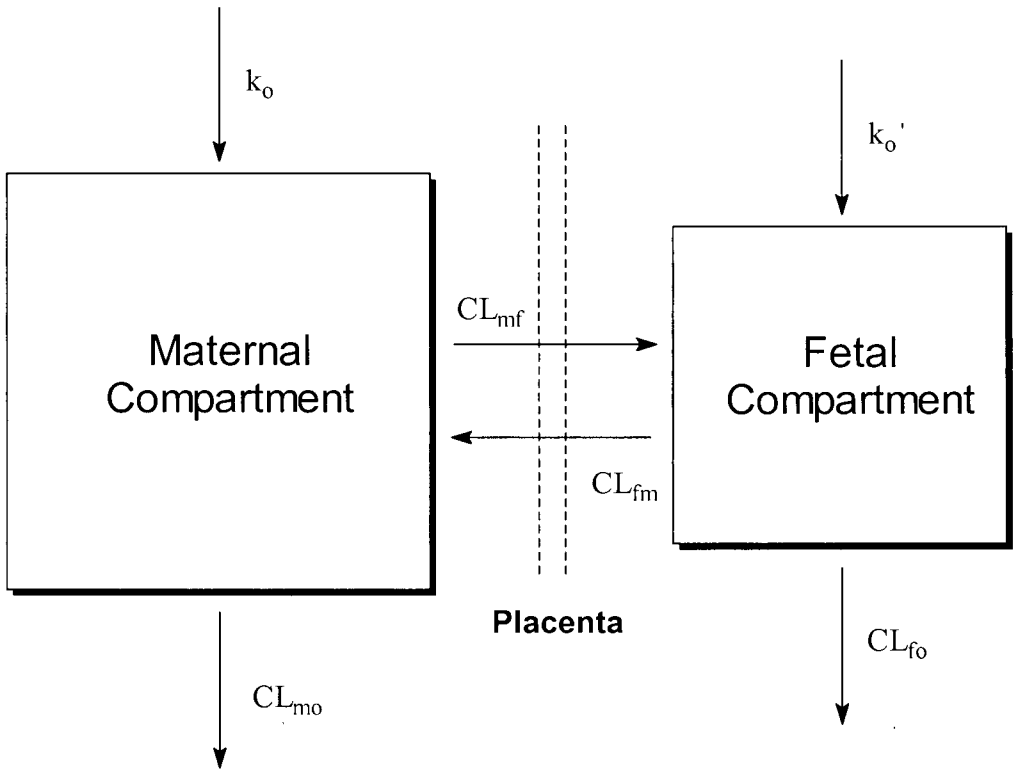


FIGURE 6.13 A representation of various placental and non-placental drug clearances in the 2-compartment pharmacokinetic model of the maternal-fetal unit (CL_{mo} – maternal nonplacental clearance; CL_{fo} – fetal nonplacental clearance; CL_{mf} – placental clearance from the mother to the fetus; CL_{fm} – placental clearance from the fetus to the mother; k_o – drug infusion rate to the mother; k_o' – drug infusion rate to the fetus). (Adapted from Szeto et al., 1982b).

including maternal-to-fetal placental clearance (CL_{mf}), fetal-to-maternal placental clearance (CL_{fm}), maternal nonplacental clearance (CL_{mo}), and fetal nonplacental clearance (CL_{fo}). Maternal and fetal total clearances of the total drug (CL_{mm} and CL_{ff}, respectively) equal the sum of their respective placental and nonplacental clearances. All these clearance parameters were calculated from the following equations as described previously (Szeto et al., 1982b):

$$CL_{mm} = \frac{k_o}{[C_m - C_f * (C_m'/C_f')]} \quad (6.1)$$

$$CL_{ff} = \frac{k_o'}{[C_f' - C_m' * (C_f - C_m)]} \quad (6.2)$$

$$CL_{mf} = CL_{ff} * \left(\frac{C_f}{C_m}\right) \quad (6.3)$$

$$CL_{fm} = CL_{mm} * \left(\frac{C_m'}{C_f'}\right) \quad (6.4)$$

$$CL_{mo} = CL_{mm} - CL_{mf} \quad (6.5)$$

$$CL_{fo} = CL_{ff} - CL_{fm} \quad (6.6)$$

The symbol k_o and k_o' denote the drug infusion rates to the mother and the fetus, respectively. C_m and C_f are the steady-state plasma drug concentrations in the mother and the fetus after maternal FX administration, respectively, and C_m' and C_f' are the steady-state maternal and fetal plasma drug concentrations, respectively, after fetal FX administration. Ideally, maternal and fetal drug infusions should be performed in the same animal to minimize the variability between animals. However, due to the long $t_{1/2\beta}$ and nonlinear profile of FX, not only is a much longer time required for the drug to reach steady-state (~4-5 $t_{1/2\beta}$), but a long wash-out period is also necessary between infusions (ideally 5 to 7 $t_{1/2\beta}$). This makes the experimental period too long to be practically performed in sheep during late

