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DRUG MILK TO SERUM RATIO PREDICTION AND ONTOGENY OF CYP3A CLEARANCE PATHWAY AS A MODEL OF DRUG EXPOSURE IN THE DEVELOPING RAT

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ABSTRACT OF DISSERTATION

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The Graduate School

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ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements of the degree of Doctor of Philosophy in the
College of Pharmacy
at the University of Kentucky

By
Maggie Magdi Abbassi

Lexington, Kentucky

Director: Dr. Patrick J. McNamara, Professor of Pharmaceutical Sciences

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Transfer of drugs into milk and the clearance of drugs in neonates are critical determinants of the exposure of infants to drugs in breast milk. Models predicting both parameters have been proposed. The objective of this dissertation is to test two models predicting milk to serum ratio and an ontogeny clearance model predicting clearance in the neonate.

Predicted milk to serum ratio (M/S) values were generated according to the Atkinson and Begg model. The model did not adequately predict M/S when comparing the predicted values to observed values in the literature.

The Fleishaker model was also tested. The model was able to predict whether the drugs appeared in milk by passive diffusion only or whether active transport processes were involved. This model, together with appropriate animal models, is useful in understanding the mechanism of drug transfer into milk.

An ontogeny model that predicts clearance was proposed earlier by our laboratory. In order to test the model prediction and assumptions of constant microsomal protein and constant Km for an enzyme-substrate system with age, the male rat was used as an animal model. The ontogeny of Cyp3a1, Cyp3a2, Mdr1a and Mdr1b mRNA was examined in the male rat liver and intestine. The ontogeny pattern of Cyp3a2 mRNA, protein and *in vitro* Cyp3a activity were found to be similar in male rat liver. The microsomal protein content was found to vary with age in the liver. Km was found to be constant with age for the midazolam 4-hydroxylation by male rat liver microsomes. Scaling factors that extrapolate adult clearance to infant clearance were calculated from *in vitro* data. The model did not predict the *in vivo* oral clearance of midazolam for day 7 and 21 age groups from the 112 day age group (adult). The assumption that intestinal availability in the rat pups and adults was equal to unity might not be true resulting in overprediction of rat pup clearance when compared to the adult. Intestinal first pass effect for midazolam in adult rats might be significant. More experiments are needed to further test the model adequacy in clearance prediction.

Keywords: Milk to serum ratio, Cyp3a, P-glycoprotein, Ontogeny, *In vitro-in vivo* extrapolation

Maggie Abbassi

July 16, 2007

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IN THE NAME OF ALLAH, MOST BENEFICIENT, MOST MERCIFUL

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Chapter 1. Introduction

Lactation is the ideal source of nutrition for a newborn. The exposure of neonates to drugs or other xenobiotics through maternal milk has been demonstrated. No conclusive approach is currently available to determine the extent of exposure that poses a risk to the neonate, due to the lack of data about the different factors affecting the exposure level. Ethical and logistical concerns hinder the direct determination of this risk in neonates *in vivo* via clinical trials. Several *in vitro* approaches and empirical models have been proposed in the literature for this purpose. In this dissertation we examine models for prediction of two critical factors in determining infant exposure, namely the maternal milk to serum ratio of a xenobiotic and infant clearance.

Chapter 2. Background

1 Lactation:

Xenobiotic exposure imposes potential risk to lactating neonates through mother's milk. It has been shown to have numerous health and emotional benefits for both the mother and the infant. Lactation protects the infant against various infections, enhances vaccine response, protects against allergies and enhances development and intelligence (AAP 1997). As for the mother it has been demonstrated to decrease risks of breast and ovarian cancer, improve bone remineralization and enhance bonding with the baby. Moreover, there are benefits for society in terms of reduced health care costs and reduced employee absenteeism due to child illness (AAP 1997).

Healthy People 2010 sponsored by U.S. Department of Health and Human Services (http://www.healthypeople.gov) aims at the increase of breastfeeding mothers from the baseline of 1998 of 64% in the early postpartum period to 75%, 29% at 6 months to 50% and 16% at 1 year of age to 25%.

Lactating mothers frequently take medications during lactation. It has been reported that 90-99% of lactating mothers will receive a medication in the first week postpartum, 17-25% by 4 months and 5% will need chronic administration of a medication (Bennett and World Health Organization. Regional Office for Europe. 1988).

Since the medications are more than likely to appear in the mother's milk, there is always a concern about the safety of the neonate. The risk versus benefit for both the mother and the infant are usually weighed by clinicians before administering medications during breastfeeding. However, sometimes the mother opts to either temporarily or totally stop breastfeeding when taking a medication or not to take the medication altogether. There is a dearth of knowledge about the safety of most of the drugs appearing in the milk to the neonate. The extent to which many of the drugs appear in the milk as well as the newborn handling of these drugs is largely unknown. In order to afford the mother a needed pharmacotherapy during breastfeeding or the infant a much needed healthy nutrition, more studies are needed which address the risks of exposure.

The American Academy of Pediatrics (AAP) classifies drugs taken during breastfeeding in terms of infant exposure and safety into the following classes (AAP 2001):

1-Cytotoxic drugs that may interfere with cellular metabolism of the nursing infant (e.g. cyclophosphamide and cyclosporine).

2-Drugs of abuse for which adverse effects on the infant during breastfeeding have been reported. (e.g. amphetamine and cocaine).

3-Radioactive compounds that require temporary cessation of breastfeeding (e.g. Iodine 131).

4-Drugs for which the effect on nursing infants is unknown but may be of concern (e.g. anti-anxiety, anti-depressants, antipsychotic and other drugs such as chloramphenicol).

5-Drugs that have been associated with significant effects on some nursing infants and should be given to nursing mothers with caution (e.g acebutolol and lithium).

6-Maternal medication usually compatible with breastfeeding.

The list detailing the drugs that are usually compatible with breastfeeding is the longest. Most of the effects cited for all of the above classes are either possible effects anticipated in the newborn, concerns about the concentration of the drug in either the milk or infant serum post the maternal dose or acute side effects in the infant (e.g. sedation, irritability, increased bowel movement…etc.). Although many of the drugs that might be of concern (class 4) are chronic drugs taken by the mother (anti-anxiety and antidepressants), no reported or possible effects are reported in the AAP publication. The long term effects of chronically taken drugs on the infant are lacking in the literature and in publications intended for guiding clinicians in prescribing drugs during breastfeeding (Riordan and Riordan 1984; AAP 2001; Hale 2004). More studies are needed to follow up on the chronic exposure of infants to drugs via breast milk and the possible risks associated.

The Food and Drug Administration has released a draft for the Guidance to Industry on Clinical Lactation Trials in 2005 (http://www.fda.gov/cber/gdlns/clinlacstud.pdf) which recommended mother-infant pair and mother only clinical trials on drugs. While these trials would be beneficial, there are many cases in which the risk of the drug in question is unknown and many logistical

hindrances in terms of having a significant number of subjects in the study who are taking the same medication under similar conditions to have definitive results.

Although some clinical data is currently available in the literature concerning the extent of appearance of some of the drugs in the milk and the serum levels and/or toxicity in the neonate, the scope is usually narrow and most of the time there are case study reports that are far from conclusive.

2 Infant Exposure:

Drug exposure of the infant through suckling has been described in the literature in various terms: Milk-to-serum ratio, dose in the mother's milk, neonatal serum concentration and exposure indices (McNamara and Abbassi 2004).

Milk-to-serum concentration ratio (M/S) has been reported in the literature frequently as a single-time point determination, or more usefully and less frequently, after steady state is reached in the milk and serum or as a ratio of AUC_{milk}/AUC_{serum} following a single dose (Wilson 1983; Wilson, Brown et al. 1985).

M/S is a step towards an estimate of the dose to which the infant is exposed but not a real measure of infant exposure. M/S is a reflection of the distribution of drug between maternal milk and serum both by passive diffusion and active transport mechanisms. Concentraton of a drug passively diffusing into milk is a function of an unbound, unionized concentration gradient set up by drug protein-binding in milk and serum, unionized fraction in milk and serum (function of pKa of drug and pH of medium) and partitioning into milk fat. Active transport is dependent on the interaction of drug in serum and the transporter proteins present on the mammary epithelial barrier.

The absolute dose which is administered to the neonate in milk reflects gastrointestinal and not necessarily systemic exposure. The dose is a function of drug milk concentration and the volume of milk consumed by the infant per time interval. Hence, lower milk consumption in general means lower dose exposure. It has been implied in the literature that if the percentage of the infant dose is 10% or lower than that of the mother then little or no risk is expected (Begg, Duffull et al. 2002). However, such an arbitrary cut-off has no evidence based clinical trials to support it.

The neonatal serum concentration best correlates with pharmacological effects and toxicity of the drug and thus is the determinant of systemic exposure. Understanding how much drug actually gets in to the circulation determines the extent/risk of exposure assuming that the pharmacodynamic effects of a drug in the neonate or infant are the same as those in adults. Such effects have not been very well studied in neonates (Berlin 2003). To be able to estimate the neonatal serum concentration either actual measurements have to be performed or accurate predicitions are needed. Actual measurements impose both ethical and logistical hindrances. If the drug is not meant for pediatric use or not expected to be used chronically by lactating mothers, there are ethical concerns about the justification of clinical studies in neonates. However, this does not mean that neonates and infants will not be exposed to such types of drugs. If the drug is actually measured in the neonatal serum then the frequency and volume of sampling are concerns, hence scarce sampling is usually employed and the need of rigorous analysis of such data arises to be able to understand the pharmacokinetics in such a population. Predicting neonatal serum concentration entails understanding the factors influencing these concentrations.

Systemic exposure can be expressed in terms of maximum serum concentration C_{max}, average serum concentration at steady state, or area under the concentration time curve AUC after a single dose.

For simplicity average serum concentration at steady state will be discussed to elucidate the factors affecting systemic exposure. It can be described by Equation 1:

$$C_{serum}^{infant} = \frac{F^{infant}}{Cl_{systemic}^{infant}} [D^{infant}] \qquad \text{Equation 1}$$

where C_{serum}^{infant} is the infant serum steady state concentration of a given drug , F^{infant} is the oral bioavailability of that drug since the drug is orally ingested via milk, D^{infant} is the dose of the drug ingested and $Cl_{systemic}^{infant}$ is the systemic clearance of the drug in the infant. Since the dose of the drug is ingested via milk, Equation 1 can be rewritten as Equation 2:

$$C_{serum}^{infant} = \frac{F^{infant}}{Cl_{systemic}^{infant}} \left[C_{milk}^{maternal} \left(\frac{V_{milk}}{\tau} \right) \right] \qquad \text{Equation 2}$$

where $C_{milk}^{maternal}$ is the steady state concentration of the drug in the mother's milk and $\left(\frac{V_{milk}}{\tau} \right)$ is the infant's milk consumption rate. Since the mother's milk concentration can be expressed in terms of maternal serum concentration and maternal milk to serum ratio as follows:

$$C_{serum}^{infant} = \frac{F^{infant}}{Cl_{systemic}^{infant}} \left[C_{serum}^{maternal} \left(\frac{M}{S} \right) \left(\frac{V_{milk}}{\tau} \right) \right] \qquad \text{Equation 3}$$

where $C_{serum}^{maternal}$ is the steady state concentration of the drug in the mother's serum, $\frac{M}{S}$ is the milk–to-serum concentration ratio of the drug. Assuming linear pharmacokinetics in the mother and infant equation 3 can be expressed as:

$$C_{serum}^{infant} = \frac{F^{infant}}{Cl_{systemic}^{infant}} \left[\frac{F^{maternal} D^{maternal}}{Cl_{systemic}^{maternal}} \left(\frac{M}{S} \right) \left(\frac{V_{milk}}{\tau} \right) \right] \qquad \text{Equation 4}$$

Equation 4 illustrates the different factors influencing average serum concentration. How each factor affects the infant serum concentration and the interplay between these factors have been previously discussed (Bailey and Ito 1997; McNamara and Abbassi 2004). It is evident that the infant serum concentration is dependent upon maternal and infant clearance, maternal and infant bioavailability, maternal dose, maternal milk-to-serum ratio, and the rate of milk consumption. An increase in the infant dose and bioavailability without other changes would increase the systemic serum concentration. An increase in maternal dose alone, or M/S alone would also increase the concentration. However, a high M/S ratio or a high maternal dose does not imply an increase in the infant serum concentration. If the systemic clearance in the neonate for such a drug is efficient, then the infant serum concentration might still be low. Hence, both the maternal and neonatal pharmacokinetics need to be understood. Although the pharmacokinetics for the drugs available in the market is readily available, how these parameters change in lactating mothers due to hormonal changes is largely lacking.

3 Exposure Indices:

Exposure indices have been reported in the literature to assess exposure in terms of maternal dose (EI_{dose}) or maternal serum concentration (EI_{conc}) (Ito and Koren 1994; McNamara and Abbassi 2004). Exposure indices can be described by the following Equations:

$$EI_{dose} = \frac{D^{infant}}{D^{maternal}} = \frac{F^{maternal}}{Cl^{maternal}} \frac{M}{S} \frac{V_{milk}}{\tau} \qquad \text{Equation 5}$$

$$EI_{conc} = \frac{\overline{C}_{infant}}{\overline{C}_{maternal}} = \frac{F^{infant}}{Cl^{infant}} \frac{M}{S} \frac{V_{milk}}{\tau} \qquad \text{Equation 6}$$

The EI_{conc} (concentration exposure index) was proposed by Ito and Koren (Ito and Koren 1994) and is more useful since it reflects systemic exposure. Using the above indices assumes steady state concentration in the mother and infant and linear pharmacokinetics.

The use of EI_{conc} is hindered by the fact that some of the parameters used to calculate it are missing. The maternal serum concentration can be measure and is reported in the literature for some drugs. However, the reported values are usually a Cmax or levels after a single dose. The infant consumption rate has been reported to be 150 ml/kg/day (Wilson, Brown et al. 1985). Trends of milk consumption have been also reported (McNamara and Abbassi 2004). The parameters that are harder to find and that would enable us to determine the risk associated with xenobiotic ingestion in the neonate are the M/S ratio and the infant systemic clearance. Hence, we are attempting to address the problem both at the maternal and the infant levels by examining M/S ratio and infant clearance predictions by models previously proposed in the literature.

4 Milk to Serum Ratio Predictions:

Milk to serum ratios (M/S) are based on total drug concentrations in their respective spaces. It would be beneficial if M/S ratios were accurately predicted by models, especially when drug administration to nursing mothers and infants is of ethical concern. Several models have been introduced in the literature to predict M/S ratios

(Rasmussen 1958; Rasmussen 1959; Meskin and Lien 1985; Fleishaker, Desai et al. 1987; Atkinson and Begg 1990; Agatonovic-Kustrin, Ling et al. 2002). These models assume that the appearance of most drugs in milk is dependent only on passive diffusion and hence correlate the observed M/S values in the literature to the physicochemical properties of the drug. If the drug passively diffuses into milk without the aid of transport, then it is assumed that only the free unbound, unionized drug in serum is capable of diffusion. Since both serum and milk have proteins, then M/S will depend on the extent of binding in both phases. Serum and milk have different pH values: serum pH is 7.4 and milk pH is 7-7.2 (Fleishaker, Desai et al. 1987). Due to that difference in pH, the degree of ionization of a drug will be different. Milk has high lipid content while serum lipids are generally lower, hence lipid partitioning will play a role in the M/S value.

Rasmussen (Rasmussen 1958; Rasmussen 1959) demonstrated that the extent of appearance of total drug concentration in the milk not only depends on protein-binding in serum and milk, but also depends on changes in pH. Basic drugs experience more ionization in the slightly more acidic milk pH in what is called “pH trap” or “ion trap”.

Meskin and Lien (Meskin and Lien 1985) introduced a quantitative structure activity relationship (QSAR) analysis of M/S ratios for a number of drugs where the M/S ratio was reported in the literature. They correlated the log M/S ratios available in the literature with Log P (log octanol-water partition coefficient, reflecting the degree of lipophilicity of a drug), the degree of ionization of a drug (log undissociated/dissociated drug) and the square root of the molecular weight since the lower the molecular weight, the easier it is for the drug to diffuse through biological membranes. They chose the best two linear regression correlations for basic and acidic drugs. Unexpectedly, the M/S ratios for both acidic and basic drugs showed negative dependence on LogP. They explained this phenomenon by the fact that the more lipophilic the drug, the more it will bind to serum proteins and the less it will be available unbound drug for diffusion into milk. However, they concluded that the confidence in the predictive power of such regression models should not be high since the M/S ratios used to build the correlations are from a variety of sources and experimental setups which might not be rigorous. Further criticism of the model is that the predictors used in the model are relevant only to

diffusion across membranes and do not take into account steady state conditions in milk and serum (Atkinson and Begg 1990).

Fleishaker et al (Fleishaker, Desai et al. 1987) proposed a model to predict M/S ratios based on the assumptions of passive diffusion and that the drug is at steady state condition in both milk and serum. The model is described by the following equation:

$$\frac{M}{S} = \frac{f_s}{f_m}\frac{f_{un,s}}{f_{un,m}}\frac{1}{S/W} \qquad \text{Equation 7}$$

where M/S is the milk to serum ratio, f_s, f_m are the free fraction of drug in serum and milk respectively, $f_{un,s}$ $f_{un,m}$ are the unionized fractions in the serum and milk respectively and S/W is the ratio of the drug concentration in skim to whole milk. S/W reflects not only lipid partitioning, but also takes into account protein binding as well, since only unbound drug is free to partition into lipids. In the above model, it is assumed that at steady state the free unbound, unionized, non-partitioned drug concentrations in serum and milk are equal. Fractions unionized in serum and milk are calculated using a rearranged Henderson-Hasselbach equation while the serum and milk protein binding and the skim to whole milk drug concentration ratio are measured *in vitro*. The advantage of this model is that it can account for the variability in M/S ratios due to differences in protein binding and milk composition reflected by S/W ratios. However, this approach may not account for any transport component and the *in vitro* determinations for the M/S predictions can be cumbersome because of all the parameters that need to be measured.

Atkinson and Begg established an empirical model (Atkinson and Begg 1990) to predict M/S values. To be consistent, we have modified Atkinson and Begg nomenclature from M/P (milk-to-plasma ratio) to M/S. The model assumes passive diffusion of drugs as well. It proposes a phase distribution equation that is dependent on the physicochemical characteristics of the drug where all the parameters can be predicted empirically from a further set of equations developed by the authors. The phase distribution equation is:

$$M/S_{phase} = f_s \times M_u/S_u \left[\left(0.955/f_m\right) + \left(0.045 \times \text{milk lipid } P_{app}\right) \right] \qquad \text{Equation 8}$$

$$\ln M/S_{phase} = a + b_1 \ln M_u/S_u + b_2 \ln f_s + b_3 \left[(0.995/f_m) + (0.045x \text{ milk lipid } P_{app})\right] \qquad \text{Equation 9}$$

where f_m and f_s refer to the unbound fractions in milk and serum, M_u/S_u is the ratio of unbound concentrations in milk and serum predicted from the Henderson-Hasselbach equation, and milk lipid P_{app} is the partitioning of drug into the milk lipid. The coefficients of f_m and milk lipid P_{app} represent the ratios of the aqueous phase and the lipid phase in milk respectively. Since the above phase equation assumes equal contribution of each factor to the predictive power of the equation, the authors modified it to a log-transformed model (Equation 9) so that multiple linear regression analysis can be carried out and each factor weighted accordingly. The M/S values gathered from the literature to build the model were either ratios of AUC (area under the curve) in milk to AUC in serum or single point measurements that were consistent in the literature and across different studies in order to satisfy the assumption of steady state conditions. The observed M/S values in the literature were correlated to the right-hand of Equation 9 to obtain the regression coefficients for a set of acidic drugs and a set of basic drugs. The equation obtained for basic drugs was further validated and modified for better prediction with a set of drugs that were not previously used to build the model (Begg, Atkinson et al. 1992). In general the equation for acidic drugs was reported to overpredict M/S ratio while for basic drugs M/S ratio values were underpredicted.

In vivo animal M/S models exist. The benefit of *in vivo* models is to identify the extent of accumulation of drugs in milk, whether it is high or low, but may not directly predict the human M/S ratios. The advantage of using an animal model over *in vitro* systems is that it accounts for all the biological changes during lactation and all the processes that can influence the M/S ratio that are absent *in vitro* (e.g. tissue binding). Animal models that have been previously used in the literature are cow, goat, rabbit, mouse and rat. The interest in cows and goats stems from the fact that they are dairy animals and their milk is consumed by humans. In our laboratory the rabbit and rat models have been used. The rabbit model has failed since it did not exhibit active transport for drugs that were actively transported in rats and humans (McNamara, Burgio et al. 1992). The rat proved to be more constant with human observations. Drugs that have high M/S ratio in humans that cannot be explained by passive diffusion alone and have to rely on active transport exhibited similar properties in rats (Pons, Rey et al. 1990;

McNamara, Burgio et al. 1992; McNamara, Meece et al. 1996a; Kari, Weaver et al. 1997; Gerk, Kuhn et al. 2001; Gerk, Oo et al. 2001). Knockout mouse models have proved valuable in demonstrating the role of specific transporters in drug transfer into milk (Jonker, Merino et al. 2005; Merino, Alvarez et al. 2006).

The use of passive diffusion models along with the appropriate animal model can predict active transport processes of drugs into human milk. For all the other drugs where active transport is absent or not significant, passive diffusion M/S prediction models are useful in estimating M/S values where clinical trials are lacking.

5 Neonatal Drug Clearance:

In addition to M/S ratio, the infant systemic clearance is the other signifcant parameter in Equation 6 that is difficult to measure *in vivo*. For the majority of drugs systemic clearance can be described by the sum of hepatic and renal clearances (Equation 10) assuming that no other organs are involved (e.g. lungs).

$$Cl = Cl_H + Cl_R \qquad \text{Equation 10}$$

where Cl is total body clearance, Cl_H is hepatic clearance and CL_R is renal clearance.

5.1 Hepatic Clearance:

Hepatic clearance is described by the well-stirred model (Equation 11). (Rowland, Benet et al. 1973; Wilkinson and Shand 1975; Pang and Rowland 1977)

$$Cl_H = \frac{Q_H f_u Cl_{\text{int}}}{Q_H + f_u Cl_{\text{int}}} \qquad \text{Equation 11}$$

where Q_H represents hepatic blood flow, f_u is the fraction of drug unbound in plasma and Cl_{int} is intrinsic clearance. According to this model, drugs can be broadly classified into 3 types. High extraction ratio drugs are those whose hepatic clearance is limited by hepatic blood flow. In this case $Q_H << f_u Cl_{\text{int}}$ and $Cl_H \approx Q_H$. Intermediate extraction drugs have hepatic clearances of about 0.3-0.7 of hepatic blood flow and hepatic clearances are dependent on hepatic blood flow, fraction unbound and intrinsic clearance and essentially the equation applies. Low extraction drugs have hepatic

clearance dependent on intrinsic clearance which is the rate limiting step ($Q_H >> f_u Cl_{\text{int}}$) and Equation 11 reduces to $Cl_H \approx f_u Cl_{int}$. For high extraction ratio drugs, Cl_H is dependent on hepatic blood flow in the infant, values of which have been previously documented (Edginton, Schmitt et al. 2006b). For the other 2 classes, intrinsic clearance and fraction unbound are critical in determining hepatic clearance. A model has been developed to predict the unbound plasma fraction of a drug in infants (McNamara and Alcorn 2002). Intrinsic clearance is dependent on both the ontogeny of hepatic enzymes and transporters. Hepatic clearance is usually described in terms of phase I, phase II and phase III reactions. Phase I and phase II are enzymatic reactions while phase III is clearance by hepatic transporters (Ishikawa 1992). Phase I is mediated by microsomal enzymes where the drug molecule is modified by addition or removal or exposure of a functional group. Cytochrome P450 enzymes are the superfamily responsible for the majority of the phase I reactions. Phase II enzymes conjugate the drug molecule or metabolite formed in phase I. Acetylation, glucuronidation, sulfation, methylation, glutathione conjugation and amino acid conjugation are reactions by which phase II enzymes make the drug molecule or metabolite mostly more water soluble. Transporters are now found to play an important role in hepatic clearance and hence have been designed as phase 0 and phase III metabolism (Ishikawa 1992; Vavricka, Van Montfoort et al. 2002). No chemical reaction occurs; the drugs are either taken up into the hepatic cells for interaction with enzymes, phase 0, or effluxed from the cells and away from the enzymes and made available to bile for excretion, phase III. The interplay between drug transporters and metabolizing enzymes is still under investigation (Wacher, Silverman et al. 1998; Benet, Cummins et al. 2004; Kurnik, Wood et al. 2006) and has not been very well characterized.

The ontogeny of hepatic enzymes and transporters needs further investigation for a full understanding of how these ontogeny processes affect neonatal clearance. *In vitro* and *in vivo* studies (Rich and Boobis 1997; Tanaka and Breimer 1997) have been performed to measure clearance or explore the underlying processes affecting clearance ontogeny (enzyme activity, protein and RNA ontogeny). *In vivo* experiments always suffer from ethical and logistical limitations (Rowell and Zlotkin 1997). It has to be noted that most of the work cited is for term neonates. However, preterm infant

clearance has been followed. For a number of pathways in a preterm infant, it has been shown from both *in vitro* and *in vivo* experiments that activity for a specific hepatic pathway is at most half of that in a full term infant (Edginton, Schmitt et al. 2006a). In this work we are interested in following hepatic enzyme ontogeny.

5.1.1 Cytochrome P450 Enzymes and Ontogeny:

The major cytochrome P450 enzymes in the adult human liver are CYP1A2 (12.7%), CYP2A6 (4%), CYP2B6 (0.2%), CYP2C (18.2%), CYP2D6 (1.5%), CYP2E1 (6.6%), CYP3A (28.8%) (Shimada, Yamazaki et al. 1994) where the numbers represent percentages of liver cytochrome P450 content. The contributions to drug metabolism have been determined to be CYP3A (52%), CYP2D6 (31%), CYP2C9 (10%), CYP1A2 (3%), CYP2E1(2%), CYP2A6 (2%), and CYP1A2 (3%) (Goodman, Gilman et al. 1996).

To be able to understand the development of hepatic clearance in the neonate, the ontogeny processes of different enzymes need to be understood on the functional level *in vitro* and *in vivo* and on the protein and mRNA levels. The molecular ontogeny of the enzymes gives an insight regarding the triggers of developmental processes.

Ontogeny of human hepatic enzymes has been extensively reviewed (Hakkola, Tanaka et al. 1998; de Wildt, Kearns et al. 1999; de Wildt, Kearns et al. 1999; Ring, Ghabrial et al. 1999; Gow, Ghabrial et al. 2001; Hines and McCarver 2002; McCarver and Hines 2002; Alcorn and McNamara 2002c; Johnson 2003).

Cresteil (Cresteil 1998) has classified the enzymes into 3 groups according to their ontogeny pattern. Fetal enzymes where the enzymes are detected in the fetal liver (CYP3A7 and CYP4A1), early neonatal enzymes that surge within hours after birth (CYP2D6, CYP2E1), and neonatal enzymes that develop later; CYP3A4 and CYP2C develop weeks after birth and CYP1A2 is the latest enzyme to develop.

Vieira et al (Vieira, Sonnier et al. 1996) investigated the ontogeny of CYP2E1. Protein levels were absent in fetuses but rose immediately after birth then gradually increased to reach adult levels in the age group of 1 to 10 years of age. CYP2E1 RNA was detectable in fetal samples. The surge in RNA was seen in the age group 1-3 months

and reached 50% of the adult levels at 3-12 months. Chlorzoxazone hydroxylation mirrored protein levels, reaching adult activity at 1-10 years.

Lacroix et al (Lacroix, Sonnier et al. 1997) demonstrated the decline of CYP3A7 and the surge of CYP3A4 after birth. The antibody used for protein quantification was found to be non-specific. Hence, total CYP3A was quantified. Protein levels were constant from early fetal stages (12 weeks) to adulthood. Fetal CYP3A4 RNA was 10% that of the adult, then reached a plateau at 1 week. The ability to differentiate between the development of CYP3A7 and CYP3A4 was achieved by using selective substrates. The 16-α-hydroxylation of testosterone was found to be selective for CYP3A7 over CYP3A4. This activity was high in fetal stages and reached a maximum at 1 week of age then declined to reach adult levels that were 10% those of the fetus. On the other hand, the 6-β-hydroxylation of testosterone, which was selective for CYP3A4, was low in the fetus and increased after birth to 30-40% of the adult activity from 8 days to 12 months, to reach adult levels by 1 year of age.

Treluyer et al (Treluyer, Jacqz-Aigrain et al. 1991) studied the development of CYP2D6 in human liver. Protein levels in fetuses and newborns less than 1 day of age had 5% CYP2D6 levels compared to adults. Subsequently, CYP2D6 level began to rise and was about 50% that of adult in age group 28 days-5years. CYP2D6 RNA development was different, it increased to levels higher than that of the adult from 1 day of age to 5 years of age in the samples used, then declined to adult levels. The activity of dextromethorphan o-demethylation was not more than 25% that of the adult at 1 week to five years of age.

Koukouritaki et al (Koukouritaki, Manro et al. 2004) studied the human hepatic ontogeny of CYP2C9 and CYP2C19. Protein and catalytic activity agreed for each enzyme. CYP2C9 was low in early fetal stages (1-2% of adult values) to reach 30% in the third trimester. From birth to 5 months of age, 50% of the samples measured had values similar to the adult. CYP2C19 had a different pattern. At 8 weeks gestation fetal levels were 12-15% that of the adult and did not change at birth. There was then a linear increase with age until 5 months of age. Adult values were reached after 10 years of age.