gestation. Further, dynamic physiological changes that occur in the fetus during the time interval between the two separate infusions may introduce substantial errors into the data due to considerable intra-animal variability. Experiments conducted on separate groups of animals may be devoid of such problems, but the data may suffer from severe inter-animal variability. For exploratory purposes, we attempted to combine the data from the previous maternal infusion study (Chapter 5) and the present fetal infusion study to calculate the placental and nonplacental clearance values. Because unparallel groups of animals are involved with this approach, naive averaged data (i.e. use the overall mean C_{ss} to fit into the equations) instead of the standard two-stage method (i.e. estimate individual clearances then the mean across the animals) was employed for the calculations. The estimates of maternal and fetal placental and nonplacental clearances of FX along with those of other drugs studied in pregnant sheep are presented in Table 6.5. Overall, FX follows the general trend for a number of other drugs in the maternal-fetal unit in that 1) fetal weight-normalized placental and nonplacental clearances are typically higher compared to mother, 2) the capacity of fetal nonplacental drug elimination is substantially higher compared to the mother, 3) CL_{fm} has a higher magnitude compared to CL_{mf} for most drugs where estimates are available. The low CL_{mo} for FX is consistent with its long $t_{1/2\ \beta}$ and slow rate of clearance (CL_{TB}) compared to other drugs. The relatively high fetal and maternal placental clearances of FX are in accordance with its high lipophilicity as indicated by a high octanol/water partition coefficient (log P = 4.05). Kim, 2000, reported a F/M ratio approximating unity in pregnant women dosed with FX, indicating unrestricted diffusion of FX across the human placenta. The finding of a high CL_{fo}, which is comparable to CL_{fm} in magnitude, is surprising considering our findings that most of the common fetal drug elimination pathways have only

TABLE 6.5 Average values of fetal and maternal placental (CL_{fm} and CL_{mf}, respectively) and nonplacental clearances (CL_{fo} and CL_{mo}, respectively) of FX and other drugs studied in pregnant sheep during late gestation.

Drug	Clearance (ml/min/kg)				Reference
	CL_{fm}[†]	CL_{mf}[†]	CL_{fo}[†]	CL_{mo}[‡]	
Fluoxetine[¶]	**140.5**	**108.7**	**156.9**	**5.6**	---
Acetaminophen	30.5	31.1	10.8	14.6	Wang et al., 1986
Diphenhydramine	214.4	50.3	109.8	36.6	Kumar et al., 1997
Fluoxetine[$]	96.3	60.2	8.5	35.6	Kim et al., 2004
Indomethacin[§]	10.0	-	0.6	-	Krishna et al., 2002
Labetalol	23.4	7.3	27.1	30.5	Yeleswaram et al., 1993b
Methadone	101.1	32.2	70.9	26.3	Szeto et al., 1982a
Metoclopramide	103.9	72.0	27.8	46.1	Riggs et al., 1990
Morphine	19.4	8.3	42.0	39.7	Szeto et al., 1982a
Ritodrine[§]	9.2	-	4.0	-	Wright et al., 1991
Valproic Acid	17.2	23.4	6.8	1.3	Kumar et al., 2000a

[†] Clearances are reported as ml/min/kg estimated fetal body weight.

[‡] Clearance is reported as ml/min/kg maternal body weight.

[¶] Determined based on k_o=6.19 mg/h, C_m=208.2 ng/ml, C_f=76.1 ng/ml obtained from the maternal steady-state drug infusions; and k_o'=2.1 mg/h, C_m'=33.4 ng/ml, C_f'=81.2 ng/ml obtained from the fetal steady-state drug infusion in separate groups of animals.

[$] Values are determined based on *i.v.* bolus administration and presented separately for S-FX and F-FX in the original article.

[§] Determined using the Fick principle and only fetal steady-state drug infusions.

limited contribution to the overall clearance of FX in the fetus.