Sonnier and Cresteil (Sonnier and Cresteil 1998) showed the delayed ontogeny of CYP1A2 in the human liver. Protein and function represented by methoxyresorufin

demethylation where probed in human liver microsomes. At 1 month of age, CYP1A2 protein represented only 3% of the adult levels and reached 50% by one year of age. Methoxyresorufin demethylation appeared to mirror CYP1A2 protein ontogeny.

These *in vitro* experiments involved livers from infants where the exact circumstances of sample retrieval and storage are not very well documented (Stevens, Hines et al. 2003). The age groups used to characterize *in vitro* ontogeny also had wide ranges. The ages studied in the above studies were fetal less than 30 weeks, fetal greater than 30 weeks, neonatal less than 24 hours, 1-7 days, 1-3 months, 3-12 months, 9-12 months and greater than 1 year. In human studies, the available samples dictate the age ranges studied. In older children, the ranges even become wider (years). However, these wide ranges may obscure important developmental milestones especially in early stages when the infant is nursing (0-1 year). The shortcomings of such studies limit the use of and makes it difficult hard to extrapolate to *in vivo* clearance values (Burtin, Jacqz-Aigrain et al. 1994).

In vivo ontogeny of metabolism of cytochrome P450 substrates has been studied (Brash, Hickey et al. 1981; Pons, Carrier et al. 1988; Davis, Killian et al. 1989; Burtin, Jacqz-Aigrain et al. 1994; Jacqz-Aigrain, Bellaich et al. 1994; Falcao, Fernandez de Gatta et al. 1997; de Repentigny, Ratelle et al. 1998; Ito, Gow et al. 1998; Anderson, Gunn et al. 1999; Lee, Charles et al. 1999). The age ranges are usually limited to the infants that are on the investigated medication during the time of the study. The age ranges used are usually 1-7 days 7-28 days, 1-3 months and 3-12 months. Many times it is just one age range, as in the case of premature neonates that need medications directly after birth and can be observed until one month of age. Again, these wide ranges again limit the ability to fully characterize the developmental activity of a certain pathway. In addition, limited drugs are administered to neonates and hence more than one specific probe for the same pathway might not be feasible to study. The limitations of both the *in vitro* and *in vivo* studies in humans create the need for an animal model where the developmental processes can be extensively studied.

5.1.2 Phase II Enzymes and Ontogeny:

Phase II enzymes are classified into glucuronosyltransferases (UGTs), sulfotransferases (STs), N-acetyltransferases (NATs), glutathione-S-transferases (GSTs), O-,S- and N-methyltransferases (MTs) (Johnson 2003).

The development of UGTs has been reviewed by de Wildt et al (de Wildt, Kearns et al. 1999). According to their summary of UGT *in vitro* ontogeny, UGT1A1 can be described as an early neonatal enzyme. UGT1A3 in fetal and early neonatal activity is around 30% of the adult. UGT1A6 is present in the fetus up to 10% that of the adult and reaches 50% by 6 months of age. There is little known about the *in vivo* development of UGTs.

Pacifici et al (Pacifici, Franchi et al. 1988) studied fetal hepatic GSTs. The method used was non-specific to individual GST enzymes. It was reported that the fetal GST activity was around 60% that of the adult. Strange et al (Strange, Howie et al. 1989) studied the ontogeny of GSTA1, GSTA2, GSTM and GSTP1. GSTA1 and GSTA2 were detectable as early as 10 weeks gestation and increased to adult levels within 1-2 years of age. GSTM was lower during gestation and increased to adult levels at birth. GSTP1 was high in fetal samples and decreased towards birth and was not detectable in adults.

Pacifici et al (Pacifici, Franchi et al. 1988) examined sulfotransferase activity by the sulfation of 2-naphthol. The fetal activity was about 33% that of the adult. SULT1A3 expression and activity were found to be higher in fetal liver than adults (Cappiello, Giuliani et al. 1991; Pacifici, Kubrich et al. 1993). SULT1A1, however was higher in adult livers and probably had delayed ontogeny (Cappiello, Giuliani et al. 1991; Gilissen, Hume et al. 1994). SULT2A1 activity and protein increased in the second half of gestation to close to adult levels in the newborn (Barker, Hume et al. 1994).

There is little known about the ontogeny of NATs, despite their known importance in drug metabolism. Fetal livers were shown to have NAT activity of p-aminobenzoic acid that was 28% of adults (Pacifici, Bencini et al. 1986).

It is clear from the above studies that the development of cytochrome P450 and phase II enzymes in the liver is different from one enzyme to the next, even within the same family. Consequently, ontogeny of clearance of a certain xenobiotic will be

dependent on the elimination pathway and cannot be generalized for all drugs. Most of the studies targeted at studying ontogeny were *in vitro* studies and hence several approaches are taken to extrapolate the *in vitro* activities to *in vivo* clearance that can be useful in determining infant exposure.

5.2 Renal Clearance:

Renal clearance is described by three mechanisms; glomerular filtration, renal secretion and renal reabsorption. Glomerular filtration is a passive diffusion process through the glomeruli of unbound molecules. Renal secretion and reabsorption are carried out by the tubules and involve transport processes.

The ontogeny of glomerular filtration rate (GFR) in human fetuses and neonates has been studied. GFR development is dependent on nephrogenesis in utero that starts at 8-9 weeks gestation and reaches completion at 36 weeks. Postnatal GFR is dependent on blood flow (Robillard JE 1999; Kearns, Abdel-Rahman et al. 2003; Solhaug, Bolger et al. 2004). GFR is 2 to 4 ml/min/1.73m^2, normalized to adult body surface area in neonates, and is lower in preterm neonates. It increases rapidly in the first 2 weeks postnatal and then assumes a gradual increase to reach adult levels by 1 year of age (Arant 1978; van den Anker, Schoemaker et al. 1995; Kearns, Abdel-Rahman et al. 2003). However, when normalized to bodyweight, GFR in the neonate appears to have similar values to that in the adult (Rodvold, Everett et al. 1997). Renal tubular secretion represented by para-aminohippuric acid clearance follows a similar pattern (Kearns, Abdel-Rahman et al. 2003). The ontogeny of transporters in renal tubules is not very well studied. The ontogeny patterns of each transporter will affect elimination rate of the involved drugs.

5.3 Models for Predicting *In Vivo* Clearance:

5.3.1 *In Vitro In Vivo* Correlation:

In vitro in vivo correlation has been widely used to predict hepatic clearance of drugs (Hoener 1994; Iwatsubo, Hirota et al. 1997; Ito, Iwatsubo et al. 1998; Obach 1999; Zuegge, Schneider et al. 2001; Shiran, Proctor et al. 2006).

The prediction of hepatic clearance of a compound by a certain enzyme starts by determining the *in vitro* intrinsic clearance of that compound, which is independent of other physiological factors. Assuming that the clearance of this compound is independent of hepatic transporters, then the *in vitro* intrinsic clearance can be scaled to liver intrinsic clearance and *in vivo* clearance by a variety of scaling factors: liver weight, blood flow, hepatocyte number and microsomal protein yield. In addition to scaling factors, a liver model is needed to predict the *in vivo* clearance (Houston 1994). Limiting factors to *in vitro in vivo* correlations are the presence of pathways not accounted for in the correlation, interindividual variability, transport processes in the hepatocytes, invalid assumptions by the model used to scale intrinsic clearance to hepatic clearance e.g. rapid equilibrium of drug between blood and hepatocytes (Iwatsubo, Hirota et al. 1997). Other limitations are dependent on the system used to predict intrinsic clearance, e.g. microsomes versus hepatocytes.

The *in vitro* intrinsic clearance is typically calculated from enzyme kinetics, as the ratio of the terms of Michaelis-Menten enzyme kinetics parameters V_{max} (maximum enzyme rate) and K_m (substrate concentration at half the maximum velocity and indicates the molecule-enzyme affinity) in the linear range of the reaction.

Other approaches for *in vitro in vivo* extrapolation have been described (Obach 1999; Lau, Sapidou et al. 2002; Niro, Byers et al. 2003).

Obach (Obach 1999) had showed good agreement between *in vitro* predicted clearance values and *in vivo* observed values for neutral, acidic and basic compounds, using both the hepatic well-stirred model and the parallel tube model when protein binding in microsomes and blood were considered. Several other studies demonstrated the applicability of *in vitro in vivo* correlation methods for hepatic clearance prediction

(Hoener 1994; Iwatsubo, Hirota et al. 1997; Carlile, Hakooz et al. 1999; Soars, Burchell et al. 2002; McGinnity, Soars et al. 2004).

In vitro in vivo extrapolation methods were also examined for the possible application in the fields of enzymatic induction and drug-drug interactions (Burtin, Jacqz-Aigrain et al. 1994; Bertz and Granneman 1997; Kedderis 1997; Andersson, Bredberg et al. 2004; Ito, Brown et al. 2004).

These extrapolations are mostly applied to predict adult *in vivo* clearance for a certain drug. In this dissertation we are examining an ontogeny model that applies *in vitro in vivo* extrapolation priniciples to predict *in vivo* infant clearance from adult clearnance for a specific pathway.

5.3.2 Physiologically Based Pharmacokinetic (PBPK) Models:

Physiologically based pharmacokinetic (PBPK) models describe the pharmacokinetics of a drug in the context of physiologically and anatomically based body compartments. The body compartments are based on body organs, tissues, fluids and systems and the flow between the compartments is based on blood flow to the different organs (Nestorov 2003). The equations describing these models involve the description of mass transport of a compound between these compartments. Parameters used include body weight, organ weight/volume, organ blood flow, tissue composition, interstitial spaces, tissue permeability, binding and partition of the compound (Edginton, Schmitt et al. 2006b). These models assume flow rate limited drug distribution and are useful in prediction of drug concentrations in various tissues especially for lipophilic drugs. They require intrinsic clearance estimation to be able to predict hepatic clearance. A disadvantage of these types of models is that it is compound specific and hence a model needs to be developed for each compound (Andersen 1995).

5.3.3 Allometric Scaling:

Allometric scaling employs the basic allometric equation:

$Y=a\ BW^{b}$ Equation 12

where Y is the biological variable, BW is body weight, a is the allometric coefficient and b is the allometric exponent. Allometric scaling has been employed to extrapolate animal data to human data, recognizing the similarities between species in the aspect that various biological variables are correlated to body weight (Hu and Hayton 2001). *In vivo* clearance in humans has been extrapolated from animal *in vivo* clearance using allometric scaling (Boxenbaum 1982; Lave, Dupin et al. 1997; Wajima, Fukumura et al. 2003; Ward and Smith 2004; Ito and Houston 2005; Mahmood 2005). Allometric scaling has been employed to predict clearance in neonates and children as well where clearance for example can be calculated from adult clearance based on the ratios of body weights (Hayton 2000; Knibbe, Zuideveld et al. 2005; Bjorkman 2006; Johnson, Rostami-Hodjegan et al. 2006). However, in many cases, *in vitro* correlations and physiologically based pharmacokinetic (PBPK) modeling were found to be superior in predicting clearance (Ito and Houston 2005; Johnson, Rostami-Hodjegan et al. 2006).

5.3.4 Ontogeny Model:

The model examined in this dissertation is a clearance ontogeny model that was proposed in our lab to predict infant intrinsic clearance based on *in vitro* infant and adult data (Alcorn and McNamara 2002d). The model utilizes the *in vitro in vivo* correlation approach to predict *in vivo* clearance in neonates from *in vitro* data from both neonates and adults.

For a hepatically cleared, low extraction ratio drug, hepatic clearance can be described as follows according to the well-stirred model and assuming clearance by multiple pathways:

$$Cl_H = f_u\, Cl_{\text{int}} = f_u \sum_{j=1}^{n} Cl_{\text{int}(j)} \quad Q >> f_u\, Cl_{\text{int}} \qquad \text{Equation 13}$$

Intrinsic clearance has been defined in terms of enzyme kinetics as follows:

$$Cl_{int(j)} = \frac{V_{max(j)}}{K_{m(j)} + f_u C} \quad \text{Equation 14}$$

where $Cl_{int(j)}$ is the intrinsic clearance for a given pathway j, Vmax (j) is the maximal enzyme activity for this pathway and Km is the drug concentration at half maximal activity, fu is the fraction unbound of drug and C is the drug concentration. Under linear conditions $Km >> f_u C$ and hence equation 14 reduces to:

$$Cl_{int(j)} = \frac{V_{max(j)}}{K_{m(j)}} \quad \text{Equation 15}$$

To extrapolate the *in vitro* intrinsic clearance to *in vivo* intrinsic clearance, a hepatic scaling factor (HSF) is needed. *In vitro* Vmax values are normalized to mg microsomal protein. If the Vmax and Km measurements determined *in vitro* are performed in a microsomal system, then the scaling factor is mg microsomal protein per gram liver normalized to body weight. Thus Vmax is now normalized to whole body and the intrinsic clearance for the whole body (*in vivo* intrinsic clearance) is expressed as:

$$Cl^{*}_{int(j)} = HSF \frac{V_{max(j)}}{K_{m(j)}} \quad \text{Equation 16}$$

The above equation is valid for both infants and adults:

$$Cl^{*infant}_{int(j)} = HSF^{infant} \frac{V^{infant}_{max(j)}}{K_{m(j)}} \quad \text{Equation 17}$$

$$Cl^{*adult}_{int(j)} = HSF^{adult} \frac{V^{adult}_{max(j)}}{K_{m(j)}} \quad \text{Equation 18}$$

Assuming Km(j) to be similar for both infant and adult, and dividing Equation 17 by Equation 18 and rearranging, the infant intrinsic clearance is calculated as:

$$Cl^{*infant}_{int(j)} = \frac{HSF^{infant}}{HSFadult} \frac{V^{infant}_{max(j)}}{V^{adult}_{max(j)}} Cl^{*adult}_{int(j)}$$

$$= RHSF_t \frac{V^{infant}_{max(j)}}{V^{adult}_{max(j)}} Cl^{*adult}_{int(j)} \quad \text{Equation 19}$$

where $RHSF_t$ is the relative hepatic scaling factor for a given age t. Since Vmax is assumed to be proportional to the amount of active enzyme present and proportional from one substrate to the next for the same enzyme, then the ratio of Vmax of the infant to that of the adult is considered to reflect the ontogeny of activity for that pathway and is termed ontogeny scaling factor (OSF). Equation 19 can then be written as:

$$Cl^{*infant}_{int(j)} = RHSF_{(t)} OSF_{(j,t)} Cl^{*adult}_{int(j)} = ISF_{(j,t)} Cl^{*adult}_{int(j)} \quad \text{Equation 20}$$

where ISF (j,t) is the infant scaling factor and is equal to the product of RHSF and OSF, for a certain pathway j at a certain age t.

The model further proposed modeling liver and body weight as a function of age. ISF for a certain pathway would also be modeled as a function of age using existing *in vitro* data such that no *in vitro* experiments would be required to predict clearance for a certain age in which *in vitro* parameters were not determined.

Hence the model assumptions can be summarized as follows:

1. Drug has a low extraction ratio.
2. Infant and adult share the same enzymes and transporters in metabolizing the same drug.
3. Microsomal protein content normalized to liver weight is constant and independent of age.
4. Km for an enzyme-substrate system remains constant throughout development.
5. Vmax reflects the amount of functional enzyme and is proportional from one substrate to the next for the same enzyme along development.
6. Hepatic Clearance is governed by hepatic metabolic enzymes and not hepatic transporters.

The validity of assumptions 3 and 4 will be examined in this dissertation.

6 Experimental Evidence:

6.1 Rat as an Animal Model for Ontogeny Model Validation:

Rat and human cytochrome P450 orthologues share some substrate specificity and regulatory mechanisms, yet major differences exist (Zuber, Anzenbacherova et al. 2002). The rat is widely used as an animal model in toxicology studies (NTP 2007a; NTP 2007b; NTP 2007c). However, the purpose of using the rat is not to extrapolate rat ontogeny data into human ontogeny data. The rat will be used as a well studied animal model in drug metabolism to verify the assumptions of the model with defined age groups and exhaustive sampling. Performing such studies in humans is very difficult if not impossible. Thus using the rat as a model is a proof of concept for the model assumptions.

6.2 Cytochrome P450 3A and P-glycoprotein:

Cytochrome P450 3A is the focus of this research. In humans, it represents 28.8% of the liver cytochrome P-450 content and metabolizes 52% of the xenobiotics (Shimada, Yamazaki et al. 1994; Goodman, Gilman et al. 1996). In the human intestine, although CYP3A is around only 1% that of the liver, it still reperesents 82% of the total P450 content and contributes significantly to first pass metabolism for orally administered drugs (Paine, Hart et al. 2006).

P-glycoprotein (P-gp, ABCB1) is a drug efflux transporter of the ATP Binding Cassette (ABC) family. It is responsible for drug resistance in tumor cells and was first identified in drug resistant mutated Chinese hamster ovary cells (Juliano and Ling 1976). P-gp has a wide role in drug disposition in the body due to its wide expression and wide substrate specificity. It is expressed in tumor cells and in normal tissue in hepatocytes on the canalicular surface, the apical surface of renal tubular epithelial cells, brush border of intestine, placenta and the apical surface of endothelial cells forming blood-brain barrier (Thiebaut, Tsuruo et al. 1987; Cordon-Cardo, O'Brien et al. 1990). The role of P-gp in drug pharmacokinetics has been elucidated using *Mdr1a/1b* knockout mice (Schinkel,

Mayer et al. 1997), where biliary and intestinal excretion of [H^3]-digoxin were shown to be decreased. Therefore, the levels of P-gp in liver, intestine and kidney, have an effect on drug elimination.

CYP3A4 and P-gp share a wide substrate similarity (Wacher, Silverman et al. 1998) and hence the interplay of CYP3A4 and P-gp in both intestine and liver affects drug disposition (Wacher, Silverman et al. 1998; Benet, Cummins et al. 2004). Therefore, in addition to characterizing the ontogeny of Cyp3a in the rat, P-gp RNA development will also be characterized to examine if there is any correlation between the developmental pattern of their transcripts.

The equivalent rat isoforms of human CYP3A4 are both Cyp3a1 and Cyp3a2. It has been demonstrated that Cyp3a2 is sex-dependent, being predominant in adult male rat livers and absent in adult female rat livers (Ribeiro and Lechner 1992). There are substrate specificity similarities between human CYP3A4 and rat Cyp3a1/ Cyp3a2 e.g. midazolam, 6β-hydroxylation of testosterone (Kobayashi, Urashima et al. 2002) and tamoxifen (Kim, Suzuki et al. 2003). Induction by dexamethasone was observed for both human and rat CYP3A isoforms (Lu and Li 2001). Several studies focusing on the ontogeny of Cyp3a1/2 in the rat were conducted. Inducibility and postnatal development were studied (Johnson, Tanner et al. 2000; Kawai, Bandiera et al. 2000). There have been conflicting reports about hepatic enzyme levels of Cyp3a1 and Cyp3a2. Cyp3a1 was reported to be constitutive in adult male and female rat livers (Simmons and Kasper 1989), while in other reports it has been reported to be totally inducible (Gonzalez, Song et al. 1986). For postnatal development of Cyp3a2, studies have shown that protein and mRNA levels in male neonate rat livers (22 days) are comparable to (Kawai, Bandiera et al. 2000) or higher than (Wright, Edwards et al. 1997) male adult levels. Another study (Gonzalez, Song et al. 1986) reported Cyp3a2 mRNA levels to be lower than adult male levels. More studies need to be done to clarify the ontogeny of Cyp3a1 and Cyp3a2 in rats. P-gp mRNA, expressed by *Mdr1a/Mdr1b* in the rat, was detected in rat kidney, liver, small and large intestine and brain (Brady, Cherrington et al. 2002). Ontogeny of P-glycoprotein mRNA levels has been studied in male Wistar rats (Matsuoka, Okazaki et al. 1999; Rosati, Maniori et al. 2003). Characterization of parallel development of P-glycoprotein with Cyp3a in the rat is our current goal.

6.3 Midazolam as a Probe for Cyp3a:

Midazolam has been widely used as a probe for Cyp3a metabolism in human and rat *in vitro* and *in vivo* (Kobayashi, Urashima et al. 2002; Galetin and Houston 2006; Krupka, Venisse et al. 2006; Kumar, Mann et al. 2007; Matsuda, Nishimura et al. 2007; Ryu, Song et al. 2007; Zhang, Tan et al. 2007). It has the advantages of being specific to Cyp3a and not endogenous (unlike testosterone) and not affected by transporters (unlike erythromycin). Kobayashi et al (Kobayashi, Urashima et al. 2002) have shown that in rat-expressed enzyme systems the 4-hydroxylation of midazolam is specific to Cyp3a2 and formed to a less extent by Cyp3a1 (at least 2.5 fold). Midazolam has been shown to be a highly permeable substrate of P-gp (Tolle-Sander, Rautio et al. 2003) and hence its permeability is not affected by P-gp expression. Another property needed for the drug to be used as a probe to predict clearance of a certain pathway is that it should have a low extraction ratio in order to be able to calculate intrinsic clearance from *in vitro* data. Midazolam has an intermediate extraction ratio (Paine, Shen et al. 1996) and thus hepatic blood flow would have an effect on clearance. To be able to calculate intrinsic clearance *in vitro* and correlate it to *in vivo* clearance without measuring hepatic blood flow, midazolam will be administered orally. Midazolam is known to be extensively metabolized by intestinal CYP3A in humans such that the intestinal availability after oral administration (F_G) is about 0.5 (Gorski, Vannaprasaht et al. 2003). In rats, F_G has been reported to be 1 (Kotegawa, Laurijssens et al. 2002; Uhing, Beno et al. 2004; Kanazu, Okamura et al. 2005) which will enable the prediction of oral clearance based only on hepatic intrinsic clearance.

Chapter 3. Hypotheses and Specific Aims

We are proposing here to experimentally examine models that have been previously developed in our lab to predict M/S ratio and neonatal clearance. Additionally an empirical model predicting M/S ratio from the literature will be examined. The purpose is to test the validity of the underlying assumptions of the models and/or their predicitivity when compared to observed values in the literature.

To test the M/S and ontogeny clearance models we propose the following hypotheses:

Hypothesis 1:

Most drugs diffuse into milk passively and their milk to serum ratios can be readily predicted using passive diffusion models.

Specific aim 1A: To test the predictivity of the Atkinson and Begg model by calculating the M/S ratios for a number of drugs not used in building the model and comparing them to observed M/S values from the literature. The model should predict the observed M/S values provided that no active transport mechanism exists for these drugs. If successful, the model could be a great asset in assessing neonatal exposure to drugs appearing in the milk by passive diffusion without having to perform experimental measurements.

Specific aim 1B: To compare calculated M/S ratios of a number of drugs based on *in vitro* determination of serum and milk unbound fractions and skim to whole milk ratios using Fleishaker's model, to observed M/S ratios *in vivo* from the literature. If drug appearance in milk were limited to passive diffusion, the parameters in the model should account for the relatively high M/S ratio. An additional value of this model is that it can account for interindividual differences in milk and serum composition.

Hypothesis 2:

mRNA expression levels of Cyp3a1, Cyp3a2, Mdr1a and Mdr1b increase with age in male rat liver and intestine. The mRNA expression ontogeny of Cyp3a2 and Mdr1b follow the same pattern.

Specific aim 2A: Determine mRNA levels of Cyp3a1, Cyp3a2, Mdr1a and Mdr1b in rat liver and intestine in different developmental stages and in adults. Cyp3a in the rat was chosen since it is the major cytochrome p450 in the liver and intestine and is responsible for the metabolism of the majority of xenobiotics. Mdr1a and Mdr1b are the genes expressing the P-glycoprotein (P-gp) transporter that plays a major role in drug disposition in both the liver and intestine and shares similar substrate specificity with Cyp3a. RNA levels were examined since they might provide insights as to developmental triggers for a pathway, and to examine whether Cyp3a and P-gp share the same triggers. Liver and intestine are the two major organs responsible for drug elimination when the drug is orally administered, as in the case of drug exposure via lactation.

Hypothesis 3:

Cyp3a protein expression and *in vitro* activity in male rat liver follow the same ontogeny pattern as mRNA expression.

Specific aim 3A: Determine protein levels of Cyp3a normalized to microsomal protein in rat liver and intestine via specific antibodies across developmental stages to adulthood. This will help assess if protein levels follow the RNA developmental pattern.

Specific aim 3B: Measure *in vitro* activity of Cyp3a in rat intestine and liver microsomes using a specific substrate (midazolam) across developmental stages to adulthood. These determinations will enable the calculation of Vmax and Km values for Cyp3a in rat. Vmax values will allow the calculation of an *in vitro* clearance. Km values will be used to validate the assumption that Km values do not change with age for an enzyme-substrate combination.

Hypothesis 4:

Age-specific *in vivo* clearance for a drug can be predicted from an ontogeny model based on the developmental pattern of its clearance pathway based on *in vitro* measurements.

Specific aim 4A: Determine microsomal protein content in rat liver across developmental stages to adulthood. The microsomal protein content will then be normalized to liver weight and body weight to examine the assumption that microsomal protein content does not vary with age.

Specific aim 4B: Model the *in vitro* Vmax values normalized to the different scaling factors to predict *in vivo* oral clearance for Cyp3a in rats throughout development.

Specific aim 4C: Measure *in vivo* oral clearance for midazolam in rats across different developmental stages and evaluate the predictivity of the *in vivo* clearance by the model.

Chapter 4. Materials and Methods

1 *In Vitro* M/S:

1.1 M/S Prediction:

The equation used to describe the Atkinson and Begg model (Atkinson and Begg 1990; Begg, Atkinson et al. 1992)(To be consistent, we have modified Atkinson and Begg nomenclature from M/P to M/S) is:

$$M/S_{phase} = f_s \times M_u/S_u \left[\left(0.955/f_m\right) + \left(0.045 \times \text{milk lipid } P_{app}\right) \right] \quad \text{Equation 21}$$

where f_m and f_s refer to the unbound fractions in milk and serum, M_u/S_u is the ratio of unbound concentrations in milk and serum predicted from the Henderson-Hasselbach equation, and milk lipid P_{app} is $C_{lipid}/C_{ultrafiltrate}$ and calculated by:

$$\log \text{milk } P_{app} = 1.29 \log \text{oct}P_{app} - 0.88. \quad \text{Equation 22}$$

Atkinson and Begg predicted fraction unbound in milk in terms of fraction unbound in serum (Atkinson and Begg 1988) as described by
Equation 23:

$$f_m = f_s^{0.448} / (0.000694^{0.448} + f_s^{0.448}) \quad \text{Equation 23}$$

The phase distribution model equation was:

$$\ln M/S_{phase} = \ln f_s + \ln M_u/S_u + \ln\left[\left(0.955/f_m\right) + \left(0.045 \times \text{milk lipid } P_{app}\right) \right] \quad \text{Equation 24}$$

Reported regression analysis for acidic and basic drugs were obtained, respectively as:

$$\text{Acidic drugs: } \ln M/S = -0.405 + 9.36 \ln(M_u/S_u) - 0.69 \ln f_s - 1.54 \ln K \quad \text{Equation 25}$$

$$\text{Basic drugs: } \ln M/S = 0.02477 + 2.28 \ln(M_u/S_u) + 0.886 \ln f_s + 0.505 \ln K \quad \text{Equation 26}$$

where K is defined as: $(0.955/f_m) + 0.045$ milk lipid P_{app}.

To evaluate the whole model, M/S values for a number of drugs not used in building the model were calculated based on the above equations and compared to

reported M/S values in the literature. Log P and pKa were obtained from ACD/Lab, version 6.0, (Advanced Chemistry Development, Inc., Toronto ON, Canada, www.acdlabs.com, 2002). Free fraction in serum was obtained from the literature.

1.2 M/S Calculation

1.2.1 Materials:

H^3-Acyclovir, C^{14}-Ascorbic acid, H^3-Citalopram, and H^3-Nicotine were obtained from Perkin Elmer Life Sciences, Inc Boston MA. C^{14}-Ciprofloxacin was obtained from Moravek Biochemicals Inc. Brea CA. Non-labeled acyclovir, ascorbic acid, ciprofloxacin, citalopram, nicotine, potassium dihydrogen phosphate and disodium hydrogen phosphate were obtained from Sigma-Aldrich Co. St. Louis MO.

1.2.2 Samples:

The protocol of the study was approved by the University of Kentucky's Institutional Review Board. The samples were collected in the facilities of the General Clinical Research Center (GCRC) at the University of Kentucky on different days. Single blood and milk samples were obtained from eight healthy non-smoking white lactating females after signing a consent form. Milk samples were obtained by an electric pump. The first few milliliters were set aside as fore-milk, then about a 100 mL were pumped. A single 30 mL sample of blood was obtained. Volunteers were not on any current medications and were instructed to abstain from having any analgesics or antihistamines one week prior to the study and abstain from having any alcohol or caffeine 48 hours prior to the study.

1.2.3 Serum Collection:

Blood samples were left to stand for at least 45 minutes then centrifuged at 3000xg for 15 minutes. Serum was collected and stored in a glass vial at –20° C or processed immediately.

1.2.4 Skim Milk Collection:

Two 13 mL aliquots of milk samples were centrifuged in plastic centrifuge tubes at 1800xg for 15 minutes. The skim milk was collected and the top fat layer was discarded. The skim milk was again centrifuged for 15 minutes at 1800xg to ensure the least amount possible of fat in the skim milk. Skim milk was collected and kept in a glass vial.

1.2.5 Crematocrit:

The crematocrit was determined in triplicate for fresh milk samples. Whole milk from each sample was drawn in 3 capillary tubes and centrifuged at 5125xg for 15 minutes. The volume percentage of the fat layer was then determined. Two and a half milliliters each of whole milk, skim milk and serum for each of the five drugs was spiked with the non-labeled drug to obtain therapeutic levels (acyclovir 6.5 μg/mL, ascorbic acid 17.6 μg/mL, ciprofloxacin 0.21 μg/mL, citalopram 0.28 μg/mL, nicotine 0.028 μg/mL). Radio-labeled drug was added to obtain an activity of 2000 dpm/150 μL solution. The added radiolabeled drug did not significantly contribute to the mass of the nonlabeled drug.

1.2.6 Skim to Whole Milk Ratio (S/W):

Skim to whole milk ratios were obtained in triplicate by adding drug to fresh aliquots of the whole milk, mixed and then centrifuged to obtain skim milk. Concentrations of the drugs in both the whole milk and the skim milk were obtained by liquid scintillation counting. Ratios of whole to skim milk concentrations were obtained.

1.2.7 Protein-Binding:

Binding to milk and serum proteins was determined using an equilibrium dialysis method. All experiments were done in triplicate. Two-half plexiglass cells were separated by Spectra/Por® membrane tubing, molecular weight cut off 6000-8000. On one side, the 400 µl half chambers were filled with either the serum or the skim milk spiked with the drug, and dialyzed against 0.133 M sodium/potassium phosphate buffer, adjusted to either pH 7.2 or 7.4 respectively, in the other half-chamber.

Equilibrium dialysis was done in water bath at 37 °C for 8 hours for acyclovir, ascorbic acid and nicotine and for 14 hours for both ciprofloxacin and citalopram. The equilibrium time was determined from preliminary studies. One hundred and fifty microliter aliquots from both the milk or serum and the buffer sides were obtained at the end of equilibrium and the concentrations of the drugs analyzed using liquid scintillation counting. Free fraction in serum and milk (fs and fm respectively) were then calculated.

1.2.8 Unionized Fractions:

The values for unionized fraction in serum and milk ($f_{un,s}$ and $f_{un,m}$ respectively) were calculated from the following equations:

For weak acids (acyclovir, ciprofloxacin and ascorbic acid):

$$f_{un} = \frac{1}{1+10^{(pH-pKa)}} \qquad \text{Equation 27}$$

For weak bases (nicotine and citalopram):

$$f_{un} = \frac{1}{1+10^{(pKa-pH)}} \qquad \text{Equation 28}$$

where the pH is 7.4 for serum and 7.2 for skim milk.

1.2.9 M/S Calculation:

The above values were used to calculate predicted milk to serum ratio for the drugs under investigation, using the following equation (Fleishaker, Desai et al. 1987):

$$\frac{M}{S} = \frac{f_s}{f_m} \frac{f_{uns}}{f_{unm}} \frac{1}{S/W}$$ Equation 29

1.2.10 Statistics:

The 95% CI of the M/S observed values were calculated for drugs where the standard deviations were reported. In the case of the other drugs the observed M/S ratio was either reported from a case study or the standard deviation and the individual values were not reported. Drugs where active transport contributes about 50% to their transfer will have an M/S observed 50-200% of that predicted. Hence, the 95% CI of the predicted values and of the 50-200% of the predicted values were calculated as well. The observed M/S ratios were then compared to both intervals, where the later is a more rigorous approach.

2 *In Vitro and In Vivo* Cyp3a Ontogeny:

2.1 Materials:

RNAlater (RNA Stabilization Reagent) and RNeasy kit were obtained from Qiagen, (Valencia, CA). Potassium chloride (KCl), sodium phosphate dibasic (Na_2HPO_4), Tween 20, ethidium bromide and acetonitrile HPLC grade were obtained from Fisher Scientific (Fair lawn , New Jersey). Calcium chloride ($CaCl_2$), histidine, sodium edetate (Na EDTA), potassium phosphate monobasic (KH_2PO_4), potassium phosphate dibasic (K_2HPO_4), sodium citrate, phenylmethylsulfonyl fluoride (PMSF), butyrated hydroxytoluene (BHT), trizma base, tris HCl, bovine serum albumin (BSA), 2-mercaptoethanol, ammonium phosphate monobasic and 4-hydroxymidazolam were obtained from Sigma Chemicals (St. Louis, MO). Sodium chloride (NaCl) was obtained from EM Sciences and sucrose from MCB Reagents. DC Protein Assay Kit, 96-well PCR plates and i-Cycler iQ™ Optical Tape were obtained from Bio-Rad Laboratories (Hercules, CA). SYBR Green PCR Core Reagent system was purchased from Applied Biosystems (Foster City, CA). SuperScript III First-Strand Synthesis System, LDS Sample Buffer, Sample Reducing Agent, Magic MarkXP, 4-12% NuPage Bis-Tris gel, MOPS Running Buffer, PVDF membranes, NuPage Transfer Buffer were obtained from Invitrogen Life Technologies (Carlsbad, CA). NADPH Regenerating System, Cyp3a2 and Cyp3a1 Supersomes™ were obtained from BD Biosciences (Woburn, MA). Midazolam was obtained as midazolam hydrochloride for injection from Bedford Laboratories (Bedford, OH). Agarose (Seakem™ LE agarose,) was from BMA (Rockland, ME). Primary antibodies Anti-Cytochrome P450 Enzyme CYP3A1 and CYP3A2 (anti-rat raised in rabbit) were obtained form Chemicon International Inc. (Temecula, CA). SuperSignal West Pico Chemiluminescent Substrate and ImmunoPure Goat Anti-Rabbit IgG (H+L) peroxidase Conjugated secondary antibody were obtained from Pierce Biotechnology, Inc. (Rockford, IL). HPLC column SUPELCOSIL ABZ+ Plus column (25 cm X 4.6 mm, 5 µm) and gurad column SUPELCOSIL™ ABZ+Plus Supelguard® Cartridge (5 cm X 4.6 mm, 5 µm) were obtained from Sigma-Aldrich Biotechnology LP.

2.2 Methods:

2.2.1 Animals:

Male Sprague-Dawley rats were used for all experiments. The studies were done under approval of the IACUC at The University of Kentucky. Animals were procured from Harlan (Indianapolis, IN). Age groups: 1, 7, 21, 42, 77 and 112 days were used for *in vitro* experiments. Four animals per age group were used. For age groups 1, 7 and 21 days pregnant dams were ordered and male rats from litters were used. Older animals were ordered directly from the supplier. The age groups were chosen based on developmental stages: Neonatal (1 and 7 days), weanling (21 days), pubertal (42 days), young adults (77 days) and adults (112 days). Animals were kept in the College of Pharmacy animal facility for at least 3 days for acclimatization before performing experiments. Standard rat chow (Harlan Teklad Global Rodent Diet 2018) and water were provided ad libitum. Animals were kept in a controlled environment of temperature and humidity and 12 hours light-dark cycle.

2.2.2 Tissue Collection:

On the day the animals became of age (according to the date of birth observed or supplied by Harlan), animals were weighed, then sacrificed under isoflurane anesthesia by exsanguination. Whole livers were excised, washed by saline, blotted and weighed. A portion of the tissue was cut in pieces less than 0.5 cm thick and kept in at least 10 volumes of its weight of RNA*later* at 4 °C for subsequent RNA extraction. The rest of the tissue was then flash frozen in liquid nitrogen and transferred to a -80 °C freezer.

The proximal third of the small intestine was collected and kept on ice in 100 mL Weiser solution A (Weiser,1973) (KCl 1.5 mM; NaCl 96 mM; sodium citrate 27 mM; KH_2PO_4 8 mM; Na_2HPO_4 5.6 mM; pH to 7.4) with phenylmethylsulfonyl fluoride (PMSF) at 40 µg/mL and the intestinal mucosa was immediately collected as described below.

2.2.3 Tissue Processing:

2.2.3.1 Intestinal Mucosa and Microsomal Preparation:

The procedure was adapted from a number of previously used methods (Weiser 1973; Bonkovsky, Hauri et al. 1985; Cotreau, von Moltke et al. 2000; Johnson, Tanner et al. 2000).

The collected intestinal segments were flushed with fresh Weiser Solution A (Weiser 1973) on ice then dissected longitudinally and washed in Weiser Solution A. The tissue was then blotted and weighed. To collect the intestinal mucosa the dissected segment was spread on an ice cold glass plate with the mucosal side upwards. The mucosa was then gently scraped using a microscope slide. The scraped mucosa was weighed on a clean slide, and a portion weighed and kept in RLT buffer (RNeasy MiniKit) with 2-mercaptoethanol for RNA extraction, homogenized using a rotor-stator homogenizer and kept at -80 °C. The rest of the mucosa was homogenized in a 15 mL centrifuge tube in 10 mL of Weiser Solution C (Weiser 1973) (histidine 5 mM, pH 7.0; sucrose 0.25 M; NaEDTA 0.5 mM; pH to 7.4) with PMSF at 40 µg/mL by sonication, 7 cycles, 10 seconds each. The homogenates were placed into two clean ultracentrifuge tubes. The total homogenate was then centrifuged at 15,000xg for 10 minutes. The supernatant was carefully transferred to a clean centrifuge tube. For each 7 mL of supernatant collected, 1.25 mL of 52 mM $CaCl_2$ was added. Tubes were gently shaken for 20 seconds and then allowed to stand for 15 minutes on ice. Fractions were then centrifuged at 25,000xg for 10 minutes. If the pellet formed was not compact, the above step was repeated. Pellets were resuspended in 0.25 M Sucrose/0.02 M Tris buffer by sonication. The protein concentration was determined using DC Protein Assay in microplate format and the microsomal suspension aliquoted and frozen at -80 °C for later use.

2.2.3.2 Liver Microsomal Protein Preparation:

All steps are performed on ice or in the cold room at 4 °C. Half of a gram of the frozen liver tissue was homogenized in 2 mL homogenization buffer (0.154 M potassium chloride prepared in 0.25 M potassium phosphate buffer (pH 7.4). Butyrated hydroxytoluene (BHT) was directly added before homogenization to a final concentration of 2.5µL BHT/mL buffer (BHT solution 10mg/mL in 100% ethanol). Homogenization was done by a stirrer type homogenizer (Potter-Elvehjem) by performing 10 strokes. The homogenate was then poured in ultracentrifuge tubes kept on ice. The homogenizer tube and head were then rinsed with 2 mL homogenization buffer and the rinse added to the ultracentrifuge tubes. The volume of the homogenate in the ultracentrifuge tubes was then completed to 7.5 mL as recommended by the manufacturer. The tubes were then centrifuged at 6860xg (10,000 rpm) for 15 minutes, 4 ºC in the Ti 70.1 Beckman rotor. The supernatant was then transferred to clean, cooled tubes and balanced with the homogenization buffer and ultra-centrifuged at 84,035xg (35,000 rpm, Ti 70.1 Beckman rotor) for 30 minutes, 4 ºC. Two mL of 0.154 M potassium chloride solution were added to the pellet, which was resuspended in the ultracentrifuge tube with the head of the homogenizer by three strokes. The tubes were filled with the KCl solution until the recommended filling level and balanced then ultra-centrifuged at 84,035xg (35,000 rpm, 70.1 Ti Beckman rotor) for 30 minutes at 4 °C. The supernatant was discarded and the pellet resuspended in buffer (0.25 M Sucrose prepared in 0.02 M Tris buffer pH 7.4) by sonication for 5 cycles 10 seconds each. The protein concentration was determined using DC Protein Assay in the microplate format. The microsomal suspension was then aliquoted and kept at -80 °C for future use.

2.2.3.3 RNA Isolation and Quantification:

Thirty micrograms of the liver tissue stabilized in RNA*later* at 4 °C was transferred to RLT buffer (600µL RLT plus 6 µL of 2-mercaptoethanol of RNeasy MiniKit) and homogenized using a rotor stator homogenizer. The intestinal mucosa samples previously homogenized in RLT buffer and frozen at -80 °C were thawed at

37°C for 5 minutes. RNA was then extracted from the liver and intestinal mucosa samples according to the manufacturer's protocol (RNeasy Mini kit Handbook, Animal Tissue Protocol, Qiagen Valencia, CA). Briefly, the samples were mixed with equal volume of 70% ethanol, centrifuged at maximum speed and the supernatant applied to an RNeasy spin column (RNA retention column). The column was washed with RW1 and RPE buffers from the kit. The RNA was eluted using 40 µL of DNase free water. The RNA concentration of each sample was determined using NanoDrop Spectrophotometer (NanoDrop Technologies Inc.). RNA was stored at -80 °C.

2.2.3.4 RNA Integrity:

A few microliters of the RNA samples were diluted in the range of 100 to 400 ng/µL and supplied to the University of Kentucky Microarray Core Facility to check RNA integrity. Agilent chips were used and Agilent 2100 analyzer and 2100 Expert software for analysis. Integrity was reported in terms of RIN (RNA Integrity Number) from 0-10, 10 being the best quality. Absorption peaks as well as RNA bands for 18s and 28s ribosomal RNA were also reported.

2.2.4 Reverse Transcription:

The RNA reverse transcription was performed using the SuperScript III First-Strand Synthesis System. One microgram of RNA was used per reaction unless the RNA concentration did not permit (8 µL RNA solution maximum per reaction). The procedure was done according to the manufacturer's protocol. One µg of RNA , 1 µL of each of 50 µM Oligo dt, 10 mM dNTP mix and DPEC treated water to 10 uL per reaction were heated to 65°C for 5 minutes then placed on ice for at least 1 minute. The synthesis mixture (2 µL of each of 10X RT buffer and 0.1 M DTT, 4µL of 25 mM MgCl2 and 1 µL of each of RNaseOUT (40 U/µL) and SuperScript III RT (200 U/µL)) was then added and heated for 50 min at 50°C and the reaction ended at 85 °C for 5 minutes and cooled on ice. The reaction was centrifuged and 1 µL of RNase H added and incubated at 37 °C for 20 minutes. The cDNA was then stored at -20 °C for later use in quantitative-PCR.

2.2.5 Quantitative PCR

2.2.5.1 Primers:

Quantitative PCR was performed for 5 rat genes Cyp3a1, Cyp3a2, Mdr1a, Mdr1b and villin. Primers were either adopted from the literature or designed as described below.

Primer design: For primers that had no satisfactory sequences in the literature, the mRNA sequence was obtained from NCBI Gene database and using Primer 3 online software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) to design primers. For Cyp3a1, sequence alignment with Cyp3a2 mRNA was first performed using NTI Vector Suite Software version 9.0.0 (Informax) to choose a target sequence that was unique to Cyp3a1 and that would not amplify Cyp3a2 cDNA.

The primer sequences were then compared against the available genome sequences using Nucleotide BLAST (The Basic Local Alignment Search Tool, http://0-www.ncbi.nlm.nih.gov.library.vu.edu.au/blast/index.shtml) on the NCBI website to ensure that the primers were unique to the target gene sequences.

Table 1: Primer sets used for Quantitative PCR

Gene	Primer Sequence	Sequence Source	Product size
Cyp3a1 [1]	Left: AAA GAA TCT CAT ACA GCC CTA TCC Right: CTG CTG GTG GGT TCA TAT CC	NM_173144	100
Cyp3a2 [2]	Left: GCTTTCAGCTCTCACACTGGAAA Right: TCTATGGGTTCCAAGTCGGTAGA	NM_153312	89
Mdr1a [3]	Left: GATGGAATTGATAATGTGGACA Right: AAGGATCAGGAACAATAAA	NM_133401	351
Mdr1b [3]	Left: GAAATAATGCTTATGAATCCCAAAG Right: GGTTTCATGGTCGTCGTCTCTTGA	NM_012623	326
Villin [1]	Left: GATGGAATTGATAATGTGGACA Right: AGTGCTCCTTCTCGCCTATG	XM_237288	157

1-Designed by Primer 3

2-(Meredith, Scott et al. 2003)

3-(Kwan, Sills et al. 2003)

2.2.5.2 Primer Optimization:

The primer concentration was set at 1 μM and the conditions to be optimized were the annealing temperature (55-64°C) and $MgCl_2$ concentrations (2.5-4.5 μM) at two starting cDNA dilutions (10^{-2} and 10^{-4}). Male Sprague-Dawley rat liver cDNA was used for optimization of all primers and subsequently for standard curves, except for Mdr1a and villin. Male Sprague-Dawley rat brain and intestinal cDNA were used for Mdr1a and villin respectively.

The reaction mixture was as follows made from SYBR Green PCR Core Reagent system (Applied Biosystems, Foster City, CA):

1 uL of cDNA, 5 μL of 12.5 mM dNTP mix with dUTP, 5 μL of 10 x SyBR Green Buffer, 0.5 μL of Fluorescein Calibration Dye (1 μM) (Bio-Rad Laboratories, Hercules, CA), 0.25 μL of 5 U/μl Taq Polymerase, 1 μL of each of forward and reverse primers (10 μM), $MgCl_2$ (25 mM) up to the desired concentration, and DNAse free water

to complete reaction volume to 50 μL. Reaction mixtures were loaded in a 96 well plate and covered with an optical film. The reaction was then run using the iCycler Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA).

The protocol for thermal cycling was:

Cycle 1:	(1X)	
Step 1:	95.0ºC	for 05:00
Cycle 2:	(50X)	
Step 1:	95.0ºC	for 00:45
Step 2:	gradient 55-64ºC	for 01:00
Step 3:	72.0ºC	for 01:00
Data collection and real-time analysis enabled.		
Cycle 3:	(1X)	
Step 1:	95.0ºC	for 02:00
Cycle 4:	(100X)	
Step 1:	95.0ºC	for 00:30
Decrease setpoint temperature by 0.5ºC		
Melt curve data collection and analysis enabled.		

The reactions were run for 50 cycles (Cycle 2 in the above protocol) except for villin (40 cycles).

To ensure the correct product formation, the product was then run on 2-3% agarose gel with a reference DNA ladder (Invitrogen Life Technologies, Carlsbad, CA) depending on the product size and stained with ethidium bromide for UV visualization using Kodak imaging station 2000 MM and Kodak Molecular Imaging Software (Eastman Kodak Company, Rochester, NY). The optimum conditions were chosen based on the lowest threshold cycle number needed for a product to be visualized by SYBR green intercalating dye for the dilutions of the reference cDNA used given that the agarose gel showed a positive product at the correct band size and no primer pairs.

2.2.5.3 Sample Analysis:

The conditions used for each primer pair are described in Table 2. The reaction mixture and the thermal cycling protocol were similar to primer optimization except at Cycle 2 Step 2 where the temperature used was the annealing temperature. Each sample was run in triplicate using the proper dilution to fit within the standard curve. Standard curve dilutions of 1 to 10^{-4} of the proper cDNA (indicated above) were run simultaneously on the plate with the samples. Standard curve cDNA was from animals other than the test animals. Results were analyzed using the i-Cycler IQ Software System v3.1.7. Results were normalized to 1µg RNA used in the reverse transcription reaction and expressed in terms of the starting quantity of RNA in comparison to the standard curve used.

Table 2: Quantitative PCR Conditions for each primer set

Gene	Annealing Temperature (°C)	Magnesium Chloride Concentration (mM)
Cyp3a1	62	3.5
Cyp3a2	62	3.5
Mdr1a	58.2	4
Mdr1b	55	3.5
Villin	62	4.5

2.2.5.4 Statistical Analysis:

The results obtained for each gene were statistically compared to the adults (112 days) by one-way ANOVA using GraphPad Prism. If statistical significance was found a post-hoc analysis was done using Dunnett's test.

2.2.6 Western Blotting:

Primary antibody specificity was first tested before running the samples against individual Cyp3a1 and Cyp3a2 Supersomes™. The tested antibodies were rat anti-Cyp3a1 from Research Diagnostics and anti-Cyp3a2 from Daiichi and anti-Cyp3a1 and anti-Cyp3a2 form Chemicon. To further characterize the specificity/interaction of the antibodies against the respective proteins, the anti-Cyp3a1 and anti-Cyp3a2 antibodies from Chemicon were used to probe mixtures of the proteins and the net intensities of the bands were analyzed.

Liver microsomal protein and intestinal microsomal protein, 5 and 10 µg respectively, were used. Standards were 3 different amounts of Cyp3a2 Supersomes™; 1.16, 0.58 and 0.12 ng. Samples and standards were mixed with 5 µL LDS Sample Buffer and 2 µL Sample Reducing Agent and water to complete the volume to 20 µL. The mixture was then heated at 70 °C for 10 minutes in a water bath.

The samples and standards in duplicate and a protein ladder (Magic MarkXP) were then loaded on a 4-12% NuPage Bis-Tris gel and run at 150 Volts constant for 55 minutes using MOPS Running Buffer. The proteins were then transferred to PVDF membranes (for 1 hour and 15 minutes at 30 volts constant using NuPage Transfer Buffer). The membrane was then blocked for 1 hour in the blocking solution (3% Bovine Serum Albumin in Tris-Buffered Saline-Tween 20 (TBST): 10 mM tris Buffer pH 7.4, 150 mM NaCl and 0.05% Tween 20). The membrane was rinsed in TBST and incubated with a 1:1000 dilution of the primary antibody (Chemicon anti Cyp3a1 or Cyp3a2) in TBST overnight at 4 °C. For detection the membrane was rinsed 3 times for a total of 20 minutes in TBST and incubated with a 1:8000 dilution of the secondary antibody (ant-rabbit Ig-G-HRP conjugated) in TBST for 1 hour. The membrane was rinsed as described above and incubated for 5 minutes with SuperSignal® West Pico Chemiluminescent Substrate. All the above incubations were done while shaking at room temperature unless otherwise indicated. The chemiluminescent imaging was done using Kodak imaging station 2000 MM and Kodak Molecular Imaging Software (Eastman Kodak Company, Rochester, NY). Analysis was done using the Kodak Molecular

imaging software using the standards run on the same gel for a standard curve. Results for each band are expressed in mass units (ng) per 5 ug protein for the liver.

2.2.7 Activity Determination:

2.2.7.1 Vmax:

2.2.7.1.1 Microsomal Incubation:

Linearity of product formation with respect to time and intestinal or liver microsomal protein were first performed as well as the midazolam concentration at which Vmax is reached. For liver, the incubation mixture consisted of 50 µg protein per sample per 200 µL reaction mixture, 50 µL of 0.2 M Tris buffer (dilutes to 0.05 M in a final volume of 200 uL pH 7.4), 12 µL of NADPH Regenerating System (10 µL of Solution A and 2 µL of Solution B), a final concentration of 60 µM Midazolam and water to complete reaction volume to 200 µL. The reactions were done in duplicate per sample per day. The reaction mixture was similar for intestinal microsomes except that the total protein amount per reaction was 200 µg. The reaction mixture was pre-incubated for at least 5 minutes at 37 °C before starting by adding the NADPH Regenerating System and stopped after 5 minutes for the liver microsomes and 10 minutes for intestinal microsomes by adding 200 µL ice-cold acetonitrile. The incubations were done in a shaking 37 °C water bath. The reaction tubes were kept on ice for 5 minutes and then vortexed for 5 minutes. Protein was precipitated by centrifuging at 1800xg for 15 minutes. A 50 µL sample of the supernatant was then analyzed in duplicate for the 4-hydroxymidazolam metabolite using HPLC-UV. Standard Curves and controls were made of 4-hydroxymidazolam in 1:1 0.05M Tris buffer:acetonitrile. Vmax determinations were performed twice on two different days and the Vmax value was taken as the average of all determinations.

2.2.7.1.2 HPLC Analysis of 4-Hydroxymidazolam:

A LC-10ADvp Shimadzu LC pump, SCL-10Avp system controller, SIL-10ADvp auto injector, and a SPD-10Avp UV-VIS detector were used to analyze 4-hydroxymidazolam in the incubation mixture. The mobile phase consisted of 60% 0.05 M Ammonium Phosphate monobasic and 40% acetonitrile HPLC grade. The column used was an alkylamide derivatized SUPELCOSIL ABZ+ Plus column (25 cm X 4.6 mm, 5 μm). The flow rate was set at 1 mL/min and UV detection at 254 nm.

Since 1'-hydroxymidazolam metabolite is also formed in the rat, a standard of 1'-hydroxymidazolam was injected to ensure that the chromatographic method separated the 2 metabolites and that the peak obtained at 6.9 minutes was solely that of 4-hydroxymidazolam.

2.2.7.2 Km determination:

Pooled protein samples from each age group were used for the Michaelis-Menten constant (Km) determination. An equal amount of protein from each rat (N=4) was combined within an age group. Protein concentrations were determined in the pooled sample prior to the microsomal incubations to ascertain that the correct amount of protein was used. The incubations were done for liver microsomes as described under Vmax determination except that a range of midazolam concentrations (1-500 μM) were used to generate the Michaelis-Menten profile of Cyp3a enzyme kinetics. The reactions were done in duplicate for each midazolam concentration. The 4-hydroxymidazolam concentrations were determined by HPLC as described above. The Michaelis-Menten kinetics were modeled using GraphPad Prism v.4.01 (Graph Pad Software, San Diego CA) using a hyperbolic one-binding site model on data from 1-60 μM midazolam only, since Vmax is reached at 60 μM. Km and Vmax mean values and standard errors are generated from a fitted line of the data according to a hyperbolic one-binding site model (Michaelis-Menten kinetics). The Km's generated for each age group were statistically compared using the non-parametric Kruskal-Wallis test to determine if Km significantly varied with age. To further confirm if Km varied or not with age, the data for all the age

groups was fitted to the Michaelis-Menten profile using WinNonlin 4.1 (Pharsight Corporation, Mountain View, CA) simultaneously. The fitting was done twice, once assuming different Km values and once assuming a single Km value. The Vmax values for both fittings were then plotted against age for comparison.

2.2.8 *In Vivo* Clearance Determination:

2.2.8.1 Animals:

In order to test the model prediction of Cyp3a ontogeny, the *in vivo* oral clearance of midazolam was determined in 3 age groups. Animal studies were approved by the University of Kentucky IACUC committee. Male Sprague-Dawley rats ages 7, 21 days (from litters of untimed pregnant females) and adult males (112 days) were obtained from Harlan (Harlan Inc., Indianapolis, IN). Adult animal clearance was used as the reference used to predict infant clearance. Seven and 21 day old groups were chosen since the former represents a neonatal age group and the latter is at the weanling stage. Animals were kept in an environment of controlled temperature and humidity and 12-hour light, 12-hour dark cycle. Food and water were supplied ad libitum. Animals were left to acclimatize for at least 4 days before the study. On the day of the study 21 and 112 day old animals were fasted 6-8 hours before dosing, water was provided. Seven day old animals were fasted 2 to 3 hours prior to dosing and were returned to the mother's cage 2-3 hours after dosing. While away from the mother, the neonatal rats were kept on a 37 °C heating pad to avoid heat loss. Due to limited blood volume in rat pups 7 and 21 days old, a destructive sampling design was adopted where a terminal blood sample was obtained from each animal. Although multiple sampling is feasible in adults, destructive blood sampling was employed as well to avoid any difference in clearance of midazolam that could be triggered by anesthesia and catheter implantation surgery and recovery (Uhing, Beno et al. 2004). Four animals were used per sampling time point per age group. Midazolam was dosed at time zero at 20 mg/kg using oral intubation. The size of the oral tube was suitable for the age group. From preliminary studies, the following sampling times were deemed appropriate for a full pharmacokinetic profile along with

minimizing the number of animals used. For the 7 and 21 day age groups sampling time points were: 0 (no dose), 5, 10 and 30 minutes, 1, 1.5, 2 ,3 , 4 , 6 and 8 hours. For 112 day rats sampling times were 0, 10 ,20 and 30 minutes, 1, 1.5, 2, 3, 4 and 6 hours. At the time of sampling the animals were anesthetized using isoflurane inhalational anesthesia and blood was collected by cardiac puncture. Blood samples were kept at room temperature for at least 30 minutes after which serum was collected by centrifugation at 3000xg for 15 minutes at 4 °C then serum samples were stored at -20 °C until analysis.

2.2.8.2 LC/MS/MS Assay of Midazolam in Serum:

2.2.8.2.1 Sample Preparation:

Midazolam concentration in rat serum was determined by an LC/MS/MS method. Serum samples were prepared by solid phase extraction. Either 25 (7and 21 days) or 50 (adults) µL of the serum sample was extracted. The serum sample was diluted with 470 µL of water and 70 µL of 100 ng/ml of flurazepam in acetonitrile as the internal standard. The sample was loaded by vacuum on preconditioned Oasis HLB solid phase extraction columns (Waters), the column was then washed with 1 mL of each of water and 5% methanol in water. The sample was then eluted by 2 X 1 mL of methanol. The methanol eluate was then evaporated under nitrogen gas and the residue was reconstituted in either 100 or 200 µL of mobile phase (80% aqueous and 20% organic).

The mobile phase used for the assay was 1 mM ammonium formate in water, pH adjusted to 3.8 using formic acid, as the aqueous phase and acetonitrile as the organic phase. Gradient elution was used starting at 80% aqueous, 20% organic.