Using a modified approach described for labetalol by Yeleswaram et al., 1993b, a different set of placental and nonplacental clearance values for FX (Table 6.5) have been obtained based on the *i.v.* bolus data (Kim et al., 2004). CL_{mo} obtained from our infusion experiments is much lower than that resulted from the *i.v.* bolus experiments (5.6 versus 35.6 ml/min/kg), a finding that is consistent with the nonlinear nature of FX pharmacokinetic where inhibition occurs upon prolonged drug administration. Accumulation of FX occurred in the maternal circulation in a disproportional manner secondary to enzyme inhibition, therefore more drug was available for placental transfer, which resulted in higher CL_{mf} and CL_{fm} compared to those estimated from the *i.v.* bolus data. Our infusion data yielded a CL_{fo} value that is substantially higher than that obtained following *i.v.* bolus administration (156.9 versus 8.5 ml/min/kg). There is no objective reason for this discrepancy, however it is speculated that a change in CL_{fo} might have occurred upon long-term infusion, which was associated with a decrease in CL_{mo} compared to acute FX exposure. However, it should be pointed out that in both *i.v.* infusion and *i.v.* bolus experiments, the administration of FX to the mother or the fetus took place on two separate occasions. The concentrations of FX achieved following maternal or fetal FX administrations were not identical (e.g. maternal FX concentrations following maternal FX administration were higher compared to those obtained after fetal FX administration) regardless of whether the same (the case for the *i.v.* bolus studies) or different (the case for the infusion studies) animals were used. This approach of calculating the placental and nonplacental clearance is problematic especially when clearance of FX is concentration dependent. Further experiments that involved simultaneous maternal and fetal infusions, which eliminate variability due to inter-occasional

and inter-subject differences, should allow better measurements of actual fetal and maternal nonplacental clearances. However, the technical difficulties of synthesizing sufficient amounts of stable isotope labeled FX (e.g. deuterated FX) for animal dosing must first be overcome before such experiments are possible.

Nevertheless, our data suggest that a significant portion of fetal drug elimination remains unexplored for FX. In the previous chapter it was proposed that a possible route of fetal drug elimination might involve uptake into the lungs, which act as a reservoir for many amine drugs including FX due to lyzosomal trapping (Daniel and Wöjcikowski, 1997). Uptake of FX from the blood circulation to the gut may also be possible. Recently our lab found that the AUC of diphenylmethoxyacetic acid, a metabolite of diphenhydramine, was significantly lower when diphenhydramine was given as *i.v.* bolus versus portal venous (*p.v.*) bolus injection in nonpregnant sheep (Kumar et al., 1999b). It was also found that the C_{ss} of diphenhydramine was 50% lower when the drug was given by *p.v.* infusion versus *i.v.* infusion of the drug (Kumar et al., 1999b). These data suggest the presence of gut extraction of diphenhydramine from the systemic circulation and that this functions as one of the major elimination pathways of diphenhydramine in sheep. However, the question of whether the fetal gut can function as an elimination organ remains to be elucidated. The mechanism of this gut uptake phenomenon is unknown and may involve active transporter systems such as P-glycoprotein. In the previous chapter we discussed the possibility of FX being a P-glycoprotein substrate and hence it would seem reasonable to speculate that gut uptake could potentially function as an elimination route in the fetus.

The presence of alternate pathways of biotransformation of FX to other metabolites apart from NFX (see Figure 1.3 in chapter 1) in the fetus is also possible. In humans, FX

undergoes phase II metabolism to form glucuronide conjugates which comprised 7-15% of the total radioactivity excreted in urine. In sheep, however, only low levels (< 0.5 % of total administered dose) of FX glucuronide conjugates were identified in adult sheep urine and virtually none was found in amniotic or fetal tracheal fluids (Kim et al., 2004). The later suggests that glucuronidation of FX does not occur in the fetus. FX also undergoes *O*-dealkylation to form *p*-trifluoromethylphenol (TFMP) in both humans and rats (Urichuk et al., 1997). The extent of FX metabolism via this pathway is not well understood compared to NFX. However, an *in vitro* human microsomal study revealed that a significant portion of FX can be *O*-dealkylated to TFMP (Liu et al., 2001). Table 6.6 lists the literature values for the kinetic parameters for FX *N*-demethylation and *O*-dealkylation in human liver microsomes. The kinetics of TFMP formation share remarkably similar affinity, capacity and intrinsic clearance characteristics to those of R-NFX. These data indicate that *O*-dealkylation is equally important to *N*-demethylation in the metabolism of FX. It has been shown that CYP2C19 and CYP3A4 are the major cytochrome P450 isoforms responsible for FX *O*-dealkylation. Although both isoforms are not reported to be expressed in human fetal liver (Treluyer et al., 1997; Hines et al., 2002), CYP3A7, an isoform of cytochrome P450 which is expressed specifically in human fetal liver, is estimated to account for approximately 50% (30-85%) of total P450 content in human fetal hepatic microsomes (Yang et al., 1994). Various studies have shown that both isoforms share similar catalytic activities and substrate specificities (Shimada et al., 1996; de Wildt et al., 1999a; Chen et al., 2000). Therefore, it is very likely that the fetal liver is also capable of biotransforming FX to TFMP. In fact, we have evidence from our preliminary analysis that TFMP was formed and concentrated in urine collected from fetal lambs. Further experiments are in process to

TABLE 6.6 Comparative kinetic parameters for FX *N*-demethylation and *O*-dealkylation in human liver microsomes.

Metabolic Pathway	K_m (μM)	V_{max} (pmol/min/mg)	V_{max}/K_m[‡] (μl/min/mg)	Reference
N-demethylation to R-NFX	5.2	37.0	7.2.	Ring et al., 2001
N-demethylation to S-NFX	0.18	3.4	19.3	Ring et al., 2001
O-dealkylation to TFMP	4.6	34.8[†]	7.6	Liu et al., 2001

[†] Original units were converted from pmol/min/nmol to pmol/min/mg using the conversion factor of 0.3 nmol/mg P450 protein (Woolf, 1999).

[‡] The ratio of V_{max}/K_m is equivalent to intrinsic clearance.

delineate the significance of formation of TFMP and its contribution to the overall elimination of FX in fetal and adult sheep.

Results from the maternal FX *i.v.* infusion experiments demonstrated that FX decreased uterine artery blood flow transiently and altered fetal blood gas status, which resulted in fetal hypoxemia and respiratory academia (Morrison et al., 2002). It was postulated that this effect involves FX-mediated inhibition of serotonin reuptake by platelets, resulting in a transient increase in maternal plasma serotonin following onset of maternal drug administration. The present study, however did not reveal much changes in fetal arterial blood gas status when FX was administered directly to the fetus via *i.v.* infusion. This could be due to high variability between animals such that a larger number of animals may be necessary to show a statistically significant difference (n=5 for fetal infusion compared to n=13 for maternal infusion). Nevertheless, the overall changes in the blood gas variables tended to be less with the fetal FX infusions and also to occur a bit later. For example, P_{O_2} dropped 2.8 mm Hg in 45 minutes after infusion in fetal infusion compared to 5.5 mm Hg in 5 minutes following maternal infusion; O_2 saturation fell 12.9 % in 4 hours after fetal infusion compared to 18.5 % in 5 minutes after maternal infusion; P_{CO_2} increased 3 mm Hg in 4 hours following fetal infusion compared to 4.5 mm Hg in 30 minutes post-dose. The delays in the occurrence of these effects suggest that it was the FX in the maternal circulation that elicited the changes via reductions in uterine blood flow as opposed to a similar effect on umbilical blood flow. In addition, the lower maternal FX and NFX concentrations following fetal FX infusion compared to those following maternal FX infusion (Figure 6.14) seem to correlate well with the milder effects on fetal blood gas status following fetal drug infusion.