2.2.8.2.2 LC/MS/MS Conditions:

LC/MS/MS was performed on a Varian Prostar Model 210 LC system coupled to a 1200 L Quadropole MS/MS Varian mass spectrometer equipped with an electrospray-ionization source (Varian Inc., Palo Alto, CA). A Fusion RP-80A, 4 µm, 50 x 2 mm column (Phenomenex, Torrance, CA) was used for separation. The mobile phase

consisted of solvent A (1 mM ammonium formate pH 3.8) and solvent B (acetonitrile), using linear gradients of 20-90% B (v/v) over 13 min. The flow rate was 0.25 ml/min. Positive–ion mass spectra was acquired. Positive-ion ESI was performed using an ion source voltage of 40 V and a capillary offset voltage of 86.0 V. Nebulization was aided with coaxial nitrogen sheath gas provided at a pressure of 20 psi and a capillary temperature of 300 °C. Mass spectra were recorded in a tandem MS mode with a collision Argon gas at a pressure of 2.02 m Torr. Selective ion monitoring (SIM) width was set at 0.7 amu for *m/z* 326 (M^+, midazolam) and monitoring its first daughter ion at *m/z* 291. Peak areas of midazolam peaks in serum samples were normalized based on the area of the recovered internal standard peak (flurazepam).

Initially there was a problem encountered with the analysis. The sensitivity greatly dropped after 10-13 injections as determined from the drop of the peak area of the internal standard. Repeated injection of the same control revealed that sensitivity dropped disproportionately for midazolam and flurazepam such that the concentration of a control appeared to significantly drop when injected twice once at the beginning of the run and another at the end of the run. To rule out that this disproportionate drop with time is due to a stability problem of midazolam, the same control was injected the next day after the ionization chamber of the mass spectrometer was cleaned with methanol. The midazolam concentration and the sensitivity appeared to be regained. To further explore if cleaning the ionization chamber was the solution to the disproportionate sensitivity drop, controls were injected (13 injections), the ionization chamber was cleaned with methanol after allowing it to cool, and meanwhile the LC flow was not interrupted and was diverted away from the mass spectrometer. After cleaning, the same controls were reinjected for a second run. This was performed for a total of three runs and two cleanings inbetween in the same day. It appeared that the concentration of the controls for the three runs where within a 20% range. This procedure was applied to sample analysis by injecting all the controls in the first run along with samples and then two of the controls were reinjected in the subsequent two runs along with the rest of the samples to ensure that sensitivity was not significantly dropping for midazolam. The runs were deemed acceptable if the concentrations of the controls where within 20%.

2.2.8.2.3 Pharmacokinetic Analysis:

Non-compartmental analysis of the concentration-time profile for all the age groups was performed using WinNonlin 4.1 (Pharsight Corporation, Mountain View, CA). The model independent parameters: AUC, Cmax and tmax were determined. For model dependent parameters, clearance (Cl/F), volume of distribution (V_d) and absorption rate constant (K_A), a one compartment, first order absorption model was assumed. Analysis was performed using NONMEM (University of California, San Fransisco) using population kinetics approach since each time point comes from a different animal (limited sampling).

2.2.8.3 Midazolam Serum Protein Binding:

Midazolam serum protein binding was determined by ultrafiltration. Blank serum from 7, 21 and 112 day old animals (not exposed to midazolam) was pooled from at least 3 animals per age group. The serum was spiked with midazolam to a final concentration of 5000 ng/mL. For ultrafiltration, disposable Millipore Centrifree ® micropartition devices (Millipore Corporation, Bedford, MA) were used, containing YMT memebranes. The devices were loaded with 400 µL of serum in duplicate for 7 day age group and triplicate for the other two age groups. Centrifugation was performed in a Sorvall RT 6000D centrifuge at 37 °C for 20 minutes at 2000xg. The free fraction was determined by assaying midazolam in the ultrafiltrate using HPLC.

HPLC Analysis of Midazolam in Ultrafiltrate:

A LC-10ADvp Shimadzu LC pump, SCL-10Avp system controller, SIL-10ADvp auto injector, and a SPD-10Avp UV-VIS detector were used to analyze midazolam in the ultrafiltrate. The mobile phase composition was obtained by contacting Ultrafine Chemicals (Manchester, UK). The mobile phase consisted of 60% 0.05 M Ammonium Phosphate monobasic and 40% acetonitrile HPLC grade. The column used was an

alkylamide derivatized SUPELCOSIL ABZ+ Plus column (25 cm X 4.6 mm, 5 μm) with an alkylamide gurad column SUPELCOSIL™ ABZ+Plus Supelguard® Cartridge (5 cm X 4.6 mm, 5 μm. The flow rate was set at 1 mL/min and UV detection at 254 nm.

The free fraction was calculated by dividing the ultrafiltrate concentration by the initial concentration in serum.

2.2.9 Model Prediction:

Clearance was predicted according to the ontogeny model described in the background (Alcorn and McNamara 2002d). The model assumptions are:

1. Drug has a low extraction ratio.
2. Infant and adult share the same enzymes and transporters in metabolizing the same drug.
3. Microsomal protein content normalized to liver weight is constant and independent of age.
4. Km for an enzyme-substrate system remains constant throughout development.
5. Vmax reflects the amount of functional enzyme and is proportional from one substrate to the next.
6. Hepatic Clearance is governed by hepatic metabolic enzymes and not hepatic transporters.

The assumption that Km does not vary with age was tested in the previous section. The assumption that microsomal protein content normalized to liver weight was examined by plotting microsomal protein content (mg/g liver) against age and a one-way analysis of variance (ANOVA) was performed to determine any significant difference between age groups. If a significant difference exists, Dunnett's post-hoc test was performed to compare different age groups to adults (112-day group).

Midazolam is a drug cleared by hepatic Cyp3a metabolism(Gorski, Hall et al. 1994; Thummel, Shen et al. 1994). Although, it was shown to be a substrate for P-gp, P-gp does not affect the permeability of midazolam intracellularly (Tolle-Sander, Rautio et al. 2003) since it is a highly permeable drug (log P= 2.68). Hence, midazolam satisfies

most of the requirements for clearance prediction by the above model. However, midazolam clearance is affected by hepatic blood flow as well as intrinsic clearance (intermediate extraction drug). To be able to circumvent hepatic blood flow, oral clearance (Cl_{po}) will be calculated for midazolam as follows:

$$Cl = Cl_H + Cl_R \quad \text{Equation 30}$$

$$Cl_{midazolam} = Cl_H = Q_H \ f_u \ Cl_{int} / Q_H + f_u \ Cl_{int} \quad \text{Equation 31}$$

$$Cl_{po} = Cl_H / F \ = Cl_H / F_H \ . F_G \quad \text{Equation 32}$$

where F is the oral bioavailability, F_H is the fraction extracted by the liver and F_G is the fraction extracted by gut or intestine. Assuming that F_G=1 then F=F_H, then F and oral clearance can be calculated as follows:

$$F = F_H = Q_H / Q_H + f_u \ Cl_{int} \quad \text{Equation 33}$$

$$Cl_{po} = (Q_H \ f_u \ Cl_{int} / Q_H + f_u \ Cl_{int}) / Q_H / Q_H + f_u \ Cl_{int}$$
$$= f_u \ Cl_{int} \ = Cl_H = Cl_{midazolam,po} \quad \text{Equation 34}$$

Thus oral systemic clearance can be calculated from *in vitro* data.

The following equations describe the ontogeny model approach to calculate intrinsic clearance as described in the background:

$$Cl_{\text{int}(j)}^{infant} = ISF_{(j,t)} \, Cl_{\text{int}(j)}^{adult} = RHSF_{(t)} OSF_{(j,t)} Cl_{\text{int}(j)}^{adult} \quad \text{Equation 35}$$

where $RHSF_{(t)} = \dfrac{HSF_{(t)}}{HSF_{(adult)}}$ $\quad HSF = \text{MP(mg/g Liver)} \dfrac{\text{LW}}{\text{BW}}$ $\quad OSF_{(j,t)} = \dfrac{V_{\text{max}}^{\text{inf}\,ant}}{V_{\text{max}}^{adult}}$

Clearance will be calculated according to the following 2 models:

Model 1 $\quad Cl_{(j)}^{infant} = OSF_{(j,t)} \, Cl_{(j)}^{adult} = \dfrac{V_{max}^{infant}}{V_{max}^{adult}} Cl_{(j)}^{adult}$ $\quad$ Equation 36

Model 2 $\quad Cl_{(j)}^{infant} = ISF_{(j,t)} \, Cl_{(j)}^{adult} = RHSF_{(t)} OSF_{(j,t)} Cl_{(j)}^{adult}$ $\quad$ Equation 37

Model 2 is the ontogeny model approach. Model 1 assumes that microsomal protein normalized to liver weight and body weight is independent of age and hence RHSF=1.

2.2.10 Prediction Evaluation:

The predicted clearance for the 7 and 21 day age groups will be compared to the clearance values determined *in vivo*. Since only two age groups were characterized, a correlation analysis was inappropriate. Using a standard statistical analysis (e.g. Student's t-test) to compare the observed and predicted was also inappropriate since a statistical significance would not indicate that the prediction was not biologically or clinically useful. Hence, an approach was used such that if the predicted values fell within the 50-200% of the observed values then the prediction will be accepted as valid i.e. within twofold of the observed.

Chapter 5. Results

1 *In Vitro* M/S:

1.1 Atkinson and Begg Model Assessment

The drugs used in the following correlations were not used in building the original model. Figure 1 shows the Atkinson and Begg model (Atkinson and Begg 1990; Begg, Atkinson et al. 1992) M/S prediction for a set of acidic drugs. The model is inadequate in predicting the observed M/S values for these acidic drugs. A large number of drugs are grouped near the ordinate (M/S<0.1) making the relationship more difficult to observe. However, the model did not perform well with this set of acidic drugs. One explanation for the discrepancy for the observed and predicted is the assumption of passive diffusion as the mechanism of transfer. Two of the drugs on the graph (acyclovir M/S_{obs} =3.0 and ciprofloxacin M/S_{obs} =1.7) had a greater M/S_{obs} than the $M/S_{pred.}$ The M/S_{pred} for oxazepam was much greater (M/S_{pred} =1.2) than the observed (M/S_{obs} =0.1). Figure 2 shows the Atkinson and Begg model (Atkinson and Begg 1990; Begg, Atkinson et al. 1992) M/S prediction for a set of basic drugs. The model performed better in predicting the observed M/S values for these drugs. Two of the drugs on the graph (acebutolol M/S_{obs} =5.7 and ranitidine M/S_{obs} =3.8) had a higher M/S_{obs} than M/S_{pred}.

Figure 1: M/S predicted values using the model proposed by Atkinson and Begg (1990) , for a set of acidic drugs not included in building their model, vs. M/S observed values reported in the literature for those same drugs (acyclovir, amoxicillin, captopril, cefadroxil, ciprofloxacin, clonazepam, doxorubicin, enalapril, indomethacin, ketorolac, naproxen, oxazepam, piroxicam). The red data points are those used in the Fleishaker model evaluation in the above section. The line of unity is depicted. ($r^2 = 0.4780$). The inset represents the area surrounded by the rectangle at the origin of the graph.

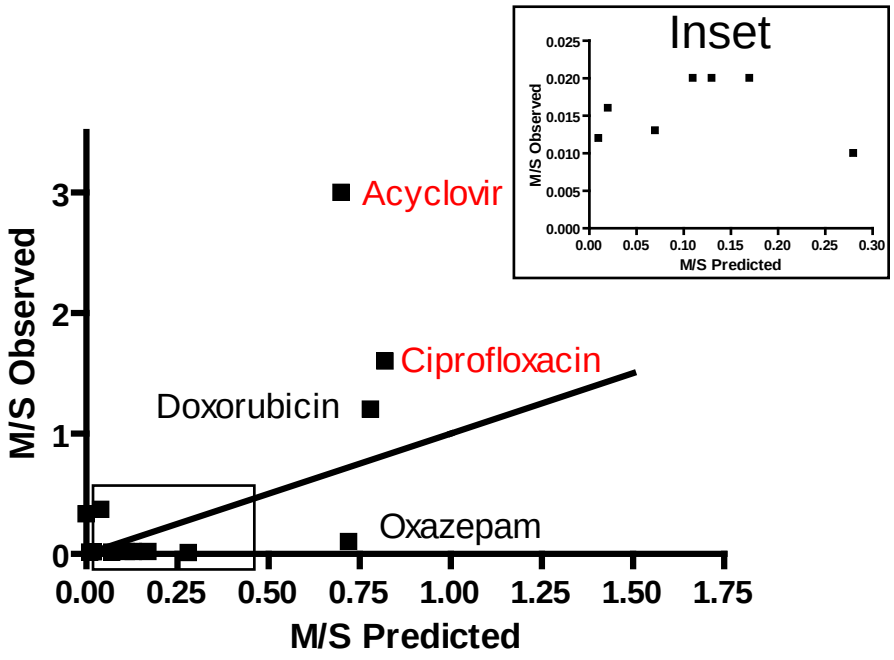
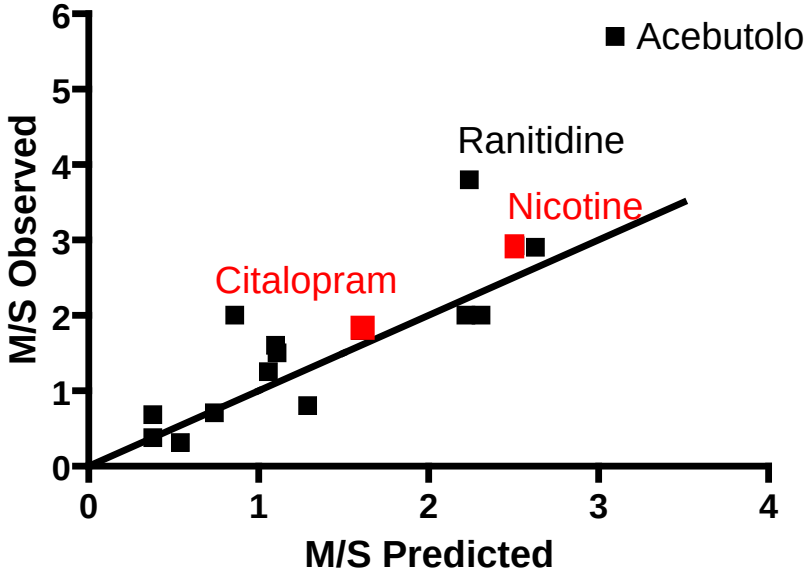


Figure 2: M/S predicted values using the model proposed by Atkinson and Begg (1990) for basic drugs, for a set of drugs not included in building their model, vs. M/S observed values reported in the literature for those drugs (chlorpromazine, clonidine, fluoxetine, fluoxetine, imipramine, labetalol, metoprolol, quinidine, ranitidine, terbutaline, timolol, trimethoprim, acebutolol, quinine, nicotine). The red data points are those used in the Fleishaker model evaluation in the above section. The line of unity is depicted. (r^2= 0.7663)



1.2 *In Vitro* M/S Calculation:

The skim to whole milk ratio (S/W), free fraction in serum (f_s) and free fraction in milk (f_m) from the above experiments for ciprofloxacin, citalopram, nicotine, acyclovir and ascorbic acid are shown in Table 3.

With the possible exception of citalopram, most of the measured parameters have small intersubject variability. The larger variability seen in the S/W ratio for citalopram can be explained in terms of greater lipophilicity of citalopram (logP= 2.51, ACD labs v.6.0) and the intersubject variability of the fat content in milk reflected by crematocrit values (average 6.75% and coeffeicient of variation 13.4%)(Figure 3).

The measured S/W ratio was low for citalopram and was inversely related to the crematocrit values indicating partitioning into milk fat. The ratio $f_{un.s}/f_{un,m}$ was 1.58. The observed M/S values (1.8, 1.52, 3.00) fell within the confidence interval of the predicted value (M/S_{pred} = 1.83) (Figure 4) indicating that the high M/S ratio could largely be attributed to partitioning into milk fat as well as ion-trapping in milk.

The M/S_{obs} for ciprofloxacin has been reported to be 1.6 whereas the predicted value is 0.85 (Figure 5). The predicted value was almost half of that observed. However, using a more stringent criterion of the 50-200% confidence interval (Edwards, Rudy et al. 2003), the M/S_{obs} ratio lies within that range (0.679-4.59). The S/W ratio and $f_{un.s}/f_{un,m}$ were close to 1. Ciprofloxacin was more bound in serum than in milk. Accordingly, the M/S_{obs} can not be readily explained by physicochemical properties measured.

The 95% CI for M/S_{obs} for nicotine (2.60- 3.24) overlapped with the 95% CI for the 50-200% M/S_{pred} (0.58-2.9), with the M/Sobs value (2.92) being outside that range (Figure 6). M/S_{pred} was 1.29. The S/W ratio was equal to 1, the $f_{un.s}/f_{un,m}$ was equal to 1.4 and the $f_s/f_{,m}$ was equal to 0.9. The high M/S observed might partially be, but not fully, explained by ion-trapping (nicotine being a base) in milk and the slightly higher binding to milk proteins.

For acyclovir the M/S_{obs} was reported in the range of 2.2 to 3.3; whereas the diffusion model prediction (M/S_{pred}) was 1.01 (Figure 7). The S/W ratio, $f_{un.s}/f_{un,m}$ and $f_s/f_{,m}$ were all equal to 1. There is no explanation for the high M/S_{obs} in terms of the measured parameters.

Ascorbic acid *in vitro* data predict an M/S ratio of 0.63, which was tenfold less than the observed value of 6.4 (n=57) (Figure 8). As an acid its accumulation in milk cannot be explained by ion-trapping ($f_{un.s}/f_{un,m} = 0.6$). Moreover, there was insignificant binding in both serum and milk and almost no partitioning into milk fat was observed (S/W=1).

Table 3: Measured parameters included skim to whole milk partitioning ratio (S/W), fraction unbound to serum (f_s) and skim milk (f_m) as well as estimated fraction unionized in serum ($f_{un,s}$) and milk ($f_{un,m}$) in human samples obtained from 8 lactating female volunteers.

	Conc [1] (µg/ml)	pKa [2]	S/W	f_s	f_m	$f_{un,s}$	$f_{un,m}$	Ref
Citalopram								
Mean	0.28	9.57	0.58	0.48	0.77	0.01	0.004	3
SD			0.10	0.06	0.12			
Ciprofloxacin								
Mean	0.21	8.38	0.91	0.72	0.91	0.91	0.94	4
SD			0.09	0.05	0.06			
Nicotine								
Mean	0.028	8	1.04	0.88	0.97	0.2	0.14	5
SD			0.08	0.04	0.03			
Acyclovir								
Mean	6.5	9.51	1.01	0.99	0.97	0.99	1	6
SD			0.05	0.06	0.04			
Ascorbic Acid								
Mean	17.6	4.37	1.03	1.05	0.98	0.0009	0.0015	7
SD			0.04	0.09	0.04			

1-Therapeutic serum concentration as obtained from the references.

2-pKa values were obtained from ACD labs v. 6.0

3- (Jensen, Olesen et al. 1997; Spigset, Carieborg et al. 1997; Rampono, Kristensen et al. 2000; Schmidt, Olesen et al. 2000)

4-(Giamarellou, Kolokythas et al. 1989)

5-(Luck and Nau 1984; Dahlstrom, Lundell et al. 1990)

6-(Lau, Emery et al. 1987; Meyer, de Miranda et al. 1988; Bork and Benes 1995; Sheffield, Fish et al. 2002)

7-(Ortega, Quintas et al. 1998)

Figure 3: Correlation of skim to whole milk concentration ratio of citalopram with crematocrit in human subjects (n=8).

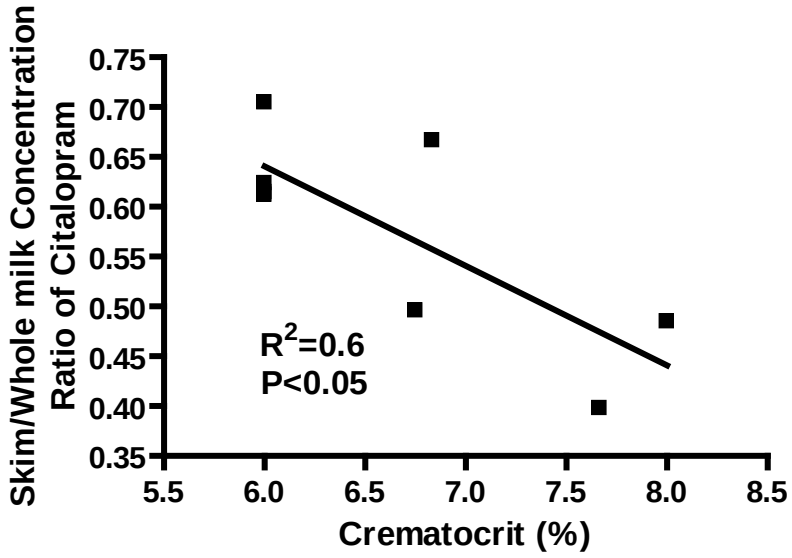


Figure 4: Citalopram. ▲ Predicted M/S (lower and upper limits of the 95% confidence intervals of the 50% and 200% values of predicted M/S respectively). ◆ Predicted M/S (95% CI). ■ Observed M/S (Jensen, Olesen et al. 1997; Spigset, Carieborg et al. 1997; Rampono, Kristensen et al. 2000; Schmidt, Olesen et al. 2000).

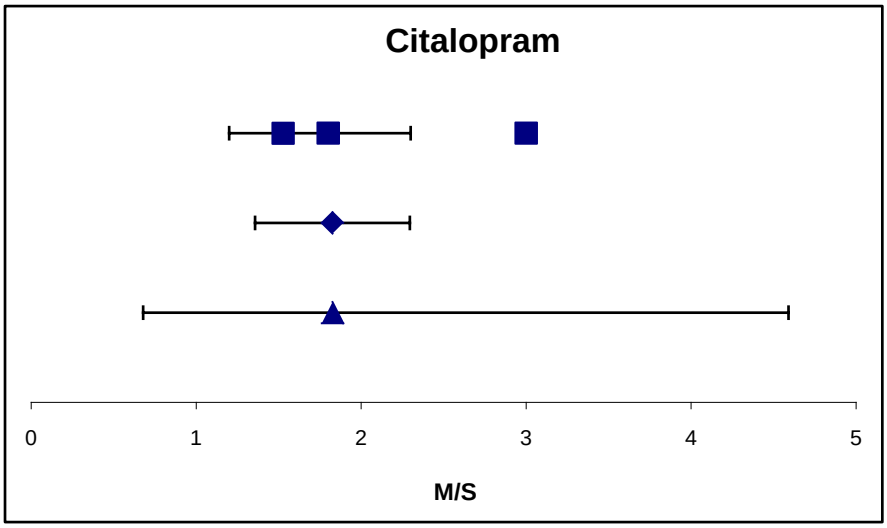


Figure 5: Ciprofloxacin. ▲ Predicted M/S (lower and upper limits of the 95% confidence intervals of the 50% and 200% values of predicted M/S respectively). ◆Predicted M/S (95% CI). ■ Observed M/S (Giamarellou, Kolokythas et al. 1989).

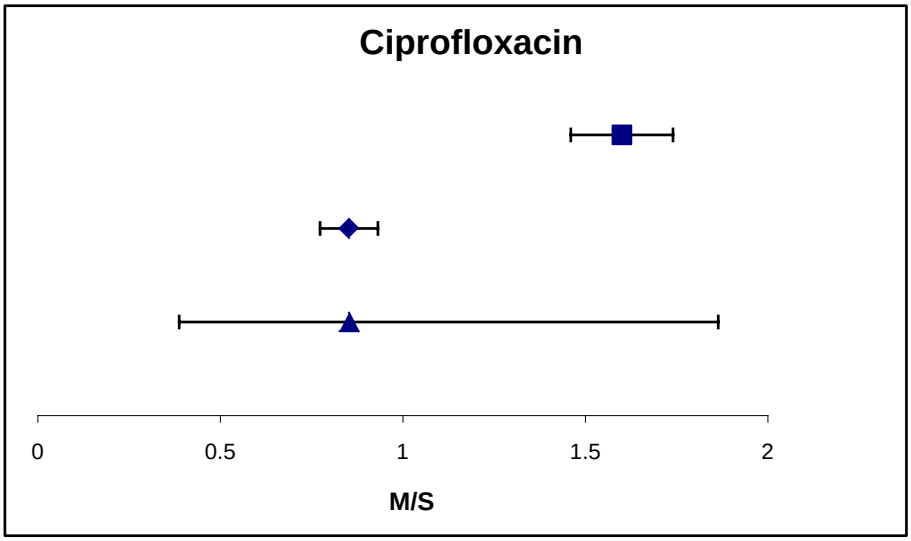


Figure 6: Nicotine. ▲Predicted M/S (lower and upper limits of the 95% confidence intervals of 50% and 200% values of predicted M/S respectively). ◆Predicted M/S (95% CI). ■ Observed M/S (95% CI) (Luck and Nau 1984; Dahlstrom, Lundell et al. 1990).

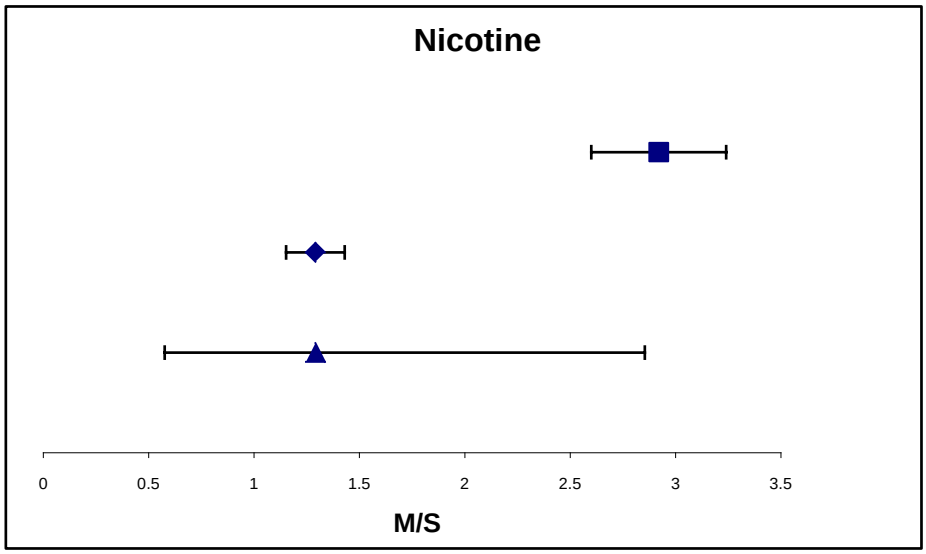


Figure 7: Acyclovir**.** ▲Predicted M/S (lower and upper limits of the 95% confidence intervals of the 50% and 200% values of predicted M/S respectively). ◆Predicted M/S (95% CI). ■ Observed M/S (Lau, Emery et al. 1987; Meyer, de Miranda et al. 1988; Bork and Benes 1995; Sheffield, Fish et al. 2002).

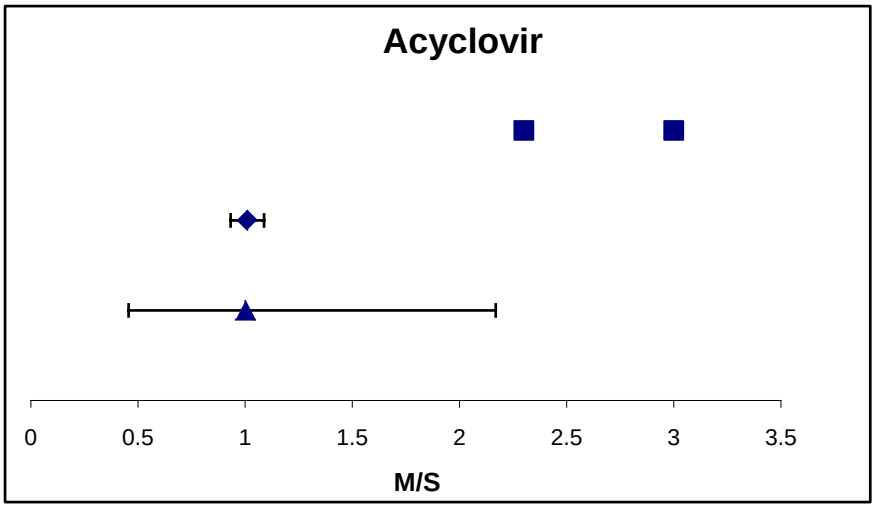
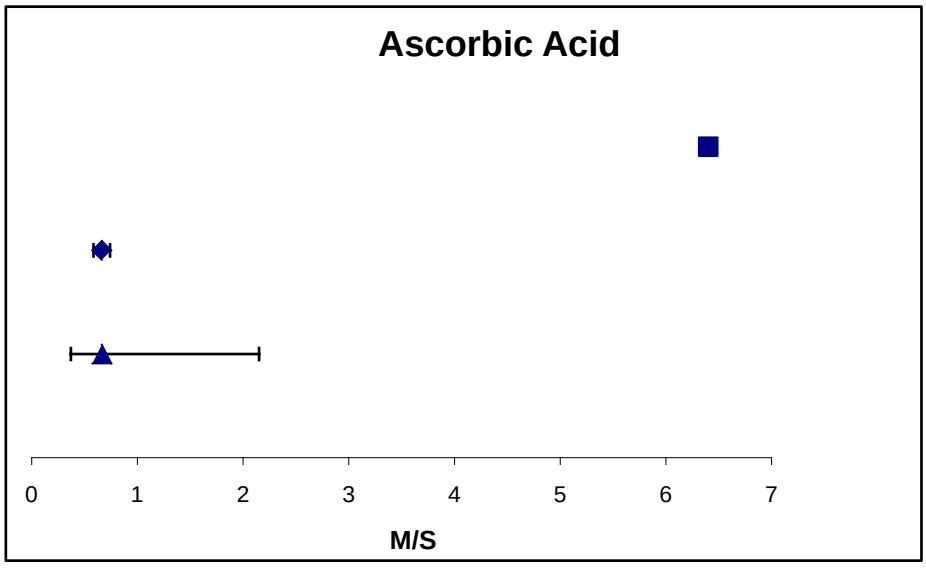


Figure 8: Ascorbic acid. ▲ Predicted M/S (lower and upper limits of the 95% confidence intervals of the 50% and 200% values of predicted M/S respectively). ◆ Predicted M/S (95% CI). ■ Observed M/S (Ortega, Quintas et al. 1998).