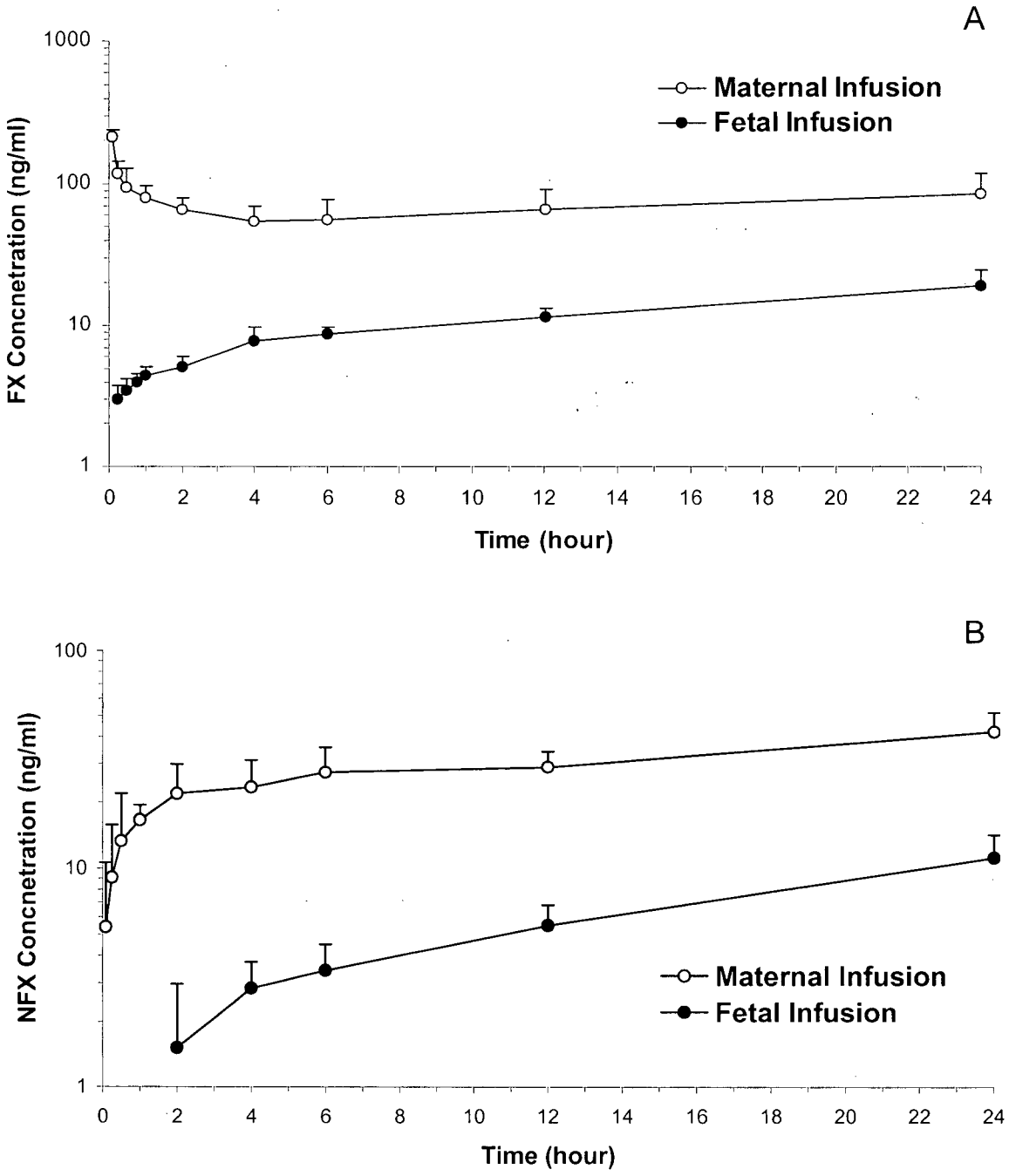


FIGURE 6.14 Mean maternal plasma concentration versus time profiles of (A) FX and (B) NFX during the initial 24 hours following maternal or fetal FX *i.v.* infusion.

6.4 Summary

In summary, this study has demonstrated that FX exhibits stereoselective disposition in fetal lambs similar to that observed in adult sheep. The differential steady-state concentrations of the FX enantiomers observed in the fetal circulation is likely the consequence of stereoselective protein binding in fetal plasma and differential maternal-to-fetal placental transfer of the enantiomers preceded by stereoselective metabolism in the mother. It appears that the fetal liver has limited metabolic ability to biotransform FX to NFX. Further excretion of FX and NFX in fetal urine also does not make a significant contribution to their overall clearance (CL_{TB}). Compared to maternal and nonpregnant adult sheep, weight normalized CL_{TB}, CL_{renal}, and Vd_{ss} are significantly higher in the fetus. Unbound fractions of FX and NFX are also higher in the fetus owing to the lower concentration of plasma protein in fetal plasma. Preliminary calculations for maternal and fetal placental and nonplacental clearances indicate that both placental and nonplacental routes are equally important in the fetal elimination of FX. Lung uptake via lyzosomal trapping, gut uptake by active transporters, and hepatic biotransformation to TFMP are potential routes of fetal nonplacental elimination of FX. However, this is only speculative at this stage and requires experimental data to be conclusively accepted. Alterations in fetal blood gas status were observed following fetal *i.v.* infusion of FX, although none of these achieved statistical significance due to variability between animals. Nevertheless, the data reveal that peak effects on fetal blood gas status were delayed and had a milder intensity compared to those observed following maternal *i.v.* infusion of FX, which supports the hypothesis of FX-elicited changes via reductions in uterine artery blood flow as opposed to an effect on diminishing umbilical blood flow.

CHAPTER 7

OVERALL SUMMARY AND CONCLUSIONS

FX is one of the most popular SSRIs used for the treatment of depression. Its use in treating depressed pregnant woman has increased substantially in recent years (Altshuler, 2002). A considerable amount of attention in the literature regarding the use of FX in pregnancy has been devoted to the assessment of birth outcomes following *in utero* FX exposure while studies that focus on pharmacokinetics during pregnancy are relatively sparse. In terms of the latter studies, none have addressed the stereoselective pharmacokinetics of FX nor the impact of pregnancy-related physiological changes on stereoselective drug disposition (Pohland et al., 1989; Heikkinen et al., 2003). Our laboratory is the first to initiate the study of stereoselective pharmacokinetics and placental transfer of FX and NFX enantiomers using the pregnant sheep model. Previous experiments were conducted using a single *i.v.* bolus FX administration, and primarily focused on the acute exposure and effects of the drug in both the mother and fetus late in gestation (Kim et al., 2004). In the present study, experiments were conducted to investigate the stereoselective pharmacokinetics of FX and NFX under steady-state condition in nonpregnant sheep and pregnant sheep at late gestation. The exposure of FX and NFX enantiomers to the fetus at steady-state was assessed by evaluating their placental transfer, and distribution in amniotic and fetal tracheal fluid. The pharmacokinetics of FX and NFX in fetal lambs were also studied in an attempt to explore the intrinsic components of fetal drug elimination via nonplacental routes. A summary of all the animal studies performed and the corresponding pharmacokinetic data are presented in Table 7.1.