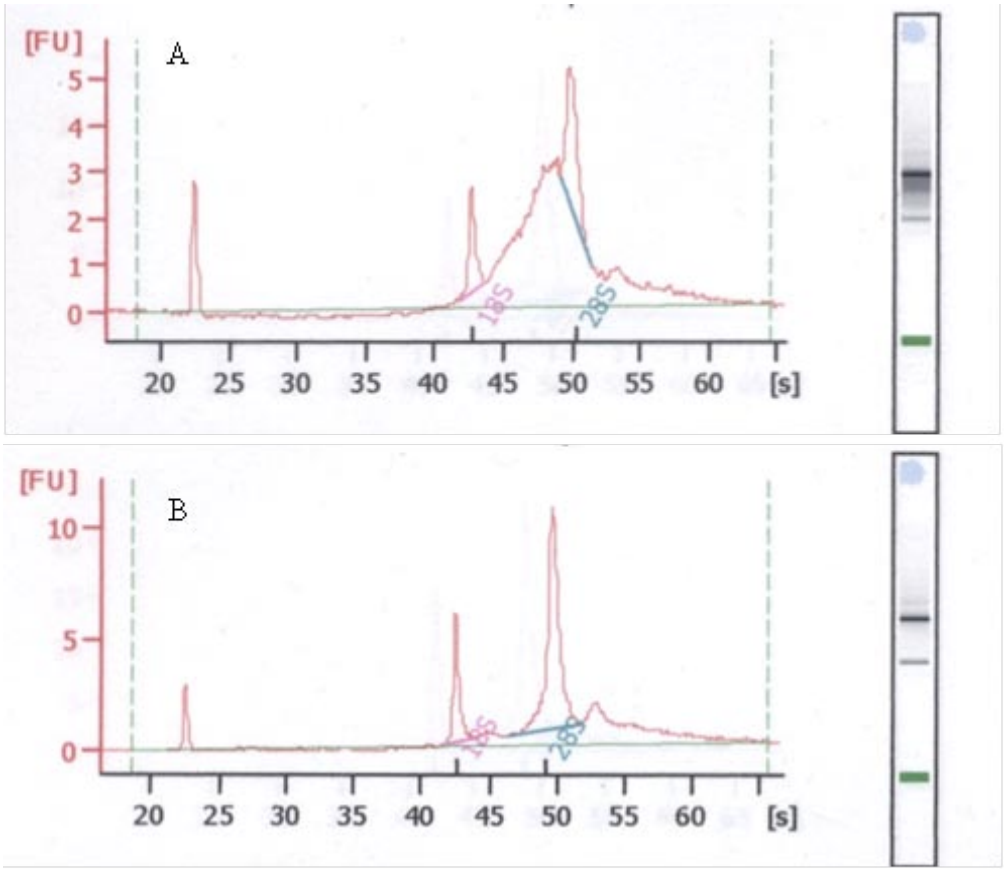


2 *In Vitro and In Vivo* Cyp3a Ontogeny:

2.1 RNA Integrity Results:

RNA integrity was checked to ensure that the RNA samples analyzed for ontogeny patterns were not degraded and thus undermining the results obtained. RNA integrity results from the University of Kentucky Microarray Core Facility were reported in terms of RNA integrity number (RIN), a parameter designed by Agilent Technologies to report the degree of integrity of the sample. Fluorescence versus time of ribosomal RNA peaks as well as RNA bands were also reported. During analysis the RNA samples in the chip flow through micro-channels and are separated according to size and detected by fluorescence, hence a fluorescence chart is generated for RNA peaks as well as RNA bands in the sample compared to a ladder. The sharper the RNA peaks, the less fragmented is the RNA in the sample, as shown by the RNA bands, the higher the RIN number, and the better the quality of the sample (Figure 9). The highest possible RIN number is 10. For the liver samples, only one sample failed to show a RIN number even though the RNA peaks were sharp and the bands showed no fragmentation; this sample was not excluded. The RIN for liver RNA samples ranged from 9.6 to 10 with an average of 9.9. For the intestinal mucosa RNA samples, rat number 3 in the 21 day age group was excluded from RNA integrity analysis and subsequent PCR reactions due to the very low amounts of RNA recovered from the sample. Rat number 1 in the 112 day age group had a RIN of 6.1 and the RNA peak was broad and the bands showed fragmentation, hence the sample was excluded from further analysis. One more sample (Rat number 3, 7 day age group) had a RIN of 8.3 but RNA integrity was not compromised and hence was not excluded. The rest of the intestinal mucosa RNA samples had RIN ranging from 9.2-10 with an average of 9.7.

Figure 9: RNA integrity results for 2 samples. The graphs show RNA peaks detected by fluorescence, the y-axis representing fluorescence units (FU) and the x-axis representing scanning time in seconds. To the right of each graph is a plot of RNA bands to show whether there is fragmentation. A is RNA from intestinal mucosa of a 112 day old rat. RIN was 6.1 and the sample was excluded from analysis. The 28s peak is broad and RNA bands on the right show fragmentation. B is RNA liver sample from a 112 day old rat. RIN was reported to be 10. The RNA fluorescence peaks are sharp and the RNA bands to the right show no fragmentation.



2.2 Quantitative PCR Primer Optimization Results:

The conditions for each primer are listed under the methods section. Representative PCR time course, gels and standard curves generated for each gene under those conditions are presented in Figures 10- 12.

Figure 10: A representative PCR time course for Mdr1a standard curve using rat brain cDNA.

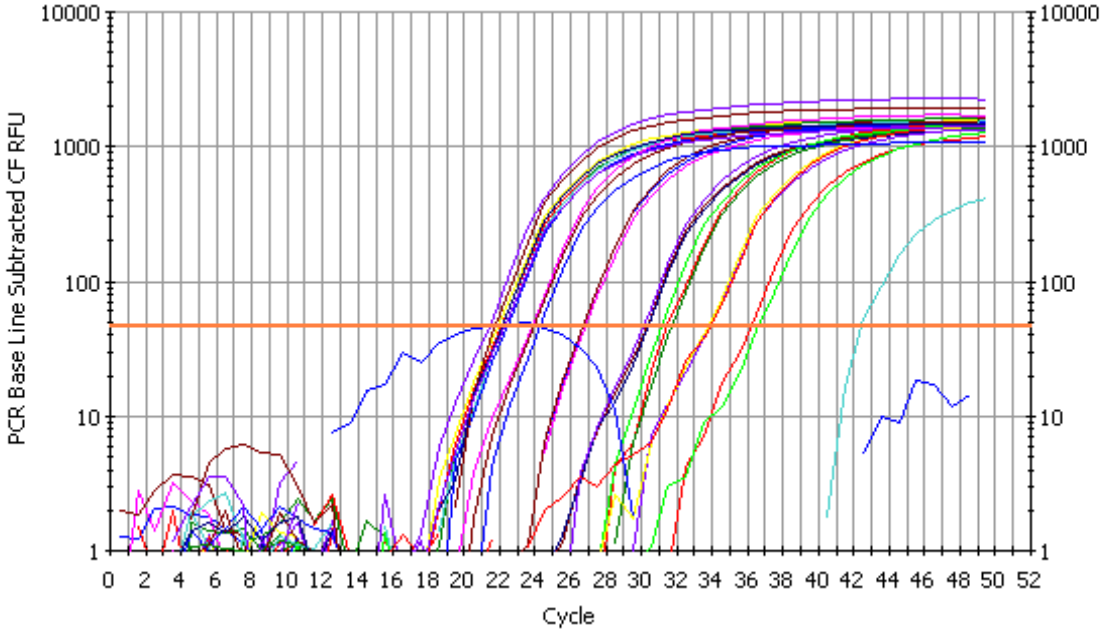


Figure 11: Ethidium bromide stained agarose gels for quantitative PCR products using primers and conditions listed in the methods section. Single bands were detected at the appropriate size for each product.

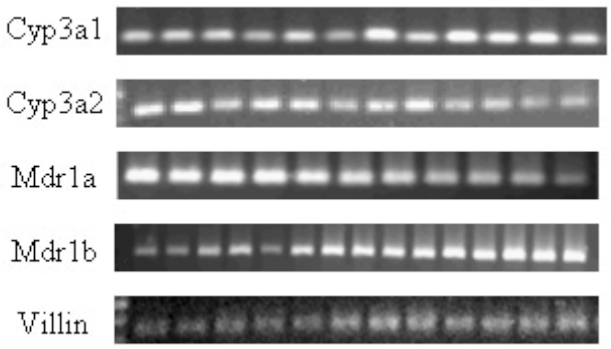
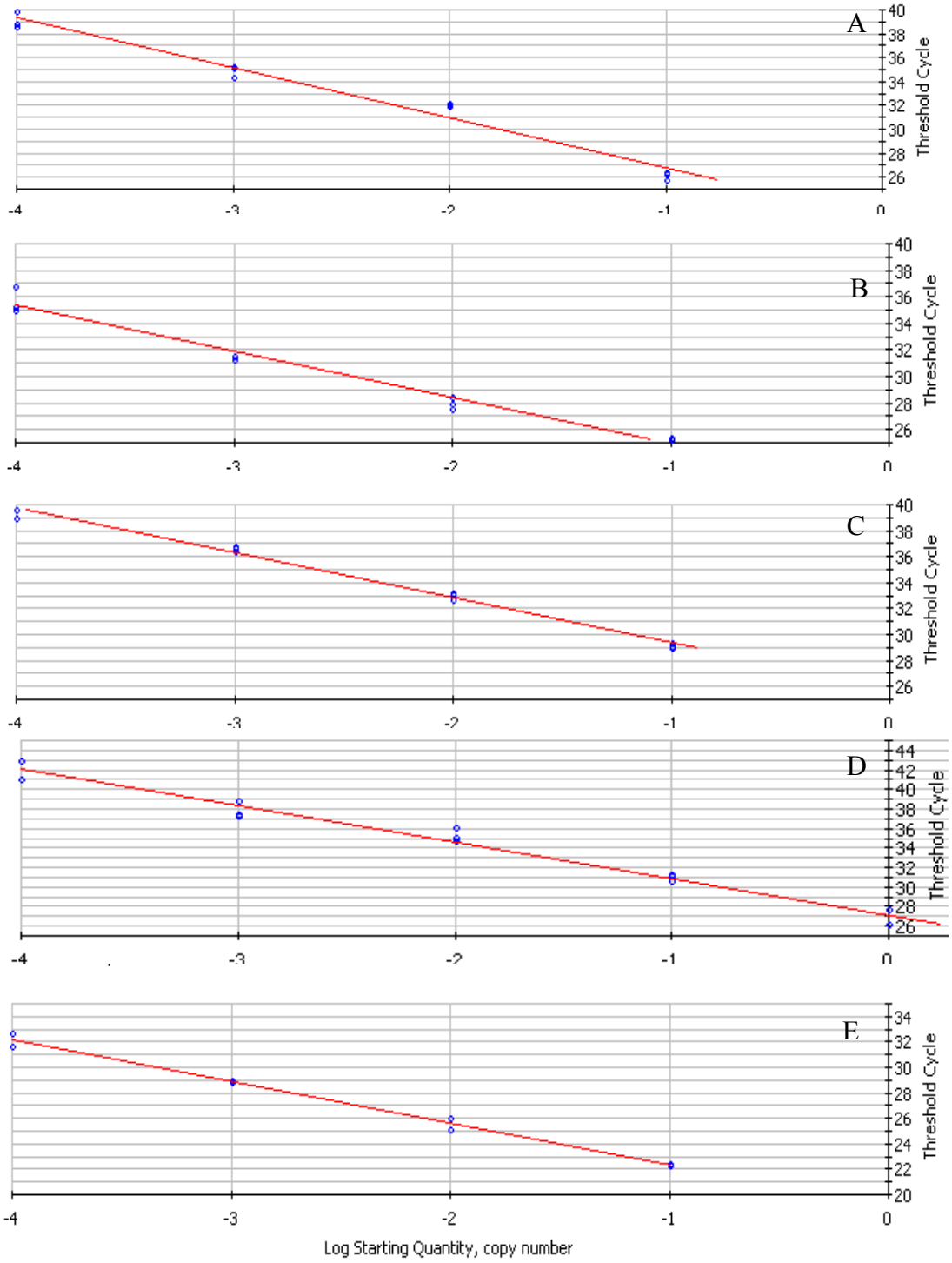


Figure 12: Standard curves for quantitative PCR using primers and conditions listed in the methods section. A-Cyp3a1 using male rat liver cDNA (r^2=0.988). B-Cyp3a2 using male rat liver cDNA (r^2=0.989). C-Mdr1a using male rat brain cDNA (r^2=0.995). D-Mdr1b using male rat liver cDNA (r^2=0.988). E- Villin using male rat



2.3 Quantitative PCR Results for Liver and Intestinal Samples:

2.3.1 Hepatic Expression of Cyp3a1 and Cyp3a2, Mdr1a and Mdr1b Genes:

The development of mRNA expression of Cyp3a1, Cyp3a2, Mdr1a and Mdr1b in the male Sprague-Dawley rat liver was determined using quantitative-PCR of cDNA reverse transcribed from the RNA at 1, 7, 21, 42, 77 and 112 days, 4 animals per age group. The developmental pattern of Cyp3a1 expression (Figure 13) was high at birth and decreased by 1 week of age to adult levels and was stable throughout different age groups. The variability was highest for the 1 day age group and minimal in the rest of the age groups.

Cyp3a2 mRNA expression (Figure 14) was low at one day of age and increased untll almost reached adult levels at 42 days. However, the 21 day age group showed an average expression lower than the 7 day and 42 day age groups and was significantly lower than the 112 day age group ($P<0.05$). The only other age group that had significantly different Cyp3a2 mRNA expression than the 112 day old group was the 1 day age group ($P<0.01$). Variability was highest for the 77 and 112 day age groups. A similar trend was seen in preliminary data (data not shown).

Mdr1a liver expression (Figure 15) was high at one day of age and decreased subsequently. The highest variability was noticed at 1 day of age and it was the only group that had significantly higher expression of Mdr1a mRNA than the 112 day age group ($P<0.05$).

Mdr1b (Figure 16) expression was unique. The expression was the same for all age groups except for the 42 day age group where the expression was the highest and most variable. The expression at 42 days of age (puberty) was significantly higher than the 112 day age group ($P<0.01$). This unique pattern for Mdr1b mRNA expression confirms the results obtained from preliminary experiments (data not shown).

Figure 13: Relative mRNA expression of Cyp3a1 in male Sprague-Dawley rat liver. Normalization was done to total RNA used in the reverse transcription reaction and starting quantities are relative to a male rat liver standard curve dilutions from 0 to 10^{-4} run on the same plate as the samples. Starting quantities are expressed as mean ± standard deviation of 4 animals. (** $P<0.01$ when compared to 112 day group, one-way ANOVA followed by Dunnett's test)

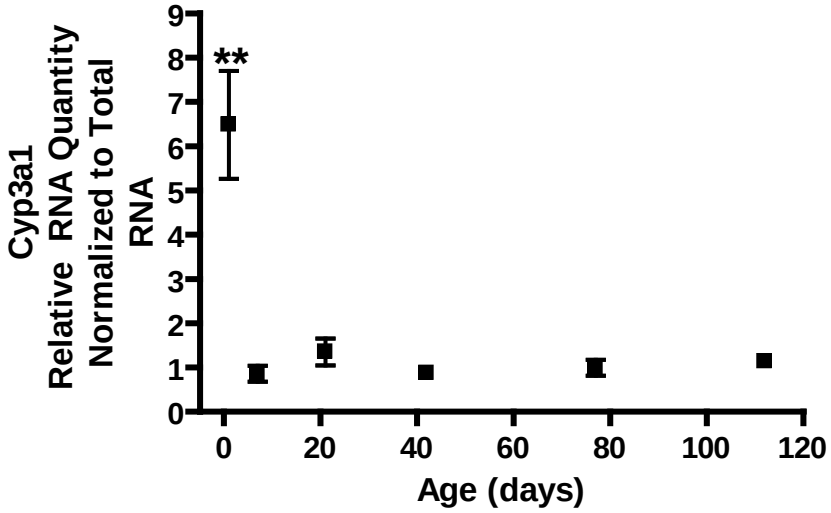


Figure 14: Relative mRNA expression of Cyp3a2 in male Sprague-Dawley rat liver. Normalization was done to total RNA used in the reverse transcription reaction and starting quantities are relative to a male rat liver standard curve dilutions from 0 to 10^{-4} run on the same plate as the samples. Starting quantities are expressed as mean ± standard deviation of 4 animals. (** P<0.01, * P<0.05 when compared to 112 day group, one-way ANOVA followed by Dunnett's test)

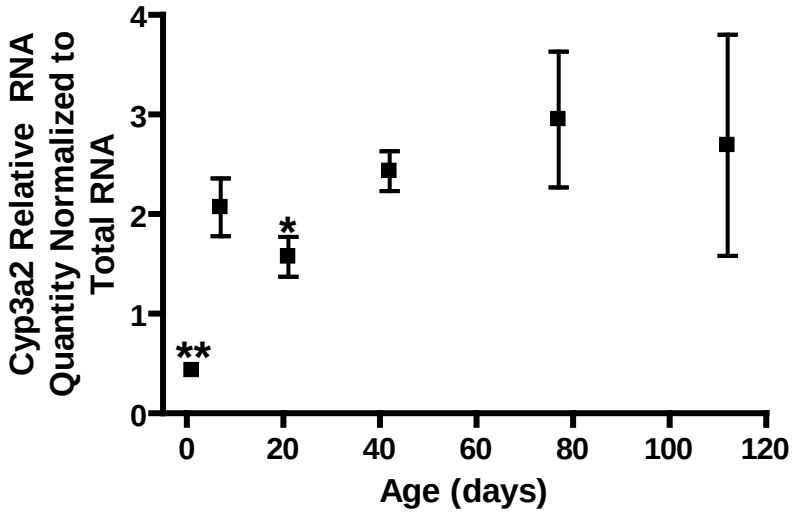


Figure 15: Relative mRNA expression of Mdr1a in male Sprague-Dawley rat liver. Normalization was done to total RNA used in the reverse transcription reaction and starting quantities are relative to a male rat brain standard curve dilutions from 0 to 10^{-4} run on the same plate as the samples. Starting quantities are expressed as mean ± standard deviation of 4 animals. (* $P<0.05$ when compared to 112 day group, one-way ANOVA followed by Dunnett's test)

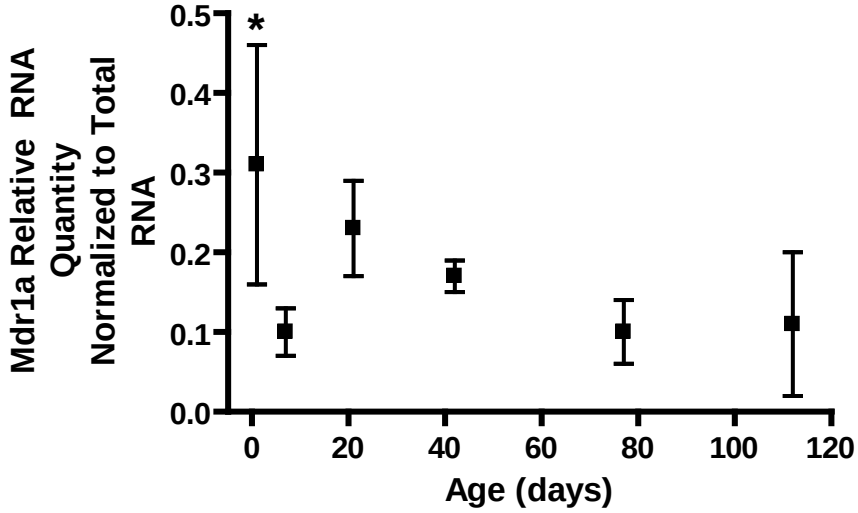
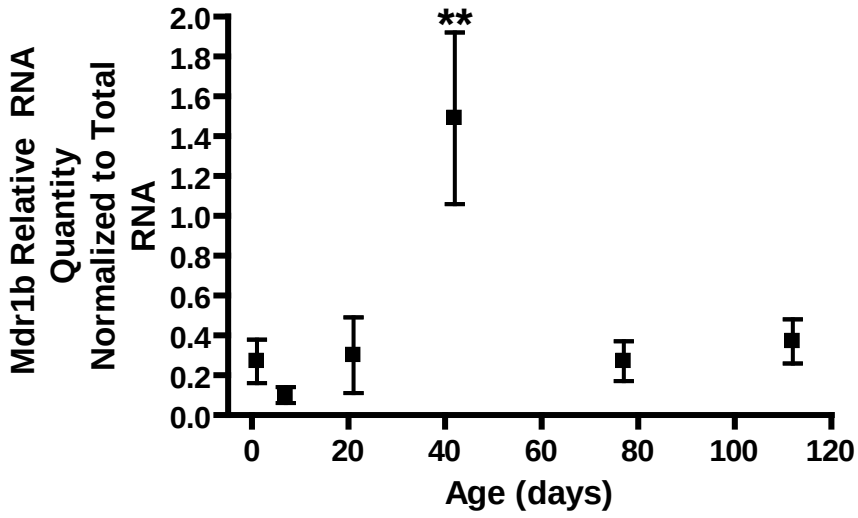


Figure 16: Relative mRNA expression of Mdr1b in male Sprague-Dawley rat liver. Normalization was done to total RNA used in the reverse transcription reaction and starting quantities are relative to a male rat liver standard curve dilutions from 0 to 10^{-4} run on the same plate as the samples. Starting quantities are expressed as mean ± standard deviation of 4 animals. (** P<0.01 when compared to 112 day group, one-way ANOVA followed by Dunnett's test)



2.3.2 Intestinal Mucosa Expression of Villin, Cyp3a1, Cyp3a2 , Mdr1a and Mdr1b Genes in Male Rat:

Villin gene expression was used as a marker for epithelial intestinal mucosa (Maroux, Coudrier et al. 1988) to assess inter-animal and inter-age variability of the intestinal mucosa collected due to variability in the scraping method (Figure 17). Although inter-animal variability was pronounced especially for the 112 day group, the overall coefficient of variation across all animals was 15.5 % and thus the scraping method was considered adequate for intestinal mucosa collection and comparison across different age groups.

Intestinal mucosa Cyp3a1, Cyp3a2, Mdr1a and Mdr1b mRNA expression levels were determined using quantitative PCR in 4 age groups 7, 21, 42 and 112 days. Since both the liver and the intestinal samples were compared to the same standard curves although run on different plates, the magnitude of expression can still be compared. Unlike the liver, Cyp3a1 mRNA in intestinal mucosa (Figure 18) tended to increase with age and was more than 1000-fold lower than in the liver at 112 days old (significantly different $P=0.0001$).

Cyp3a2 mRNA expression was detectable in all of the intestinal samples but was not quantifiable in the linear range of the quantitative-PCR reactions except for the 7 day age group, indicating much lower expression than in the liver. Mean relative expression of Cyp3a2 in the liver ranged from 0.43 to 2.69 for the age groups. In the intestine the highest expression was in the 7-day age group and the average was 0.0002.

Mdr1a mRNA (Figure 19) increased with age. The expression at 7, 21 and 42 days were significantly lower than the expression at 112 days. At 112 days, the intestinal mucosa mRNA relative levels for Mdr1a were more than 150 fold higher than in the liver.

Relative Mdr1b expression (Figure 20) in the intestinal mucosa was comparable to the liver except for the 42 day old age group. The 42 day age group Mdr1b intestinal expression seemed lower than the other age groups but was not significantly different from the 112 day age group when all the age groups where compared using one-way

ANOVA. In contrast, the expression of hepatic Mdr1b in the 42 day age group was significantly higher than the rest of the age groups.

Figure 17: Relative mRNA expression of villin in male Sprague-Dawley rat intestinal mucosa. Normalization was done to total RNA used in the reverse transcription reaction and starting quantities are relative to a male rat intestinal mucosa standard curve dilutions from 0 to 10^{-4} run on the same plate as the samples. Starting quantities are expressed as mean ± standard deviation of 3 animals for 21 and 112 day age groups and of 4 animals for the 7 and 42 day age groups.

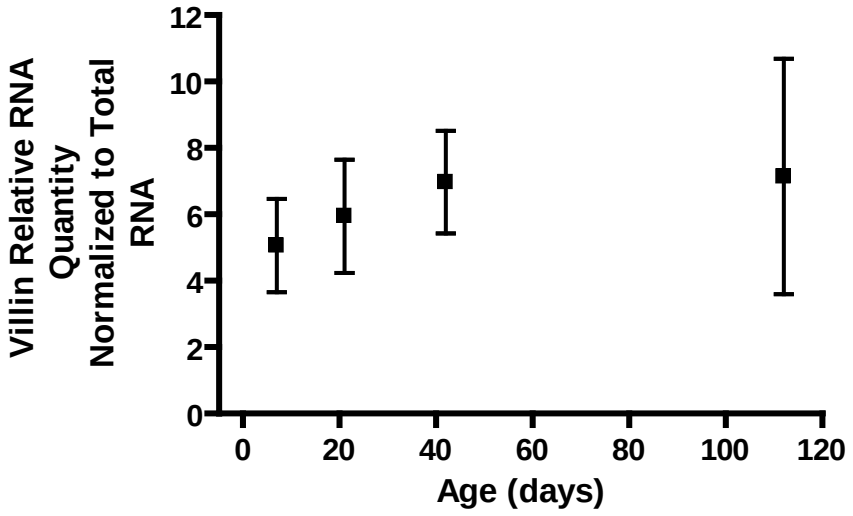


Figure 18: Relative mRNA expression of Cyp3a1 in male Sprague-Dawley rat intestinal mucosa. Normalization was done to total RNA used in the reverse transcription reaction and starting quantities are relative to a male rat liver standard curve dilutions from 0 to 10^{-4} run on the same plate as the samples. Starting quantities are expressed as mean ± standard deviation of 3 animals except for the 42 day age group where n=4.

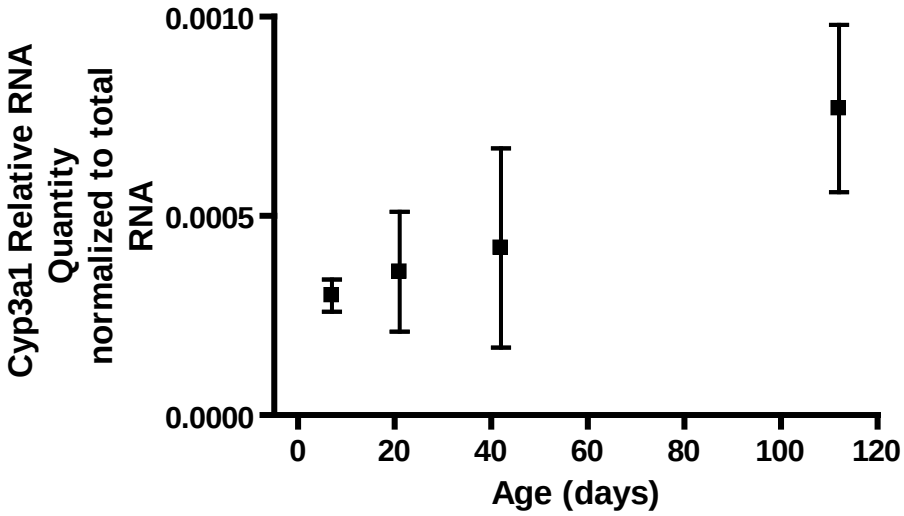


Figure 19: Relative mRNA expression of Mdr1a in male Sprague-Dawley rat intestinal mucosa. Normalization was done to total RNA used in the reverse transcription reaction and starting quantities are relative to a male rat brain standard curve dilutions from 0 to 10^{-4} run on the same plate as the samples. Starting quantities are expressed as mean ± standard deviation of 3 animals for 21 and 112 day age groups and of 4 animals for the 7 and 42 day age groups. (** P<0.01, * P<0.05 when compared to 112 day group, one-way ANOVA followed by Dunnett's test)

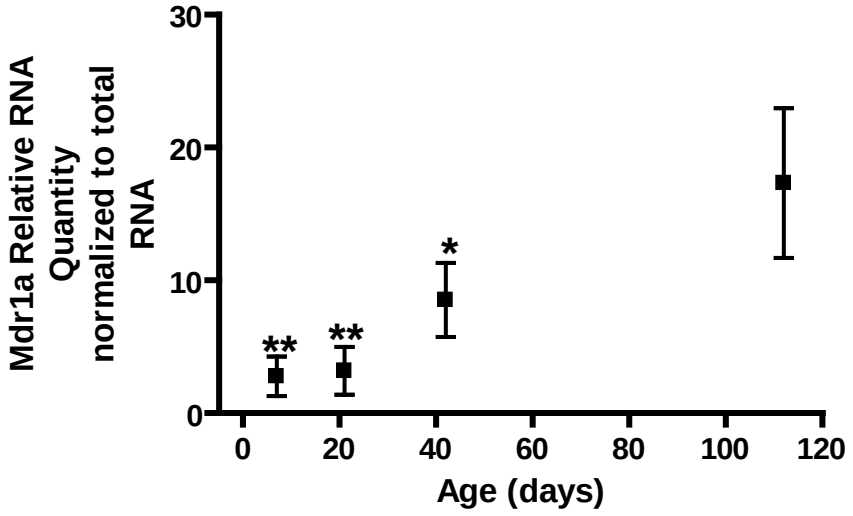
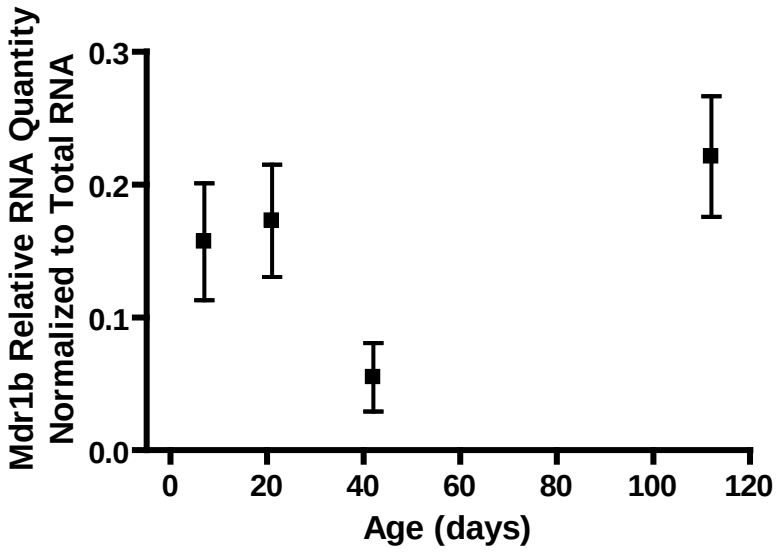


Figure 20: Relative mRNA expression of Mdr1b in male Sprague-Dawley rat intestinal mucosa. Normalization was done to total RNA used in the reverse transcription reaction and starting quantities are relative to a male rat liver standard curve dilutions from 0 to 10^{-4} run on the same plate as the samples. Starting quantities are expressed as mean ± standard deviation of 3 animals for 21 and 112 day age groups and of 4 animals for the 7 and 42 day age groups.