TABLE 7.1 Mean (%CV) summary of plasma pharmacokinetic parameters for fluoxetine and norfluoxetine in sheep.

Chapter No./ Study Title	Dosing/ # of Animals	Analyte	$T_{1/2\beta}$ (h)	MRT (h)	C_{ss} (ng/mL)	AUC (μg*h/L)	Vd_{ss} (L/kg)	CL_{TB} (L/h/kg)	CL_{renal} (mL/h/kg)	f_u (%)	F/M ratio
Chapter 3 Steady-State Stereoselective PK Study in Nonpregnant Adult Sheep	70 mg *i.v.* LD, then 6.92 mg/h *i.v.* infusion × 8 days n=6	R-FX	18.4 (42)	24.6 (28)	35.8 (21)	6086 (22)	44.9 (25)	1.91 (32)	0.97 (41)*	2.42 (34)	n/a
		S-FX	14.8 (22)	34.9 (25)	115.2 (27)	18056 (30)	22.7 (37)	0.68 (41)	0.66 (58)*	0.71 (32)	n/a
		R-NFX	26.0 (22)	124.8 (4)	74.4 (28)	13658 (30)	n/a	n/a	1.46 (71)*	2.02 (23)	n/a
		S-NFX	21.4 (20)	108.5 (3)	68.8 (29)	12265 (26)	n/a	n/a	1.07 (53)*	1.14 (31)	n/a
	35 mg *i.v.* LD, then 3.46 mg/h *i.v.* infusion × 8 days n=5	R-FX	20.1 (42)	33.4 (60)	12.6 (37)	2289 (38)	76.7 (43)	2.55 (27)	2.64 (136)	4.33 (35)	n/a
		S-FX	19.7 (45)	44.1 (50)	49.4 (54)	8080 (53)	31.0 (44)	0.79 (35)	0.57 (67)	2.37 (55)	n/a
		R-NFX	31.0 (11)	126.1 (13)	31.9 (50)	6566 (49)	n/a	n/a	0.99 (108)	4.15 (39)	n/a
		S-NFX	26.1 (18)	105.7 (9)	25.9 (33)	5130 (35)	n/a	n/a	0.81 (93)	3.00 (38)	n/a
Chapter 4 Steady-State Stereoselective PK Study in Pregnant Sheep	70 mg *i.v.* LD, then 6.92 mg/h *i.v.* infusion × 8 days (Maternal Infusion) n=5	R-FX	31.9 (46)	40.2 (52)	41.1 (21)	7630 (23)	48.2 (52)	1.20 (27)	n/a	2.71 (18)	n/a
		S-FX	35.9 (51)	57.8 (42)	167.2 (46)	29343 (48)	20.2 (54)	0.38 (58)	n/a	0.83 (31)	n/a
		R-NFX	68.0 (26)	160.0 (8)	**	17999 (29)	n/a	n/a	n/a	1.94 (25)	n/a
		S-NFX	52.1 (45)	125.0 (10)	**	16502 (43)	n/a	n/a	n/a	1.38 (30)	n/a
Chapter 5 Placental Transfer Study at Steady-State	70 mg *i.v.* LD, then 6.92 mg/h *i.v.* infusion × 8 days (Maternal Infusion) n=5	R-FX	35.0 (67)	103.6 (13)	14.7 (44)	3059 (41)	n/a	n/a	n/a	n/a	0.39 (23)
		S-FX	36.7 (63)	99.2 (12)	61.4 (54)	12497 (57)	n/a	n/a	n/a	n/a	0.41 (20)
		R-NFX	54.4 (38)	94.5 (21)	**	5148 (38)	n/a	n/a	n/a	n/a	0.29 (31)
		S-NFX	52.5 (43)	93.0 (32)	**	5927 (43)	n/a	n/a	n/a	n/a	0.36 (25)
Chapter 6 PK Study in Fetal Lambs under Steady-State Condition	2.1 mg/h *i.v.* infusion × 4 days (Fetal Infusion) n=4	R-FX	48.9 (25)	25.4 (41)	33.4 (19)	3244 (16)	463.4 (13)	18.87 (23)	52.1 (97)	4.79 (14)	3.16 (31)
		S-FX	43.4 (15)	20.7 (26)	47.7 (6)	4938 (12)	245.3 (9)	12.25 (18)	33.8 (107)	1.27 (31)	2.72 (33)
		R-NFX	62.7 (12)	129.0 (10)	**	1313 (22)	n/a	n/a	30.3 (55)	6.41 (11)	0.62 (21)
		S-NFX	46.8 (12)	108.5 (7)	**	1100 (26)	n/a	n/a	25.1 (84)	5.09 (10)	0.62 (21)

* n=3 for this parameter; ** Steady-state was not reached at the end of infusion period
Other abbreviation used: *i.v.* = intravenous, LD = loading dose, n/a = not applicable, PK = pharmacokinetic.

In the nonpregnant animal study, enantiomers of FX and NFX were found to exhibit significant pharmacokinetic differences under steady-state conditions, confirming the observations made in previous *i.v.* bolus experiments (Kim et al., 2004). For FX, the R-enantiomer has a lower C_{ss} level but a longer $t_{1/2\beta}$ than the S-enantiomer while for NFX, both the R- and S-enantiomers have similar C_{ss} levels but $t_{1/2\beta}$ is slightly longer for R-NFX compared to S-NFX. FX and NFX also display stereospecific protein binding, with free fractions of the R-enantiomers that are about 2 to 3-fold higher than those of the S-enantiomers. In comparison to humans, quantitative differences exist but qualitatively the overall stereoselective disposition of FX and NFX are similar between sheep and humans. Using the literature reported value for hepatic blood flow in sheep, CL_{int}, a measurement of metabolic enzyme activity, was estimated for each FX enantiomers based on the present sheep data. It was found that CL_{int} of R-FX is about 50% higher than that of S-FX, which agrees with the findings from *in vitro* human microsomal metabolism studies (Stevens and Wrighton, 1993; Ring et al., 2001). The difference in CL_{int} between FX enantiomers indicates that in addition to stereoselective protein binding stereoselective metabolism also plays a pivotal role in the overall stereoselective disposition of FX. Comparison of pharmacokinetic parameters from the present infusion experiments to those from the previous *i.v.* bolus experiments (Kim et al., 2004) reveals a disproportional change in pharmacokinetics of FX in sheep upon long term administration, characterized by a decrease in CL_{TB} and increase in dose normalized AUC values. Interestingly, stereoselectivity is also evident in this dose-related phenomenon, with S-FX being more affected compared to R-FX. Similar dose-related changes in FX pharmacokinetics has been reported in humans previously (Altamura et al., 1994). It is generally believed this nonlinear nature of FX

pharmacokinetics is primarily due to CYP2D6 inhibition (Stevens and Wrighton, 1993; Ring et al., 2001), which infers not only that the source of nonlinearity observed in sheep is due to enzyme inhibition but also that sheep may possess enzyme(s) that are analogous to those in humans. Taken together the data from the *i.v.* infusion and *i.v.* bolus experiments (Kim et al., 2004), the stereoselective disposition of FX may involve the following mechanisms: 1) differential protein binding, 2) stereoselective metabolism, and 3) stereoselective enzyme inhibition.

The pregnant sheep study demonstrates that the stereoselective pharmacokinetics of FX and NFX under steady-state conditions are similar to those observed in nonpregnant animals. No remarkable changes in the S/R ratio of FX and NFX were observed. From the previous single *i.v.* bolus experiments (Kim, 2000), clearance of FX was observed to be higher in pregnant sheep compared to nonpregnant animals. Similar observations have been made in humans and CYP2D6 induction is likely the cause of increased drug clearance during pregnancy (Wadelius et al., 1997). However, this increase in clearance was not observed in our long-term infusion experiments. Instead, a decrease in clearance was found. It is speculated that the potent enzyme inhibitory effects of FX and NFX may have abolished any increased enzyme activity during pregnancy under steady-state conditions. The findings of decreased CL_{TB} of FX in pregnant sheep at steady-state together with the increased dextromethorphan/dextrorphan metabolic ratio in CYP2D6 PMs during pregnancy (Wadelius et al., 1997) suggests that the activity of CYP3A4, an enzyme secondary to CYP2D6 that is involved in the metabolism of both FX and dextromethorphan, is possibly attenuated during pregnancy. Future studies focused on the effect of pregnancy on the expression of drug metabolizing enzymes are imperative. *Ex vivo* equilibrium dialysis studies with sheep

plasma showed no difference in protein binding between pregnant and nonpregnant sheep for either FX or NFX. This finding is consistent with other reports regarding the lack of significant change in α_1-acid glycoprotein concentration during pregnancy (Wood and Wood, 1981; Krauer et al., 1984). However, the relationship between unbound fraction and concentration of the binding protein does not always correlate as exemplified by the alteration of binding of other α_1-acid glycoprotein bound basic compounds like lidocaine and propranolol during pregnancy (Wood and Wood, 1981).