2.4 Expression of Cyp3a protein:

2.4.1 Antibody specificity:

The specificity of the antibodies purchased from Research Diagnostics, BD Gentest (Daiichi manufactured) and Chemicon was tested against rat Cyp3a1 and Cyp3a2 Supersomes. As shown in Figure 21, anti-rat Cyp3a1 from both Research Diagnostics and Chemicon were shown to be non specific, although the interaction with Cyp3a2 protein seems to be weaker with Chemicon's antibody. Daiichi anti-rat Cyp3a2 was non specific as well. Anti-rat Cyp3a2 from Chemicon was specific, bands were only detected for Cyp3a2 protein and none for Cyp3a1 protein. To further confirm the Chemicon antibody specificity, Cyp3a1 and Cyp3a2 Supersomes mixtures were prepared where one of the proteins was constant in amount and the other was varied. The interaction of the mixtures with each antibody was plotted against the net intensity of the bands. Figure 22-A shows that when the Chemicon anti-Cyp3a2 antibody was used, the net intensity of the bands increased when Cyp3a1 protein was constant and Cyp3a2 protein increased. The net intensity was constant when Cyp3a2 protein was constant and Cyp3a1 protein varied in the mixture. These data further confirm the specificity of anti-Cyp3a2 for Cyp3a2 protein. For the Cyp3a1 antibody (Figure 22-B), the net intensity increased when either of the proteins was increased and the other kept constant showing the lack of specificity of this antibody. Due to the cross-reaction of the anti-Cyp3a1 antibody, Cyp3a1 protein ontogeny could not directly be addressed.

Figure 21: Characterization of cross-reactivity of Cyp3a antibody. Rat anti-Cyp3a1 from Research Diagnostics and anti-Cyp3a2 from Daiichi and anti-Cyp3a1 and anti-Cyp3a2 from Chemicon were used to probe equal protein amounts (~1.16ng) of rat Cyp3a1 and Cyp3a2 Supersomes.

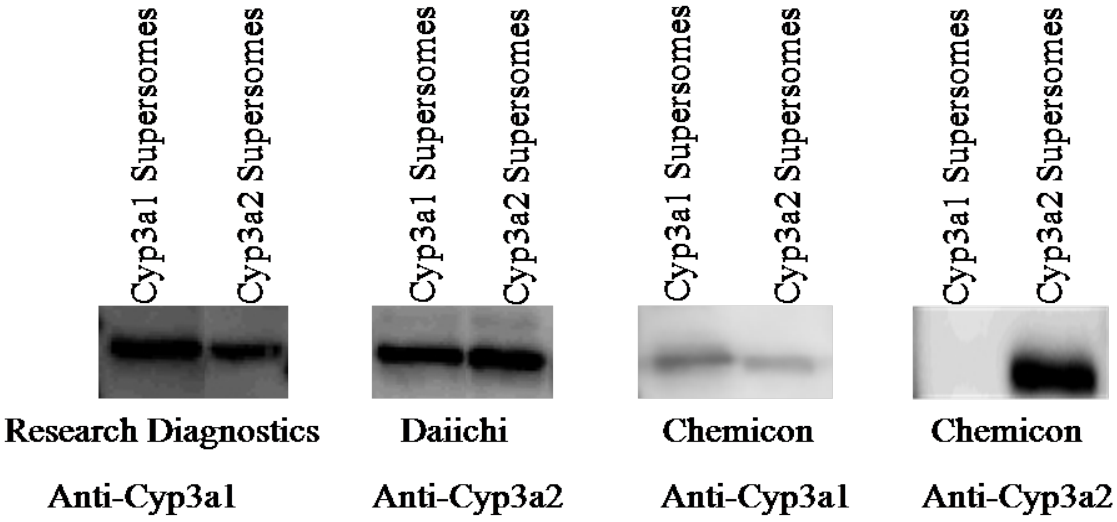
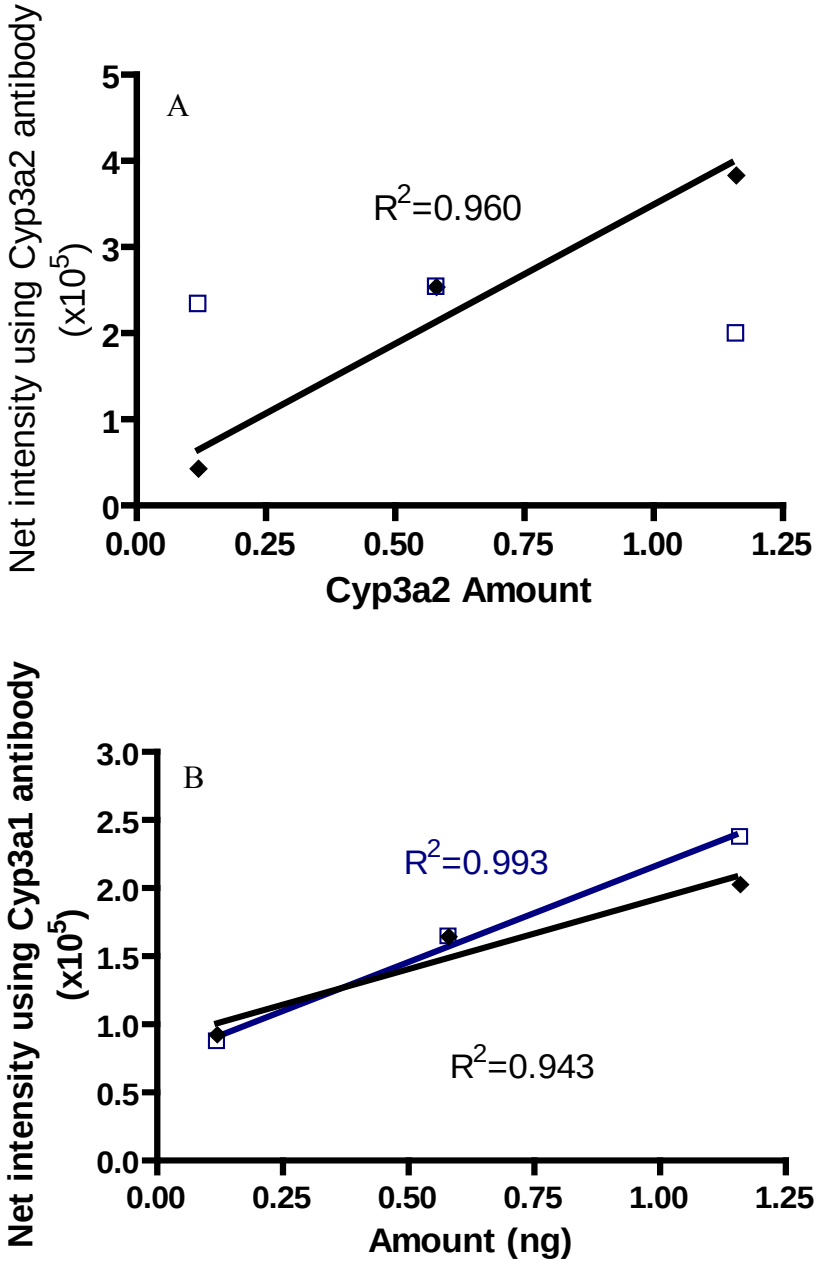


Figure 22: Interaction of Cyp3a1 and Cyp3a2 Supersomes mixtures with anti-rat Cyp3a2 (A) and anti-rat Cyp3a1 (B) antibodies from Chemicon.

◆ Cyp3a2 Varying Amounts with Constant 0.58 ng Cyp3a1 Amount.

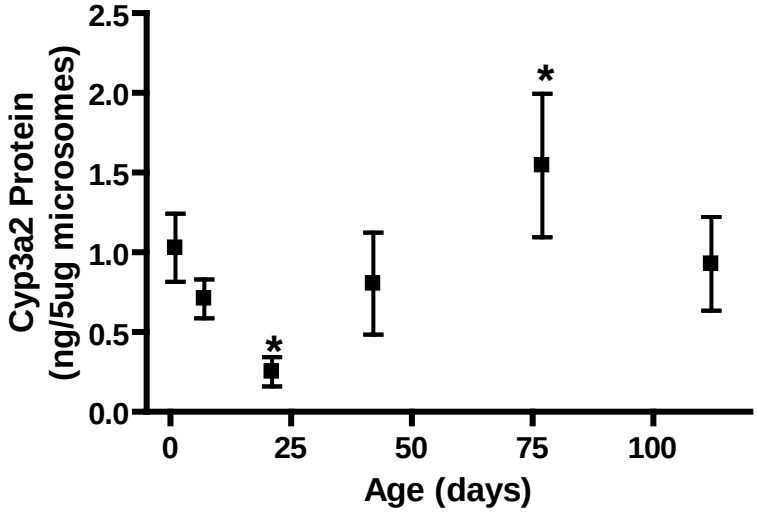
□ Cyp3a1 Varying Amounts with Constant 0.58 ng Cyp3a2 Amount.



2.4.2 Cyp3a2 Expression in the Liver:

Western blotting for liver microsomal protein using a specific anti-rat Cyp3a2 antibody was used for the determination of the ontogenic pattern of Cyp3a2 in the developing rat liver. Age groups used were 1, 7, 21, 42, 77 and 112 days, 4 animals per group except for the 1 day group which had 3 animals. Cyp3a2 protein content was normalized to the total amount of microsomes loaded and determined from a Cyp3a2 Supersomes™ standard curve. The results are plotted against age (Figure 23). The amounts and standard curves were calculated based on duplicate loading of samples and standards except for the 42 day age group, which was determined based on only a set of loading. At 21 days of age, the Cyp3a2 content was the lowest observed among the age groups under test and was significantly lower than in the 112 day age group. Cyp3a2 mRNA expression was also the lower at 21 days of age (Figure 14). The expression at 77 days of age was significantly higher than at 112 days. Since anti-Cyp3a1 antibody was not specific, data from liver microsomal blots probed with anti-Cyp3a1 cannot provide useful information on Cyp3a1 ontogeny in male rat liver.

Figure 23: Cyp3a2 protein expression in male Sprague-Dawley rat liver microsomes (mean of 4 animals ± standard deviation). Each animal sample was loaded in duplicate. For the 42 day old sample, only one set of the duplicate loading was used for concentration calculation. (* $P<0.05$ when compared to 112 day group, one-way ANOVA followed by Dunnett's test)



2.4.3 Cyp3a2 And Cyp3a1 Expression in the Intestine:

Intestinal mucosa microsomal Cyp3a2 expression had much lower intensity than liver microsome Cyp3a2, despite the increased protein loading for intestine (10 μg) compared to liver (5 μg) (Figure 24). Probing with the primary antibody anti-rat Cyp3a1 from Chemicon, for age groups 7, 21 and 42 days, produced stronger bands than Cyp3a2. Although the antibody is non-specific, the lack of an adequate Cyp3a2 response in 7 day old rats indicates the presence of only Cyp3a1 protein. Representative blots for 7 and 42 days group are shown in Figure 25.

Figure 24: Cyp3a2 western blotting of liver and intestinal mucosa microsomes using Chemicon anti-Cyp3a2, N=4, each animal sample was loaded in duplicate. The numbers above each band represents an animal microsome sample.

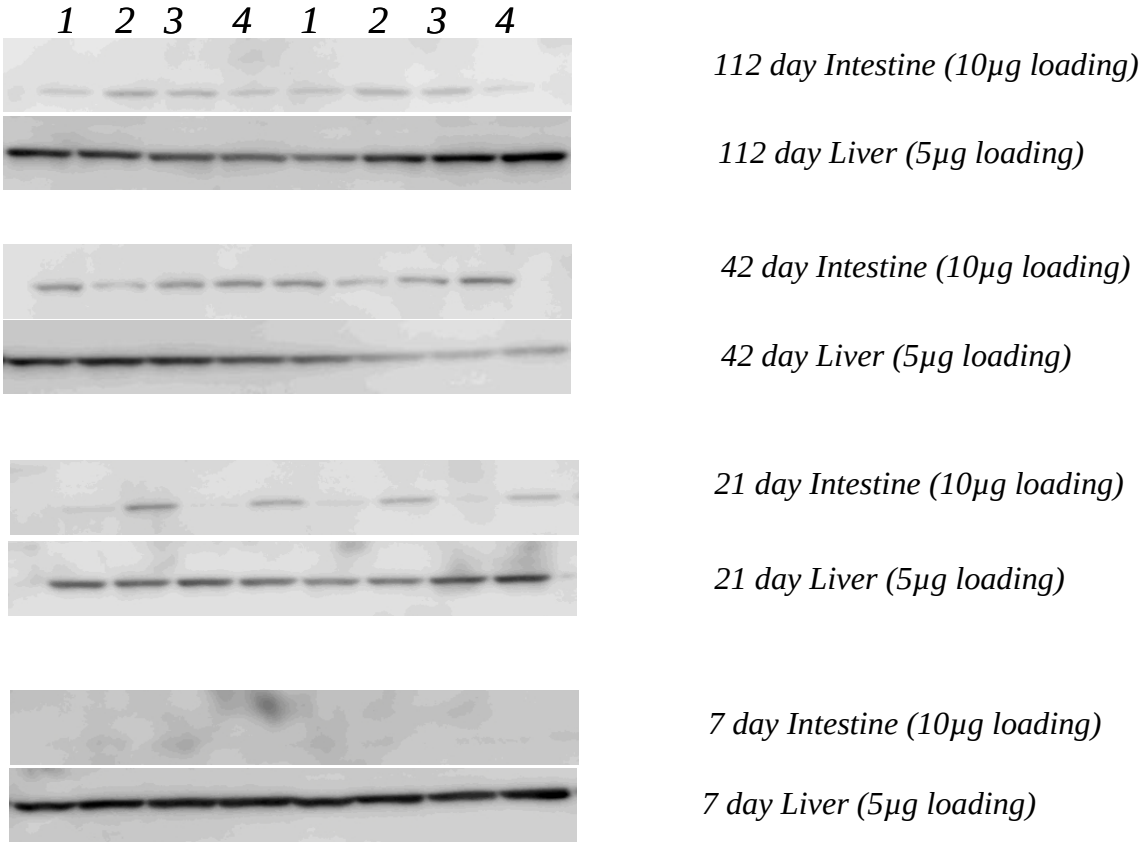
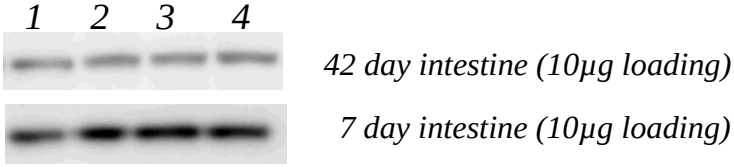


Figure 25: Cyp3a1 western blotting of male rat intestinal mucosa microsomes. The numbers above each band represents an animal microsomal protein sample.



2.5 Developmental Activity of Cyp3a

2.5.1 Developmental Activity of Cyp3a in Male Rat Liver:

The *in vitro* activity of Cyp3a across developmental ages was determined by the 4-hydroxylation of midazolam in male rat liver microsomes, 4-hydroxymidazolam being the major midazolam Cyp3a metabolite in male rat livers. 4-Hydroxymidazolam was measured in the reaction mixture using HPLC. The 1-hydroxymidazolam metabolite did not interfere with the 4-hydroxymidazolam peak (Figure 26). A representative standard curve is shown in Figure 27. Time and protein linearity studies showed the reactions to be linear over a time frame of 1-10 minutes and protein amount of 15-75 μg per 200 μL reaction (Figure 28). The reactions were carried out for 5 minutes using 50 μg microsomal protein and 60 μM midazolam as determined from preliminary studies. The midazolam concentration of 60 μM was based on plotting the reaction velocity (V) against substrate concentration (0-500 μM). The velocity starts to decrease at midazolam concentrations greater than 60 μM due to auto-inhibition which has been documented in the literature (Houston and Kenworthy 2000). Vmax normalized to time and microsomal protein was initially low and came to a minimum at 21 days (weaning) and then increased to reach adult levels by day 77 (Figure 29). The activity was significantly lower in all the age groups compared to 112 day old group except for 77 days. When Vmax was normalized to Cyp3a2 protein amount obtained from the western blot, Vmax was constant with age except for the 21 day age group which was significantly higher (Figure 30).

Figure 26: HPLC chromatograms of 4-hydroxymidazolam (A) and 1- hydroxymidazolam (B). Retention times 6.9 and 8.2 minutes respectively.

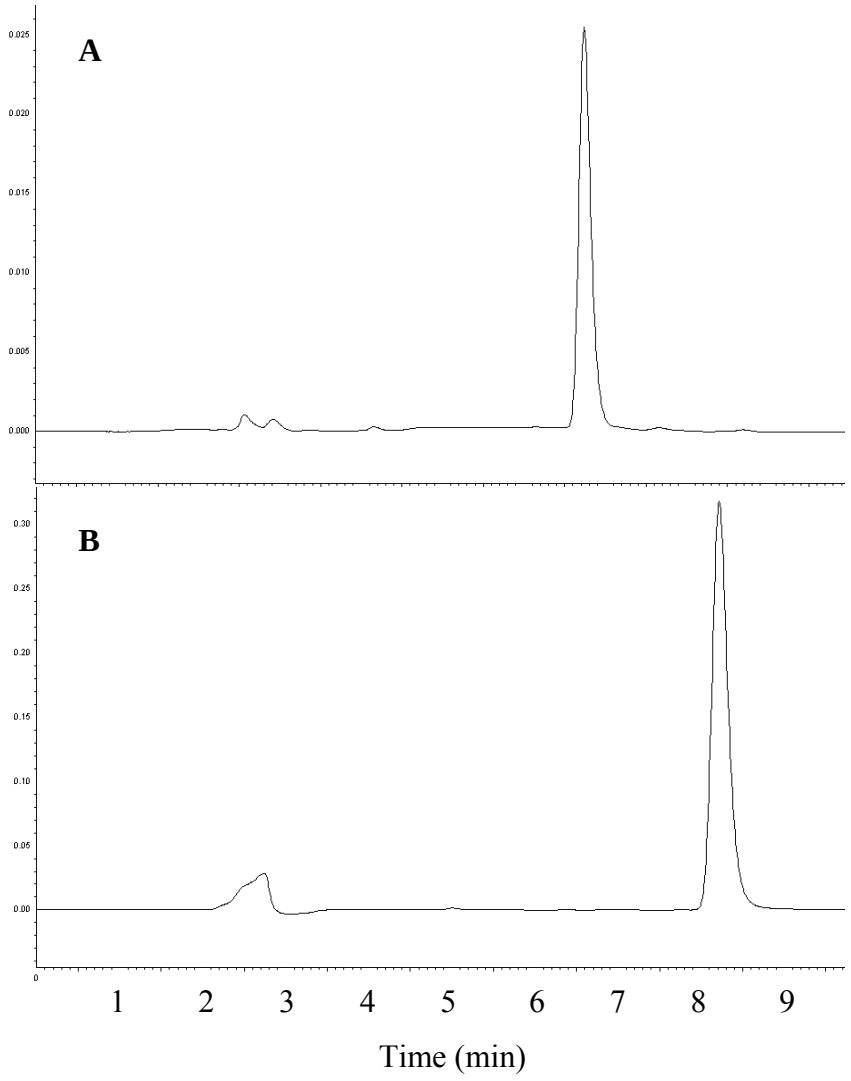


Figure 27: HPLC standard curve of 4-hydroxymidazolam. Five standards were determined in triplicate.

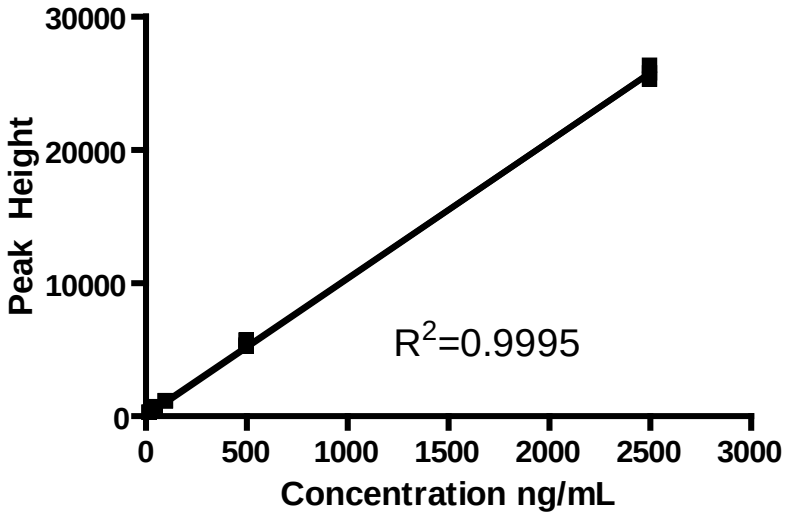


Figure 28: Linearity with time and protein amount of the 4-hydroxylation of midazolam by male rat liver microsomes. Time linearity (A) was done at constant protein amount. Protein linearity (B) was done for 5 minutes of incubation at midazolam concentration of 120 μM.

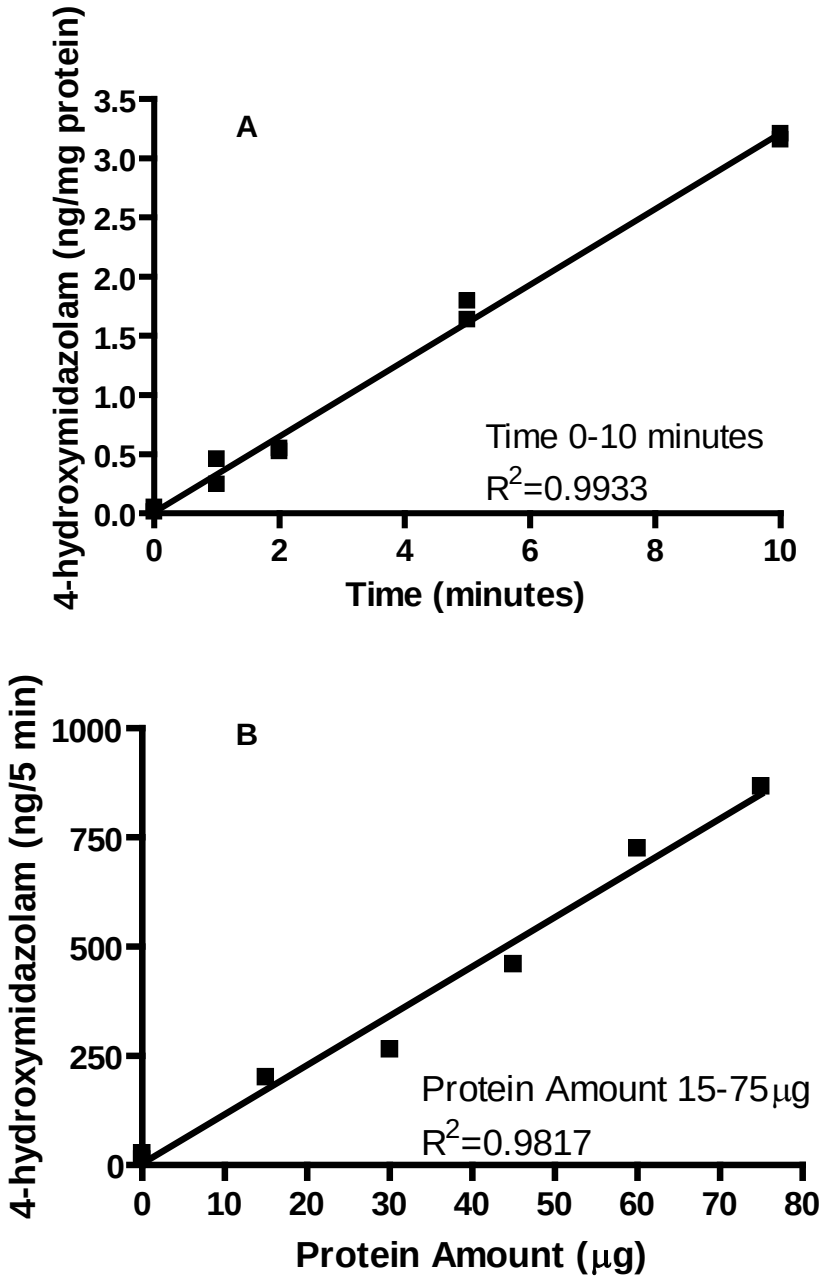


Figure 29: Vmax ontogeny for the 4-hydroxylation of midazolam in male rat liver. Midazolam (60µM), 50µg microsomal protein and 5 minutes incubation. (n=4, ** P<0.01, * P<0.05 when compared to 112 day group, one-way ANOVA followed by Dunnett's test).

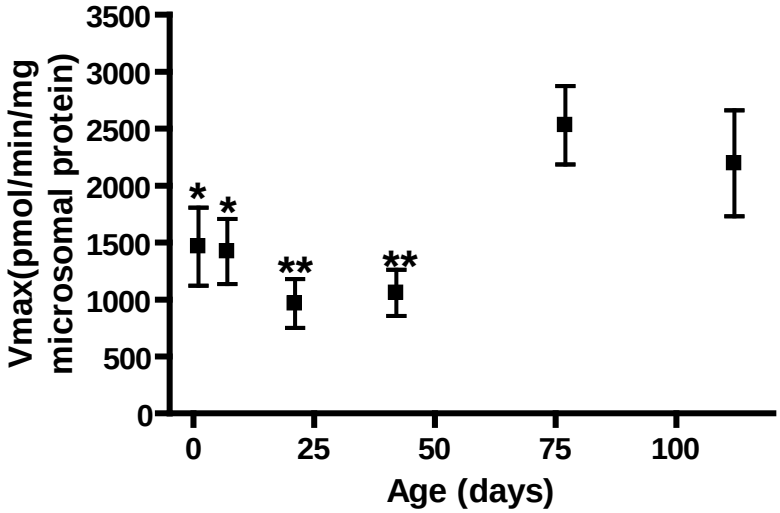
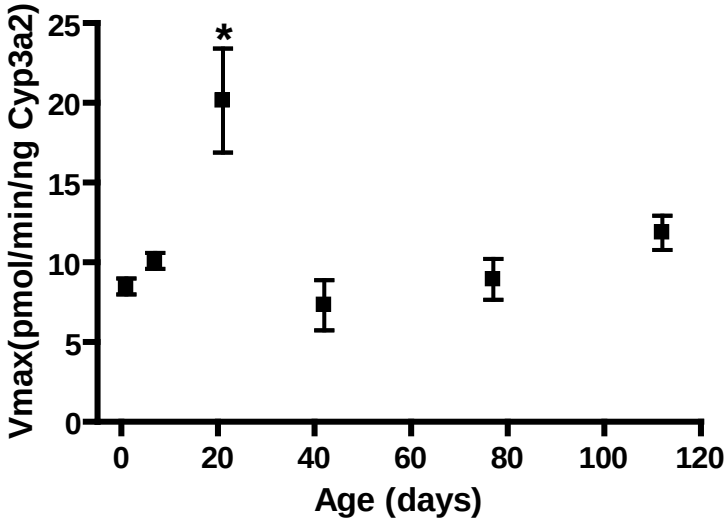


Figure 30: Vmax ontogeny for the 4-hydroxylation of midazolam in male rat liver normalized to Cyp3a2 protein. Midazolam (60µM), 50µg microsomal protein and 5 minutes incubation. (n=4, * P<0.05 when compared to 112 day group, one-way ANOVA followed by Dunnett's test).