Analysis of fetal plasma from the 8-day maternal FX infusion study for FX and NFX concentrations revealed that both the parent and metabolite exhibit a moderate degree of placental transfer upon chronic administration, with a F/M AUC ratio of about 0.40 and 0.33, respectively. The diffusibility of a drug across biological membranes is usually well predicted based on its physicochemical properties. Correlation analyses between F/M ratios and 1) partition coefficient, 2) unionized fraction (estimated from pKa), or 3) molecular weight from 37 different compounds (including FX and NFX), however, fail to reveal any significant relationships, implying that physicochemical properties alone cannot predict the ultimate extent of placental drug transfer. When the concentration is corrected for the difference in free fraction between the maternal and fetal compartment, the F/M ratio still remains less than unity, which suggests that other nonplacental routes of elimination for FX are present in the fetus. Analyses of amniotic and fetal tracheal fluids show that excretion of FX and NFX into these fluids result in levels not exceeding those in fetal plasma and significant accumulations are therefore unlikely. There are possibilities for fetal renal excretion, active placental transport, fetal metabolism, and placental metabolism although previous *in vivo* and *in vitro* experiments (Kim, 2000) suggest that the latter elimination

mechanism is probably of minor significance. In comparison to humans, the extent of placental drug transfer in sheep is generally less extensive, which probably due to the lower permeability characteristics of the sheep placenta. Attempts were made to draw correlations between animal and human placental drug transfer data. Of the nineteen compounds for which placental transfer has been studied in both sheep and humans, fourteen show a significant correlation for F/M ratios between these two species. While this correlation needs to be validated with more data, and the reasons for the other five compounds not following the correlation requires further elaboration, there appears to be an opportunity to establish a model for animal-to-human extrapolation in terms of placental transfer. Comparison of F/M ratios between enantiomers was also made. The F/M ratio does not differ between R-FX and S-FX, while the ratio is slightly higher (~25%) for S-NFX compared to R-NFX, indicating that NFX distributes between the mother and the fetus in a stereoselective manner. A clinically important consideration for this difference in F/M ratio lies in the fact that S-NFX is 20-times more active than R-NFX in terms of 5-HT reuptake inhibition (Wong et al., 1990), and hence the fetus is actually exposed to more pharmacological active species than one would normally predict based on racemic data. The mechanism behind this difference in S/R ratio between the mother and the fetus remains largely unexplored, but may involve stereoselective fetal drug elimination or stereoselective carrier mediated transfer (e.g. P-glycoprotein).

In the last experiment, the effect of FX on fetal blood gas status, as well as the stereoselective pharmacokinetics of FX and NFX enantiomers were studied following direct fetal FX administration via *i.v.* infusion. Alterations of fetal blood gas status, characterized by decreases in P_{O_2} and O_2 saturation, and increases in P_{CO_2} and pH, were noted following

fetal *i.v.* infusion of FX, and persisted for up to 24 hours before returning to pre-infusion levels. Due to variability between animals, the majority of these effects did not reach statistical significance. Nonetheless, the data reveal that the maximal effects on fetal blood gas status were slightly delayed and less intensive compared to those observed following maternal *i.v.* infusion of FX, which strongly suggests that it was the maternal circulating FX that elicited these changes via reductions in uterine artery blood flow as opposed to an effect on umbilical blood flow. The stereoselective pharmacokinetics of FX and NFX in fetal lambs were similar to those observed in adult sheep, but the extent of stereoselectivity in terms of the S/R ratio is less. The differential steady-state concentrations of the FX enantiomers observed in the fetal circulation is likely due to stereoselective protein binding in fetal plasma and differential maternal-to-fetal placental transfer of the enantiomers preceded by maternal stereoselective metabolism. Based on the F/M ratios for NFX (~0.62) following fetal FX infusion, it appears that the fetus has limited metabolic capability to biotransform FX to NFX, if any. It is not fully known, however, whether other metabolic pathways such as the formation of TFMP via *O*-dealkylation are functional in the fetus. The elucidation of the possible formation of TFMP in the fetal liver may provide important new information on the ontogeny of drug metabolizing enzymes during the fetal period. Renal excretion of FX and NFX do not appear to make a significant contribution to the overall clearance in the fetus (<1% of CL_{TB}). In comparison to maternal and nonpregnant adult sheep, weight normalized CL_{TB}, CL_{renal}, and Vd_{ss} are significantly higher in the fetus. Unbound fractions of FX and NFX are also higher in the fetus owing to the lower concentration of plasma protein in fetal plasma. In attempt to quantitatively assess the intrinsic capability of the fetus to eliminate FX, maternal and fetal placental and nonplacental

clearances were estimated based on the 2-compartment model for maternal-fetal unit (Szeto et al., 1982b). The results indicate that both placental and nonplacental routes are equally important in fetal elimination of FX. It is apparent that other elimination routes are present in the fetus but as of now these remain poorly understood. Among these, lung uptake via lyzosomal trapping, gut uptake by active transporters, and hepatic biotransformation to TFMP are potential routes of fetal nonplacental elimination of FX, but none of these has been systemically examined. Finally, the importance of stereoselectivity for the study of these nonplacental clearance mechanisms cannot be overemphasized.

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