2.5.2 Developmental Activity of Cyp3a in Male Rat Intestine:

The reaction was determined to be linear within 5-15 minutes and 50-400 µg microsomal protein at 60 µM. Midazolam 4-hydroxylation in intestinal mucosa microsomes was minimal under the current experimental conditions (200 µg protein, 10 minutes and 60 µM midazolam). Activity in the intestine was only noticed in several animals in the 42 and 112 day age groups and was only in the range of 30 pmol/min/mg protein (more than 80 fold lower than the liver) and none was seen in the 7 and 21 day age groups. These results are consistent with the Cyp3a RNA and protein expression in the intestinal mucosa compared to the liver. Hence, midazolam metabolism in the intestine by Cyp3a was considered negligible for modeling purposes.

2.5.3 Km Vmax Development in Rat Liver:

Km and Vmax for the 4-hydroxylation of midazolam in the pooled rat liver microsomes were modeled using Michaelis-Menten kinetics in GraphPad Prism. The assay was performed at midazolam concentrations of 0-500 µM (Figure 31). When the reaction velocity (V) is plotted against substrate concentration, the velocity starts to decrease at midazolam concentration greater than 60 µM due to auto-inhibition, which has been documented previously in the literature (Houston and Kenworthy 2000). Therefore, modeling was restricted to the range of 0-60 µM (Table 4, Figure 32). Table 4 lists fitted parameters of Michaelis-Menten kinetics. The average and standard error are generated from the 95% confidence interval of fitting curves in Figure 32. To determine whether Km's significantly varied with age, the modeled Km values were statistically tested with Kruskal-Wallis test and the differences were not significantly different (P value = 0.4060). Thus, the model assumption that Km does not vary with age appears to be valid. To further confirm that Km does not change with age, the data was fit to the Michaelis-Menten kinetics using WinNonlin v.4.1 (Pharsight Corporation, Bedford, MA) twice. One fit was done assuming different Km values and the other assuming a single Km value for all the age groups. The resultant Vmax values from both fits are plotted in Figure 33.

Figure 31: Midazolam 4-hydroxylation by pooled male rat liver microsomes (pooled from 4 animals for each age group). Midazolam Concentrations 0-500 µM.

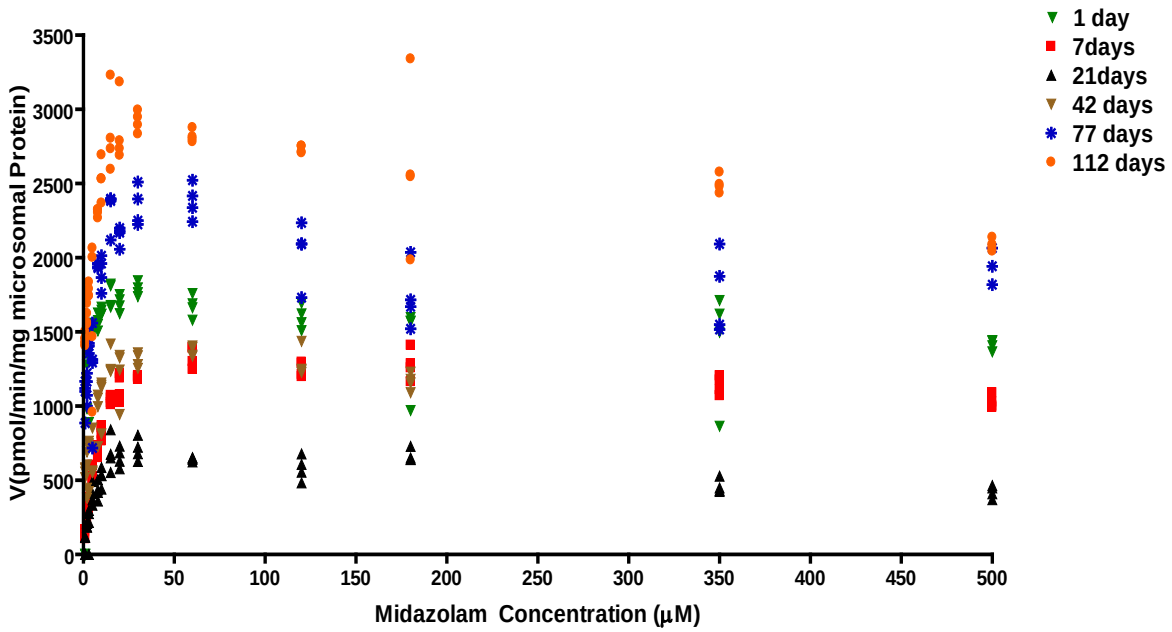


Figure 32: Michaelis-Menten fit of midazolam 4-hydroxylation by pooled male rat liver microsomes. Midazolam concentrations 0-60 µM.

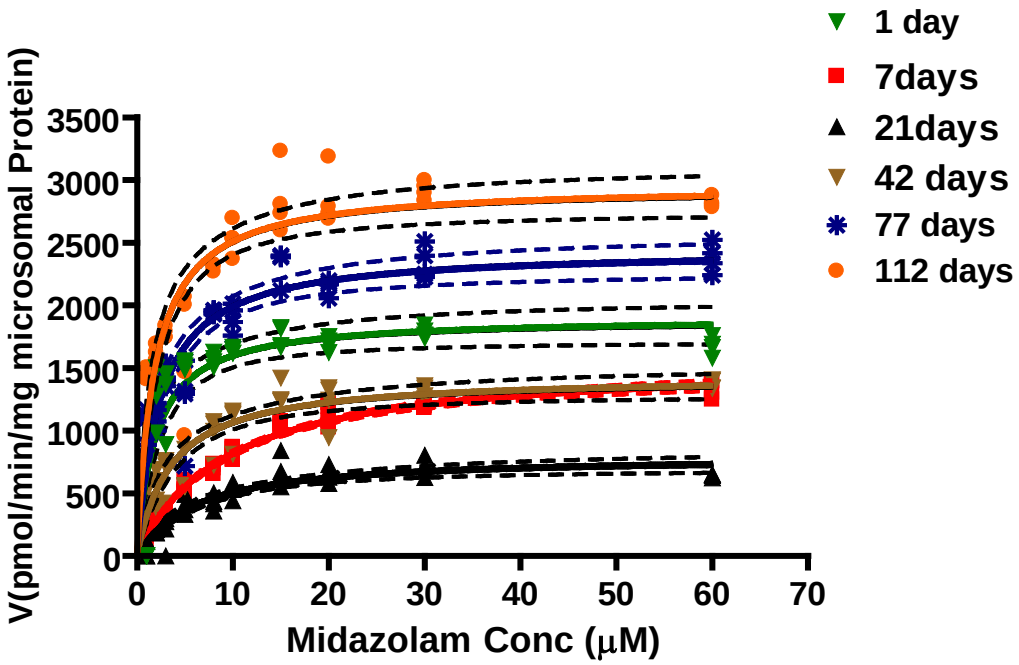
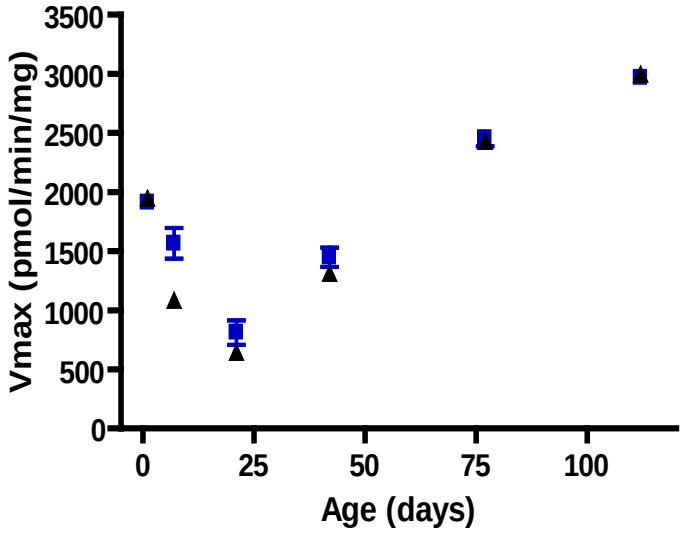


Table 4: Michaelis-Menten fit parameters of midazolam 4-hydroxylation by pooled male rat liver microsomes (mean ± standard error) using GraphPad Prism. Midazolam concentrations 0-60 µM.

Age (days)	Vmax ± SE (pmol/min/mg)	Km ± SE (µM)
1	1910 ± 86	1.95 ± 0.41
7	1590 ± 34	9.36 ± 0.56
21	810 ± 45	6.00 ± 1.00
42	1450 ± 62	3.55 ± 0.58
77	2460 ± 80	2.34 ± 0.33
112	2970 ± 93	1.82 ± 0.28

Figure 33: Michaelis-Menten fit of Vmax of midazolam 4-hydroxylation by pooled male rat liver microsomes (mean ± standard deviation) using WinNonlin assuming different or same Km values. Midazolam concentrations 0-60 µM.

■ Different Km values ▲ Same Km values



2.6 *In Vivo* Clearance Determination:

Oral midazolam (20 mg/kg) was administered to 7, 21 and 112 day rats and serum samples were collected by destructive sampling to be able to calculate *in vivo* clearance for the different age groups. Serum samples were analyzed for midazolam using LC/MS/MS. Standard curves of different ranges were needed to accommodate the concentration ranges in the different age groups. A representative chromatogram and standard curve are shown in Figure 34. The pharmacokinetic profiles are shown in Figure 35. The model independent parameters were obtained by non-compartmental analysis in WinNonlin v 4.1 (Table 5). Maximum concentration (Cmax) and area under the concentration-time curve (AUC) appeared to increase with age. To characterize clearance more accurately the data was analyzed using NONMEM (University of California, San Francisco) by fitting to a one compartmental, first order absorption model. Since destructive sampling was used the data is comprised of one observation per subject (rat), hence it would be more appropriate to employ population kinetics approach to analyze the data. NONMEM employs nonlinear mixed effects method for population kinetics. It adds the advantage of estimating interindividual variability. The average oral clearance values obtained by NONMEM for 7, 21 and 112 day old rats are 3.7, 5.2 and 64 L/hr/kg respectively (Table 6).

Figure 34: LC/MS/MS analysis of midazolam in rat serum using flurazepam as internal standard. A-Representative standard curve. B-Midazolam chromatogram. C-Flurazepam chromatogram.

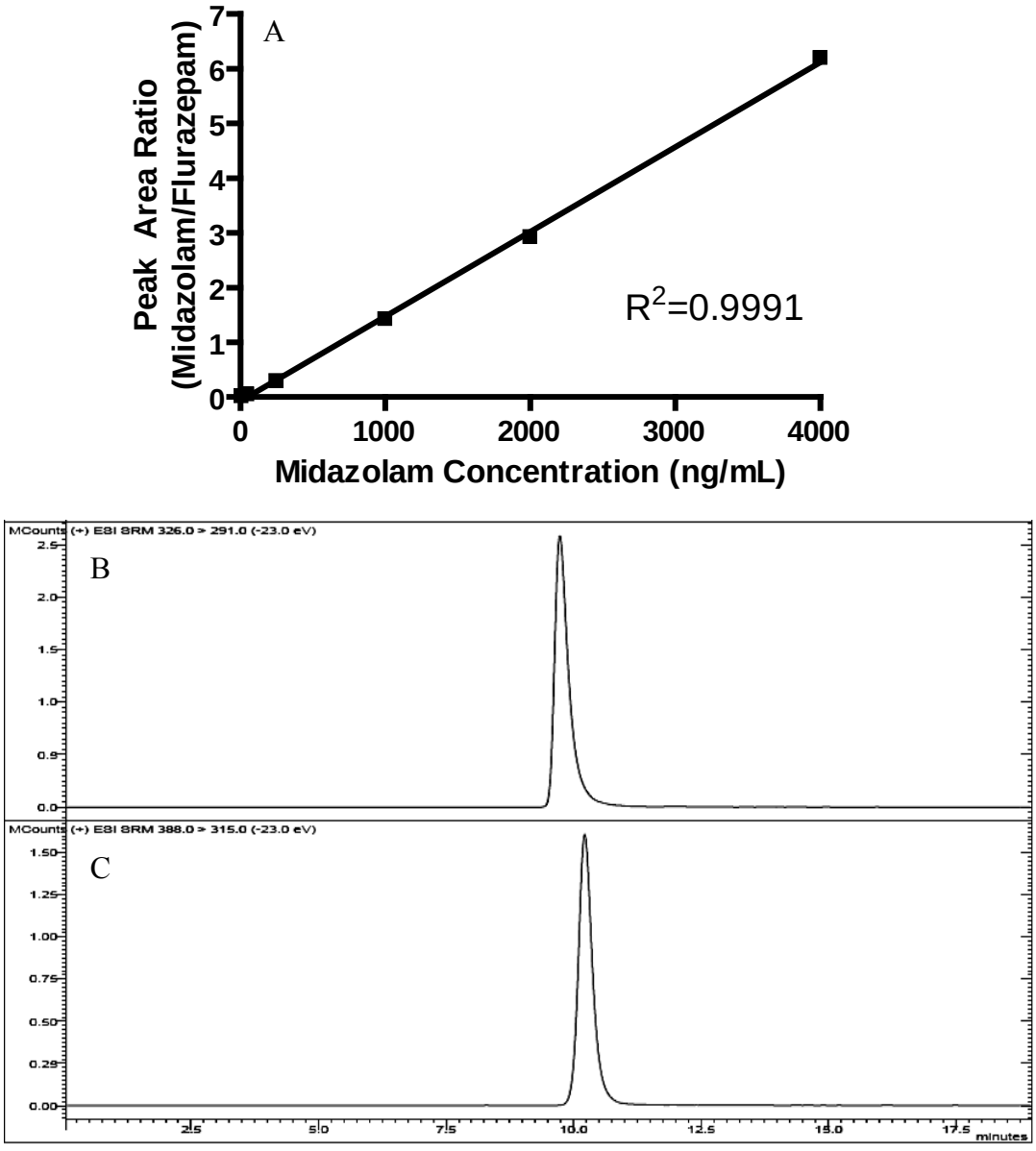


Figure 35: Pharmacokinetic profiles of oral midazolam (20mg/kg) in male rats ages 7, 21 and 112 days (Four animals per time point, destructive sampling).

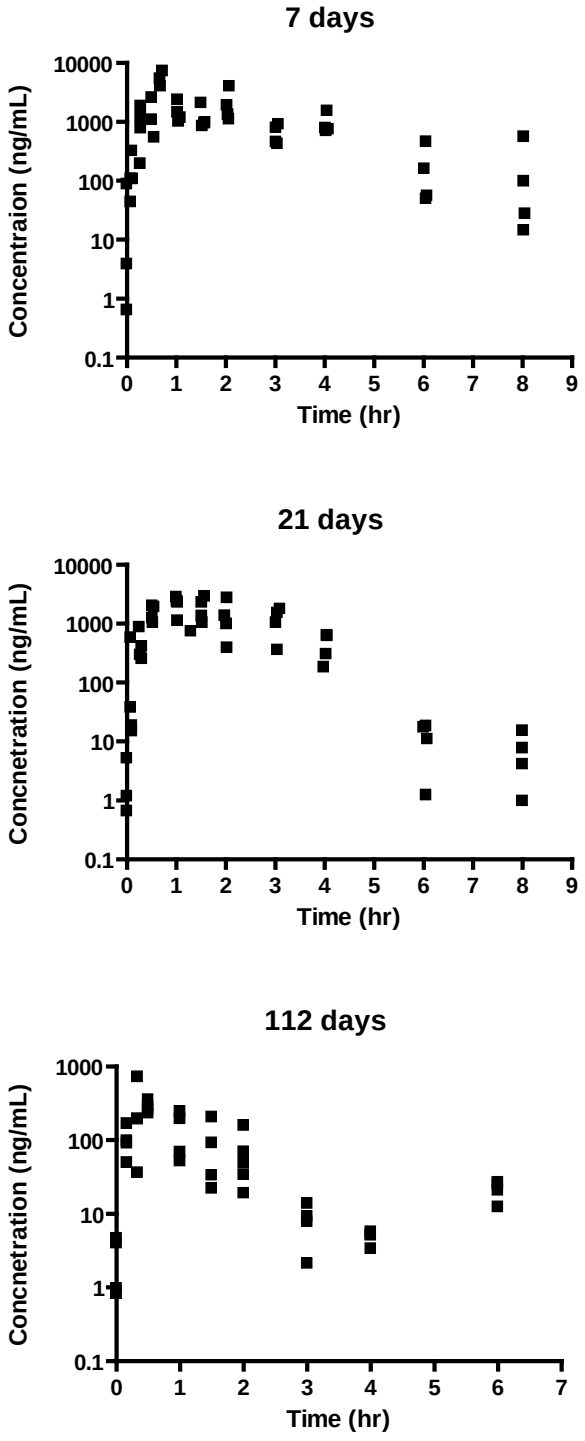


Table 5: WinNonlin non-compartmental estimates of model independent pharmacokinetic parameters of ora lmidazolam (20 mg/kg) in male rats.

Age (days)	T1/2 (hr)	Tmax (hr)	Cmax (ng/mL)	AUC (0-∞) (hr*ng/mL)
7	1.75	0.72	7244	7554
21	0.65	1.57	2889	5377
112	1.12	0.33	722	429

Table 6: NONMEM calculated pharmacokinetic parameters (mean± SD)

Age (days)	Cl/F L/hr/Kg	V/F L/Kg
7	3.71±1.06	3.46±1.64
21	5.2±1.66	4.31±0.69
112	64±15	67±22

2.6.1 Protein Binding:

Protein binding of midazolam in serum was measured using an ultrafiltration method. The unbound fractions of midazolam in rat serum for 7, 21 and 112 days are 0.08, 0.05 and 0.09 respectively.

2.7 Model Prediction:

To test the model assumption that microsomal protein does not vary with age, microsomal protein per gram liver was calculated and compared by one-way ANOVA to the adult (112-day) group. All age groups were significantly different from the 112-day age group (Figure 36). Micosomal protein normalized to liver weight increased with age except for the 21 day age group (weaning) that was higher than the rest of the age groups. Hepatic scaling factor was calculated and results are shown in Table 7. Infant scaling factor (product of relative hepatic scaling factor and ontogeny scaling factor, Equation 35) was calculated and its coefficient of variation was calculated according to the following formula for ratios (Table 8):

$$CV_{X/Y} = \sqrt{\left(\frac{SD/\sqrt{n}}{\overline{X}}\right)_X^2 + \left(\frac{SD/\sqrt{n}}{\overline{Y}}\right)_Y^2} \qquad \text{Equation 38}$$

Where $CV_{X/Y}$ is the coefficient of variation of the ratio, X is the numerator set and Y is the denominator set , SD is the standard deviation, n is the number per group, $\overline{X}$ and $\overline{Y}$ are the averages for the X and Y sets respectively.

The scaling factors were used to calculate the predicted clearance values according to Model1 and Model 2 (Equations 36 and 37) as described in the methods section. Model 1 predicts clearance based on enzyme activity only assuming that microsomal protein does not vary with age. Model 2 is the ontogeny model equation where prediction is based on the infant scaling factor. The adult clearance value used for prediction is the 112 day age group average clearance obtained from the *in vivo* study (64 L/hr/Kg). Predicted values are displayed in Table 9.

Figure 36: Developmental pattern of microsomal protein content per gram liver in male Sprague-Dawley rats. Mean ± Standard Deviation. (** P<0.01, * P<0.05 when compared to 112 day group, one-way ANOVA followed by Dunnett's test)

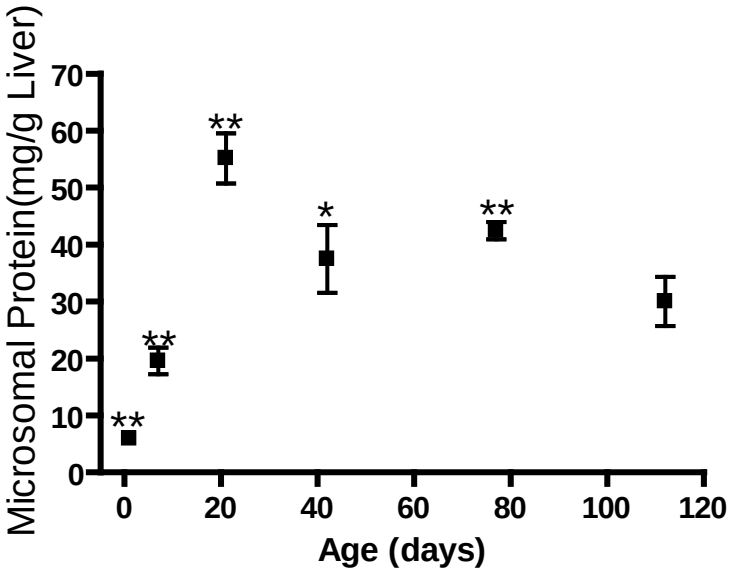


Table 7: Hepatic scaling factor calculated for the different age groups (mean ± standard deviation). $HSF = MP(mg/g\ Liver)\frac{LW}{BW}$

Age (days)	HSF
1	0.24 ± 0.05
7	0.69 ± 0.11
21	2.16 ± 0.13
42	1.70 ± 0.25
77	1.47 ± 0.17
112	0.97 ± 0.12

Table 8: Infant Scaling factor calculated for the different age groups. CV is the coefficient of variation for the ISF value as calculated from Equation 38

$$ISF = \frac{HSF^{infant} * V_{max}^{infant}}{HSF^{adult} * V_{max}^{adult}}$$ Equation 38

Age (days)	ISF	CV
1	0.19	0.20
7	0.45	0.13
21	0.97	0.16
42	0.82	0.14
77	1.79	0.14
112	1	0.18

Table 9: Predicted oral clearance of midazolam in male rats. Mean (95% confidence interval). Model 1 predicts clearance based on enzyme activity only assuming that microsomal protein does not vary with age. Model 2 is the ontogeny model equation where prediction is based on the infant scaling factor.

Age	Cl/F (L/hr/Kg)	
days	Model 1	Model 2
7	42 (32-51)	29 (23-35)
21	28 (24-32)	62 (48-76)

2.8 Prediction Evaluation:

After examining the microsomal protein across the different age groups it is clear that Model 1 would not be the model of choice for clearance prediction since microsomal protein changes with age and needs to be accounted for. Model 2 (the ontogeny model) did not predict the *in vivo* oral clearance. The criterion for accepting the prediction was that the predicted value falls within the 50-200% range of the observed value. For the 7 day age group the predicted value for oral clearance is 29 L/hr/Kg while the 50-200% range of the observed value is 1.86 – 7.42 L/hr/Kg. For 21 day age group the predicted value is 61 L/hr/Kg and the 50-200% range of the observed value is 2.6 – 10.4 L/hr/Kg. Using the 50-200% criterion, the model failed to predict the *in vivo* clearance. Hence looking at the model assumptions, if Km was not truly constant then ignoring its value in the prediction would bias the model. Predicted clearance values were recalculated using Vmax and Km in the ontogeny scaling factor (OSF) for infants and adults and the model predictions were 5.73 and 19.05 L/hr/Kg for the 7 and 21 day age groups respectively. These values are more predictive of the observed oral clearance than when predicted by the other 2 models. The other possibility is that F_G is lower in the adults ($F_G << 1$) than in the pups causing both oral clearance (Cl/F) and volume of distribution (V/F) to be greater. F_G was assumed to be 1 in both pups and adults which might account for the prediction discrepancy.

Chapter 6. Discussion

The risk resulting from infant exposure to xenobiotics has been a long debated issue. Most of the drugs on the market have no indications for infant use and the consequences of their use in infants are largely unknown. Exposure to drugs or environmental chemicals through breast milk is well documented in the literature. Due to the lack of a standardized approach in assessing infant risk, the risk (to the infant) versus benefit (for the mother) ratio is sought before deciding to dose a mother with a drug while breastfeeding. Since in most of the cases, the actual dose reaching the infant and the infant's ability to clear such doses is unknown, short term and long term adverse effects in the infant are difficult to predict. Rigorous clinical trials of drug exposure in neonates or in breastfeeding mother-infant pairs have many ethical and logistical drawbacks and hence are very rarely done. Most of the information in the literature is derived from case report studies or is a compilation of retrospective data from existing cases under treatment. Hence there is no control over experimental variables or circumstances occurring during treatment that might influence the outcomes, adding variability to the data, thereby hindering scientists and clinicians from reliable conclusions. Alternatives have thus been sought to try to answer some of the questions about risk assessment of infant exposure to drugs in the neonate.

First, the parameters influencing infant exposure are determined through indices designed to identify the critical factors influencing the underlying processes (Equation 3):

$$C_{serum}^{infant} = \frac{F^{infant}}{Cl_{systemic}^{infant}} \left[C_{serum}^{maternal} \left(\frac{M}{S} \right) \left(\frac{V_{milk}}{\tau} \right) \right] \qquad \text{Equation 39}$$

An exposure index assumes that the drug-target interaction and toxicity in the neonate are related to serum concentration and are similar to the adult (Ito and Koren 1994). Infant serum concentration is dependent on dose ingested in the milk, bioavailability and infant clearance. The infant dose is in turn dependent on the amount of milk ingested and milk concentration of a drug. The milk concentration is a result of maternal serum concentration and drug distributional properties that make up the maternal milk to serum

ratio (M/S). Maternal serum concentration is a variable that might be readily determined in a controlled setting.

The milk to serum ratio is most accurate if determined experimentally under steady-state conditions, which are rarely performed. As a result, several approaches have been proposed in the literature over the years to predict the M/S ratio (Rasmussen 1958; Rasmussen 1959; Meskin and Lien 1985; Fleishaker, Desai et al. 1987; Atkinson and Begg 1990; Agatonovic-Kustrin, Ling et al. 2002). Out of these approaches, two have been chosen to be further evaluated in the current work. Both of these approaches assume passive diffusion into milk based on physiochemical properties of the drug.

The Atkinson and Begg approach has a distinct advantage of being empirical; no additional experimental work is required to make M/S predictions. The approach stems from existing literature data. If the model can predict observed M/S ratio in humans then it would be of a great value when observed values are not available.

The Fleishaker model provides a fundamental baseline model (passive diffusion) which might be able to identify those drugs undergoing active transport into milk by comparing the value predicted by the model to observed M/S *in vivo*. The basis for choosing the Fleishaker model (Fleishaker, Desai et al. 1987) is that it is derived from first principles and considers all the relevant physiochemical properties of the drug from a mechanistic approach. Moreover, it can explain interindividual variability in serum and milk protein binding and milk composition. The disadvantage of this approach is the cumbersome nature of the *in vitro* experiments needed and the logistics of obtaining human samples in order to perform the work.

Another critical component of the exposure index is the infant clearance of a drug. For the vast majority of drugs, this systemic clearance for infants is largely unknown. Most of the data in the *in vivo* literature that has been reported is from sick infants under therapy, and frequently the details of their conditions are unknown or not reported. To provide a more mechanistic perspective, some *in vitro* studies have examined enzyme activity (both phase I and II) in livers from children of various ages (Pacifici, Bencini et al. 1986; Pacifici, Franchi et al. 1988; Pacifici, Franchi et al. 1988; Cappiello, Giuliani et al. 1991; Treluyer, Jacqz-Aigrain et al. 1991; Pacifici, Kubrich et al. 1993; Barker, Hume et al. 1994; Gilissen, Hume et al. 1994; Cresteil 1998; Sonnier and Cresteil 1998; de

Wildt, Kearns et al. 1999; de Wildt, Kearns et al. 1999). However, even these *in vitro* experiments involved livers from infants where the exact circumstances of sample retrieval and storage are not very well documented (Stevens, Hines et al. 2003). In most of the cases this *in vitro* data is not directly extrapolated to *in vivo* clearance concepts. An ontogeny model (Alcorn and McNamara 2002d) was proposed from our laboratory whereby the *in vitro* data could be extrapolated to *in vivo* data through the use of scaling factors and adult clearance values. Such a model, if validated, would prove to be very beneficial for the approximation of initial estimates of clearance in neonates. To extensively evaluate the model assumptions in infants presents tremendous logistical and ethical issues associated with human experimentation that is dramatically complicated when considering these vulnerable age groups. Therefore, we examined the *in vitro – in vivo* model in rats. Clearly, rats are not humans; however, they are extensively used in preclinical studies for evaluation of metabolic pathways, toxicity and drug interactions due to many shared similarities. Moreover, the use of the rat will allow for a more detailed evaluation of the assumptions in the clearance ontogeny model. In addition to examining the basic model assumptions, the ontogeny of RNA, protein and function was followed for a major drug metabolizing enzyme (Cyp3a).

1 *In Vitro* M/S Ratio

1.1 Atkinson and Begg Model

An empirical model that accurately predicts M/S ratios for passively diffusing drugs based on physiochemical properties without need for experimental data would be of great benefit. Atkinson and Begg's model (Begg, Atkinson et al. 1992; Begg and Atkinson 1993) was evaluated for this purpose. For our analysis, drugs were selected that have not been incorporated into the orginal model building or evaluation. The drugs had reported observed M/S ratios and the other parameters were either from the literature or predicted by ACD labs software. The model did not accurately predict the observed milk-to-serum ratios (Figures 1 and 2). One explanation for the discrepancy in the observed and predicted is the assumption of passive diffusion as the dominant mechanism of drug transfer into and out of the milk. Two of the drugs (acyclovir M/S_{obs} =3.0 and ciprofloxacin M/S_{obs} =1.7) indeed have an active transport component as described earlier for the Fleishaker model. The M/S_{pred} for oxazepam was much greater (M/S_{pred} =1.2) than the observed (M/S_{obs} =0.1). For the basic drugs, two of the drugs (Figure 2) (acebutolol M/S_{obs} =5.7 and ranitidine M/S_{obs} =3.8) may have an active transport component in their transfer mechanism. However, the M/S_{pred} (2.5) for nicotine was similar to M/S_{obs} (2.9), although nicotine might have an active transport component to its transfer into milk. A review of the model in the literature (Larsen, Ito et al. 2003) found no correlation between the values of milk-to serum ratios of a number of drugs predicted by Atkinson and Begg's model and those observed in lactating women. This review lumps together acidic and basic drugs as well as drugs known to be transported by active transport. When we reanalyzed the data, the r^2 for acidic drugs improved from 0.01 to 0.7 when acyclovir and ciprofloxacin, both known to be actively transported into milk, were removed from the correlation. In addition, the calculated M/S ratios in this review were more than twice M/S_{obs} for many of the basic drugs (citalopram, flecainide, fentanyl, fluoxetine, labetalol, loaratadine, hydroxychloroquine, metoclopramide, paroxetine, propranolol, diltiazem, chloroquine, venlafaxine and verapamil.). When these drugs were removed from the correlation along with cimetidine (known to be actively

transported) and quazepam, both of which had M/S observed twice or more M/S predicted, the r^2 improved from 0.0058 to 0.59. The review was criticized for using logP instead of apparent logP at pH 7.2 and for incorporating drugs used to build the model (Doogue, Gardiner et al. 2004; Ilett and Hackett 2004). However, the authors noted that even when using the apparent log P, predictions were not improved. In the evaluation presented here, the correlation for acidic drugs was worse than for basic drugs where the M/S predicted values where actually higher in many cases than the observed values, making the model unsuitable for predicting M/S ratios when no *in vivo* data exists. One reason for the poor correlation is the active transport of some of the drugs, thus violating the basic assumption of the model that drugs appear in milk via passive diffusion. Another reason is the variability of the physicochemical parameters reported in the literature (logP, log D, serum protein binding) and used for M/S ratio predictions. Outliers might also exist for the relationship proposed to predict milk protein binding further complicating the prediction. Since building the model also involved values of physiochemical parameters from the literature for a relatively limited number of drugs, the regression relationship used for prediction might be biased by these values. It seems that a more vigorous evaluation of the regression relationship is required as well as the model suggested for millk protein binding using a larger set of drugs provided that proper M/S_{obs} data in the literature is available.

1.2 Fleishaker Model

Breastmilk is an important source of infant nutrition and inadvertently a route of xenobiotic exposure for neonates. The M/S ratio and its determinants play a major role in determining the extent of this exposure. The ability to determine the factors governing this ratio will improve the predictability of infant exposure. The Atkinson and Begg empirical model could not predict observed M/S ratios; hence a mechanistic approach is needed. The model studied here is the Fleishaker model which predicts M/S ratios based on the physiochemical properties of the drug obtained by *in vitro* experiments (unbound fractions in serum and milk and skim to whole milk ratio), or calculated (fraction unionized). The drugs selected for this model investigation have relatively high

observed M/S (M/S_{obs}) ratios as reported in the literature. If the passage of these drugs into milk is dictated by passive diffusion pathways, then those high M/S ratios would be explained in terms of their physicochemical properties. These include extensive partitioning into fat milk for lipophilic drugs (S/W ratio<1), by ion trapping in case of basic drugs ($f_{un,s}/f_{un,m}>1$) assuming that only the unionized drug can diffuse through the mammary epithelial tissue and into milk or by greater binding to milk proteins than to serum proteins ($f_s/f_m>1$). Previously, our laboratory (Alcorn and McNamara 2002) has used a subjective cutoff which considered active transport to be playing a significant role for those drugs for whom the observed M/S was twofold larger than the values predicted by the diffusion model. Thus the 95% confidence interval of the 50% and 200% values of the predicted M/S was calculated (Alcorn and McNamara 2002) to account for active transport as well as any variability associated with the observed M/S ratios, especially when observed in a single case study. If the observed value or its 95% confidence interval fall within that range or significantly overlap with it, then passive diffusion can be considered the major pathway for transport of drug into milk. Otherwise, active transport can be assumed to play the key role i.e. at least 50% of passage into milk.

Active transport of drugs into milk has been previously described in the literature (Lau, Emery et al. 1987; McNamara, Burgio et al. 1992b; Schadewinkel-Scherkl, Rasmussen et al. 1993; Oo, Kuhn et al. 1995; McNamara, Meece et al. 1996b; Gerk, Kuhn et al. 2001; Gerk, Oo et al. 2001; Oo, Paxton et al. 2001; Alcorn and McNamara 2002; Edwards, Rudy et al. 2003). An important role of Breast Cancer Resistance Protein (BCRP/ABCG2) in active transport of drugs into milk has been established (Jonker, Merino et al. 2005; Merino, Jonker et al. 2005; Merino, Alvarez et al. 2006; van Herwaarden, Wagenaar et al. 2007) for most of the drugs that were previously described to have an active transport component such as nitrofurantoin, cimetidine and acyclovir. BCRP is a member of the ATP Binding Cassette (ABC) family of transporters. Active transport is achieved by the energy obtained by ATP (adenosine tri-phosphate) hydrolysis. In addition to tumor cells, BCRP is present on the apical membrane in normal human tissues, in the liver, intestine, placenta, mammary glands and brain (Maliepaard, Scheffer et al. 2001; Cooray, Blackmore et al. 2002).

Citalopram M/S ratio was consistent with simple diffusion. The predicted M/S ratio was 1.8 (Figure 4) and the observed values were 1.8, 1.52 and 3.00 (Jensen, Olesen et al. 1997; Spigset, Carieborg et al. 1997; Rampono, Kristensen et al. 2000; Schmidt, Olesen et al. 2000). As a base and lipophilic drug, the M/S ratio is readily explained by partitioning into milk lipids and ion trapping. The high variability observed in the predicted milk to serum ratio can be partially attributed to variability in milk fats as measured by the crematocrit values (Figure 3). No evidence of active transport of citalopram is found in the literature.

Ciprofloxacin predicted value (0.85) was about half that observed (1.6) (Giamarellou, Kolokythas et al. 1989)(Figure 5). This would suggest a contribution of active transport to the drug appearance into milk. However, applying a more stringent criterion of the confidence interval of 50-200% of the observed value to account for any experimental variability *in vitro* or interindividual variability *in vivo*, the predicted M/S ratio is with in that range. There is evidence in the literature of active transport mechanisms for ciprofloxacin both *in vitro* and *in vivo* (Griffiths, Hirst et al. 1993; Griffiths, Hirst et al. 1994; Merino, Alvarez et al. 2006). Active transport of ciprofloxacin into milk was demonstrated in BCRP (BCRP-/-) knockout mice, where the M/S value was twice that in wild type mice when compared to knockout mice. This would suggest that the 50-200% criterion is perhaps too stringent to predict active transport. The ratio of the M/S in the wild type versus knockout mice was similar to the ratio between the value predicted by the Fleishaker model and the observed *in vivo* value.

The observed nicotine M/S value in humans was outside the 50-200% range, with the 95% confidence intervals overlapping suggesting a role for active transport (Figure 6). Nicotine has been shown to accumulate *in vitro* in isolated rabbit choroid plexus by both an active saturable process and an unsaturable process (Spector and Goldberg 1982) and by a transport system in LLC-PK1 cells (Takami, Saito et al. 1998). Nicotine has been also shown to interfere with the renal organic cation transport of amantadine in isolated male rat kidney tubules (Wong, Smyth et al. 1992). However, no specific transporter has been identified yet for its active transport into milk or in other tissues.

Acyclovir has an observed M/S ratio that is two to three-fold the predicted one (Figure 7). This agrees with previous data from this lab investigating acyclovir

distribution in rat milk (Alcorn and McNamara 2002), suggesting a significant role of active transport processes for the appearance of acyclovir in human milk. There is a report that rat multispecific organic anion transporter 1 (rOAT1) can transport acyclovir, and other antiviral nucleoside analogs (Wada, Tsuda et al. 2000), however, OAT1 does not appear to be expressed in human lactating mammary cells (Alcorn, Lu et al. 2002). In a recent study (Jonker, Merino et al. 2005), breast cancer resistance protein knock-out mice (BCRP-/-) were shown to have a significantly lower acyclovir M/S ratio when compared to wild type mice. In the same study (Jonker, Merino et al. 2005) lactating human mammary epithelial cells were shown to express BCRP protein, indicating that acyclovir transport mechanism into human milk is most probably mediated by BCRP as in mice.

Ascorbic acid *in vitro* data predict an M/S ratio which is tenfold less than the observed value (Figure 8). As an acid, its accumulation in milk cannot be explained by ion-trapping. Moreover, there was insignificant binding in both serum and milk and almost no partitioning into milk fat was observed, making it hard to explain its accumulation in milk by passive diffusion. It has been shown that ascorbic acid is actively transported into human neutrophils and fibroblasts by means of a carrier and in a sodium-dependent fashion (Welch, Wang et al. 1995). A human Na(+)-dependent vitamin C transporter 1 (SVCT1/SLC23A1) has been cloned and is specific for ascorbate with a Kt of approximately 75 microM (Wang, Dutta et al. 1999). SVCT1 is expressed apically in human cells (Subramanian, Marchant et al. 2004). SVCT1 was found to be expressed in human lactating mammary epithelial cells (Alcorn, Lu et al. 2002a). SVCT2/SLC23A2 and SVCT3/SLC23A3 are also human ascorbic acid transporters (Daruwala, Song et al. 1999; Strausberg, Feingold et al. 2002). There is evidence from our lab that SVCT2/SLC23A2 mRNA is expressed in a purified population of human lactating mammary epithelial cells (unpublished data).

Our results confirm that the model is valuable as an initial step in understanding mechanisms of drug transfer into milk in combination with animal models. When the M/S_{obs} is at least twice the M/S_{pred}, active transport is judged to play an important role in drug transfer. The rat, as an animal model, has proved to provide insight into transfer mechanisms into milk. To date, drugs actively transported into milk seem to share

common mechanisms of transport in rats and humans. Knockout mice are another valuable tool to prove the involvement of a certain transporter in drug transfer into milk. Further investigations are needed to identify the carriers of transport across mammary epithelial cells and to characterize the properties of these carriers *in vitro* and *in vivo*. Differences between animal models used and humans in these processes need to be well understood. Such knowledge might enable the addition of an active transport component to the model further improving the ability to predict *in vivo* M/S ratios.

The list of drugs for which evidence of active transport into milk exists, is by far shorter than the drugs currently on the market. Then it is assumed that most of the drugs appear in milk by passive diffusion. Hence, the model can be of a great value in preclinical studies when the M/S is unknown and when the candidate compound is not known to be a substrate for transporters.

2 Clearance Ontogeny

2.1 Rat as an Animal Model

Rat is widely used as an animal model in toxicology and in preclinical studies for new drug entities. As a result, drug metabolism in the rat has been extensively studied. Expressed enzymes and antibodies for rat cytochrome P450 are commercially available making it a useful animal model. Although rat and human enzymes share similarities, differences do exist in enzyme isoforms, substrate specificities, major metabolites of a substrate, inducers and inhibitors. These similarities and differences have been documented in the literature (Eberhart, Gemzik et al. 1991; Guengerich 1997; Bogaards, Bertrand et al. 2000; Gibson, Plant et al. 2002; Zuber, Anzenbacherova et al. 2002; Martignoni, Groothuis et al. 2006).

Guengerich (Guengerich 1997) classified the major cytochrome P450 subfamilies involved in drug metabolism roughly into 4 classes according to the ability to extrapolate across species:

1- Subfamilies where extrapolation seems to work, as for CYP2E1.
2- Subfamilies where extrapolation has to be interpreted carefully as for CYP1A1 and CYP1A2.
3- Subfamilies where extrapolation has to be interpreted more carefully as for CYP3A and CYP2D.
4- Subfamilies where problems seem to arise during extrapolation as for CYP2A, CYP2B and CYP2C.

Bogaards et al (Bogaards, Bertrand et al. 2000) compared the activities of 9 cytochrome P450 enzymes in the liver microsomes of 7 animal species with that of humans. Their approach was to compare Vmax (normalized to microsomal protein), Km and inhibitors. The species that was closest to humans with respect to all of the studies parameters was considered a suitable animal model for this cytochrome P450 in humans. For Cyp3a , the 6-β-hydroxylation of testosterone was utilized. Rats and mice were concluded to be the closest species and the most appropriate model for human CYP3A.

Although this study highlights the similarities between rat and human CYP3A, it is not conclusive for other CYP3A activities based on other substrates that may not share these similarities. Eberhart et al (Eberhart, Gemzik et al. 1991) studied species differences in the Cyp3A dependent metabolism of digitioxin. There were significant qualitative and quantitative differences between rat and human. Martignioni et al (Martignoni, Groothuis et al. 2006) reviewed differences in cytochrome P450 mediated metabolism between humans and four other species. They concluded that rat is not a good model for human Cyp3A since it is not induced by rifampicin. Discrepancies in Cyp3A gene inducibility between species was explained by the differences in the Pregnane-X-Receptor (PXR) ligand-binding domain. Zuber et al (Zuber, Anzenbacherova et al. 2002) highlighted the differences between rat and human CYP3A inducibility by rifampicin and metabolism of dihydropyridine calcium channel blockers. All the similarities between the inducibility of human and rat CYP3A by phenobarbitone, pregnenolone-16-α-carbonitrile and dexamethasone as well as the shared substrates were ignored. Gibson et al (Gibson, Plant et al. 2002) reviewed the differences in regulation of Cyp3a between species. They demonstrate that in addition to genetic differences, differences in the host cell environment e.g. transcription factors available, contribute to species differences in response to inducers. Although differences exist between rat and human Cyp3a and their nuclear factors, the nuclear factors Pregnane-X-Recpetor (PXR), Constitutive Androstane Receptor (CAR), Glucocorticoid Receptor (GR) are involved in xenobiotic regulation of human and rat Cyp3a.

All the studies above did not provide conclusive evidence as to the suitability of one animal model to human cytochrome P450 metabolism. It is clear that despite the differences between human and rat Cyp3a, the rat still serves as widely used animal model. In this dissertation the rat was used to test the ontogeny model assumptions for possible future application in humans, however, one needs to keep the differences in mind. It is possible that alternative animal models for ontogeny hepatic clearance may more closely mimic the human. Nonetheless the rat affords a viable approach to test the assumptions of the underlying *in vitro – in vivo* model.

2.2 *In Vitro* Ontogeny of mRNA, Protein and Activity

We hypothesized that mRNA and protein levels reflect activity levels during rat development and that Cyp3a and P-gp mRNA ontogeny follow similar patterns. To test this hypothesis mRNA, protein and activity during different male rat developmental changes were followed.

2.2.1 mRNA Ontogeny

Ontogeny of Cyp3a1, Cyp3a2, Mdr1a and Mdr1b mRNA were followed in the rat liver and intestine. In our data, Cyp3a1 mRNA ontogeny in male rat liver was significantly high at birth and had decreased to adult levels by one week of age. The expression seemed stable throughout development (Figure 13), while in male rat intestinal mucosa, it increased with age with no statistical significance between the age groups (Figure 18). Cyp3a1 ontogeny has been studied earlier. Simmons and Kasper (Simmons and Kasper 1989) showed dramatic increase of Cyp3a1 mRNA after birth in the male rat liver that further increased slowly till 110 days of age. However, the filter bound plasmid used for RNA hybridization was shown to be non-specific and the assay is at best semi-quantitative. In the same study, the intestinal Cyp3a1 was found to be 8% of the maximal level in the liver and a developmental pattern was similar to that in the liver except that the surge in Cyp3a1 RNA was seen 24 hours before birth. In a different study (Omiecinski, Hassett et al. 1990), hepatic Cyp3a1 mRNA was found as early as fetal day 15 and increased at fetal day 22. Levels from 3 weeks up to 46 days postpartum were similar. The method used was autoradiography of ^{32}P-labeled probe for southern blotting of PCR products, which is semi-quantitative, and the sex of rats was not specified. Ribeiro and Lechner (Ribeiro and Lechner 1992) showed sex- dependence of Cyp3a1 and Cyp3a2 mRNA expression, where Cyp3a1 and Cyp3a2 transcripts were absent in the female liver at 45 and 90 days of age. Cyp3a1 mRNA expression was found to be transiently high after birth up to 10 days of age but then at 25, 45 and 90 days the expression was low.

In the present study, Cyp3a2 expression seemed to increase with age (Figure 14). However when testing with one-way ANOVA only days one and 21 age groups were significantly lower than the day 112 age group. Mahnke et al (Mahnke, Strotkamp et al. 1997) found stable expression of Cyp3a2 transcripts in male Sprague-Dawley rats from 1 week to 20 weeks of age. In a second study (Ribeiro and Lechner 1992) Cyp3a2 mRNA expression was found to peak at 25 days in male Wistar rats. In a third study (Rosati, Maniori et al. 2003) Cyp3a2 transcripts peaked at 60 days in Wistar rats. Kawai et al (Kawai, Bandiera et al. 2000) showed no change in Cyp3a2 expression in male Sprague-Dawley rats from 22 to 91 days of age. Wright et al (Wright, Edwards et al. 1997) examined 21 and 84 day old rats and showed higher Cyp3a2 mRNA expression in the 21 day old group. Gonzalez et al (Gonzalez, Song et al. 1986) studied newborn 1, 2, 3, 4 and 12 weeks male rats for Cyp3a2 mRNA expression, their results show increased expression from newborn to 1 week, slight depression at 4 weeks then a significant increase at 12 weeks compared to other age groups. It is apparent that there are some discrepancies between the results from different studies, probably due to difference in the sensitivity of methods used, rat strain, and/or the age groups used to describe the ontogeny pattern. The overall ontogeny pattern from our results (Figure 14) seemed to generally agree with the studies described above in the fact that Cyp3a2 mRNA increased with age and reached adult levels early in ontogeny. Cyp3a2 mRNA expression pattern in the intestine could not be followed. Cyp3a2 was detectable in all of the samples but not quantifiable in intestinal mucosa, indicating lower expression than the liver. To date, no studies in the literature were found that followed Cyp3a2 mRNA development in the rat intestine. One study was unable to detect Cyp3a2 transcripts in 63 day old male Wistar rat intestine (Takara, Ohnishi et al. 2003).

Mdr1a developmental pattern in the liver showed a significantly different expression at 1 day of age when compared to 112 day old rats (adults) (Figure 15). In a study by Rosati et al (Rosati, Maniori et al. 2003), Mdr1a expression in the liver in Wistar rats increased with age till 150 days and did not show the current pattern. Differences in methodology where densitometric analysis of PCR products was employed or when it was not specified whether male or female rats or both were used could be sources for discrepancies between results. Mdr1a expression in the intestine showed a

different pattern (Figure 19). Expression increased with age and all age groups had expression levels significantly different from the adult. The adult levels were greater than 150 fold higher in the intestine than in the liver at 112 days. Mdr1a has been previously shown to be more highly expressed in the intestine than in the liver in male Sprague-Dawley rats (Brady, Cherrington et al. 2002). No ontogeny studies characterizing Mdr1a mRNA development in the rat intestine were found in the literature.

Mdr1b expression in the liver showed a stable expression from birth to adulthood (112 days) except for the 42 day age group which was significantly higher in expression than the rest of the age groups (Figure 16). The data from Rosati et al (Rosati, Maniori et al. 2003) showed Mdr1b expression levels peaking at 30 and 60 days and decreased at 150 days, in agreement with our results. In previous preliminary studies, we have also demonstrated that Mdr1b gene expression in male rat liver peaked at 42 days (data not shown). Paradoxically, Mdr1b expression pattern was slightly different in the intestine and no significant difference was detected among the different age groups (Figure 20). There seemed to be lower expression at 42 days but this was not statistically significantly different from the adult. The 42 day age group represents puberty in rats. It is unclear whether hormonal changes at this stage influence Mdr1b expression in both liver and intestine. Expression level in the liver and intestine was comparable. In a study by Brady et al (Brady, Cherrington et al. 2002) duodenal Mdr1b expression was about 3.6 higher than in the liver in male Sprague-Dawley rats.

It is interesting to note that mRNA ontogeny for hepatic Cyp3a1, Cyp3a2, Mdr1a and Mdr1b did not follow a similar ontogeny pattern. Due to their common substrates and role in drug disposition their common regulation was suggested (Westphal, Weinbrenner et al. 2000). However, there is only partial overlap between substrates and modulators and the interplay between P-gp and Cyp3a is unpredictable except on a case by case basis (Kim, Wandel et al. 1999).

PXR was shown to be involved in the induction of both Cyp3a and P-gp proteins (Mei, Richards et al. 2004). Although we did not follow the protein ontogeny of P-gp, mRNA expression of Cyp3a1/2 and Mdr1a/b genes did not follow a similar pattern. Different hormones are probably responsible for gene regulation during development.

The role of these hormones for P-gp needs to be elucidated to explain the difference in ontogeny pattern when compared to Cyp3a.

Cyp3a1 and Cyp3a2 share a 90% nucleotide homology (Gonzalez, Song et al. 1986). However, Cyp3a1 appears to be present in low levels throughout development while Cyp3a2 increases with age in male rats, indicating different regulation patterns throughout development. Cyp3a2 has been shown to be regulated by growth hormone and testosterone levels in male rats (Ribeiro and Lechner 1992; Kawai, Bandiera et al. 2000). Growth hormone secretion is pulsatile in male adult rats, peaking every 3 to 4 hours and with non-detectable troughs in-between. In adult females, pulses are closer, irregular and of lower levels and there is always growth hormone detected in the circulation in between peaks (Thangavel, Garcia et al. 2004). It has been implied that response to growth hormone is imprinted by sex hormones (Laz, Wiwi et al. 2004; Thangavel, Garcia et al. 2004). Cyp3a1 was also found to be affected by growth hormone levels (Shimada, Nagata et al. 1989). Cyp3a1 is induced by dexamethasone in rat liver while Cyp3a2 is not (Caron, Rioux et al. 2005). Phenobarbital and pregnenolone-α-carbonitrile induce both Cyp3a1 and Cyp3a2 (Debri, Boobis et al. 1995; Caron, Rioux et al. 2005; Jan, Mishin et al. 2006). It is possible that the interplay of growth hormone and testosterone regulate the developmental changes in Cyp3a1 and Cyp3a2 differently leading to distinct expression of the genes in males and females and across different developmental stages.

P-glycoprotein is known to be the product of Mdr1a and Mdr1b, but these genes appear to be regulated differently. Not only do they exhibit a different developmental pattern in the liver but they also show a different tissue distribution pattern in adults and the relative distribution of Mdr1a and Mdr1b is different (Brady, Cherrington et al. 2002; Mei, Richards et al. 2004). Induction by cytochrome P-450 inducers is also different between the 2 gene products and within the same gene product in different tissues (Brady, Cherrington et al. 2002) which suggest different regulation mechanisms of the two genes. Induction with dexamethasone showed concentration and tissue dependent responses in terms of magnitude and direction (induction/inhibition)(Mei, Richards et al. 2004).

Cyp3a9, Cyp3a18 and Cyp3a62 are other Cyp3a isoforms expressed in rat liver and intestine (Takara, Ohnishi et al. 2003; Matsubara, Kim et al. 2004). No commercial antibodies or expressed enzyme systems are available for any of them. Substrate specificity is largely unknown. Hence, ontogeny studies were not performed for these genes.

2.2.2 Cyp3a2 Protein Ontogeny

Cyp3a1 protein developmental expression was not followed due to the lack of commercial antibodies specific to rat Cyp3a1 as shown in the results. Cyp3a2 protein expression was followed by western blotting using rat anti-Cyp3a2 from Chemicon. Cyp3a2 expression in male rat liver microsomes was not significantly different in most of the age groups compared to adults (112 days) (Figure 23). The only 2 groups that were statistically significantly different were the 21 day old group which was lower and the 77 day old group which was higher. Visual inspection of intestinal mucosa microsomes western blots probed by the anti-Cyp3a2 antibody reveal increased levels with age (Figure 24). In a study by Wright et al (Wright, Edwards et al. 1997) Cyp3a1 and Cyp3a2 expression in the liver were examined by specific antibodies (Debri, Boobis et al. 1995) raised in rabbit, in 21, 42, 63, 84 and 105 day old groups. The protein levels of Cyp3a2 were found to be depressed between 63 and 84 days in male Sprague-Dawley rats. Cyp3a1 expression was stable throughout development. Johnson et al (Johnson, Tanner et al. 2000) examined Cyp3a development in the liver and enterocytes in male Wistar rats at 1, 10, 15, 20, 25, 30, 40, 60, 80 and 365 days. Using a polyclonal anti-Cyp3a2 antibody, hepatic Cyp3a appeared to start high at birth, decrease until 20-40 days then gradually increase to reach adult levels. These results are consistent with our data. It is not clear if they used an antibody that cross-reacted with Cyp3a1 as well or not. Intestinal Cyp3a2 protein seemed to increase at 20 days. A study by Kawai et al (Kawai, Bandiera et al. 2000) showed a stable protein expression of Cyp3a2 between 22 and 91 days of age. Lee and Werlin (Lee and Werlin 1995) showed an increase in Cyp3a hepatic expression in male Sprague-Dawley rats by 20 days of age that persisted at 4 and 18 months old. Using a polyclonal anti-Cyp3a1 antibody Gonzalez et al (Gonzalez, Song et

al. 1986) could not detect Cyp3a protein atbirth , levels were elevated at 1 week of age and were maintained throughout development. Due to differences in antibodies used and their specificities, there are some discrepancies between the ontogeny patterns of Cyp3a protein reported in different studies as well as ours. An additional factor is the difference in age groups used. Overall, Cyp3a2 protein starts lower at birth and increases with age early in development.

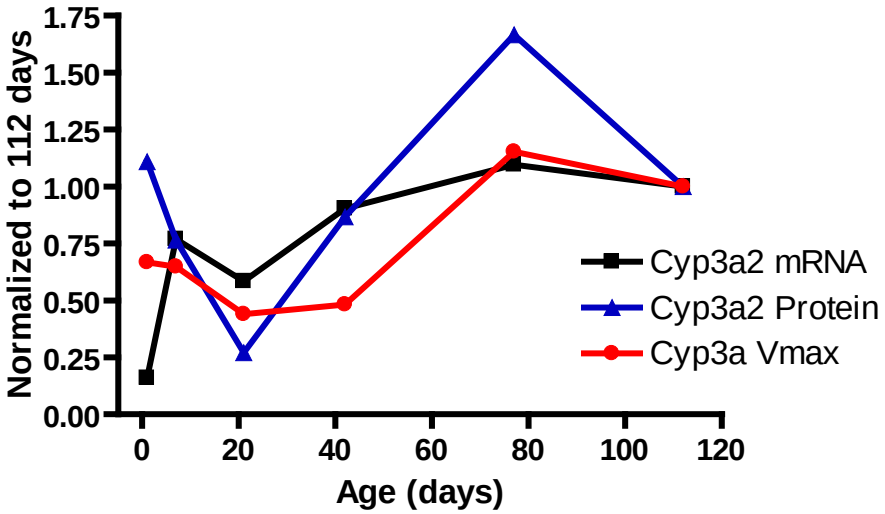
2.2.3 Ontogeny of *In Vitro* Activity

Midazolam was used as a probe for Cyp3a activity. Both Cyp3a1 and Cyp3a2 metabolize midazolam into 4-hydroxymidazolam and 1-hydroxymidazolam. Unlike humans, 4-hydroxymidazolam is the major metabolite in the rat liver. Kobayashi et al showed that 4-hydroxymidazolam production by Cyp3a2 is three-fold higher than by Cyp3a1 recombinant enzymes under similar conditions (Kobayashi, Urashima et al. 2002). Their study also showed that the 4-hydroxy metabolite was more specific to rat Cyp3a2 and Cyp3a1 compared to the 1-hydroxy metabolite, which was produced at very low levels by Cyp2C6, Cyp2C11 and Cyp2D2 using recombinant enzyme systems. These findings are consistent with other data in the literature (Kotegawa, Laurijssens et al. 2002).

The formation of 4-hydroxymidazolam was followed in rat liver and intestinal microsomes as a marker of the ontogeny of Cyp3a activity *in vitro*. The Vmax (maximal enzyme activity) normalized to incubation time and microsomal protein was significantly lower in the liver microsomes of 1, 7, 21 and 42 day old male rats when compared to adults (112days) (Figure 29). The 77-day old age group was not significantly different. The activity was also normalized to Cyp3a2 protein obtained by western blotting (Figure 30). It was constant for all the age groups except for the 21 day age group which was significantly higher. It would be expected that activity per unit functional enzyme would be constant with age. However, western blot detected total Cyp3a2 protein and not necessarily functional enzyme. In the intestine, the ontogeny pattern was difficult to follow. Using our current methods, 4-hydroxymidazolam formation was not detected in the 7 and 21-day age groups and was detected in only few animals in the 42 and 112 day

age groups. Whether the assay sensitivity or the method of preparation of microsomes played a role in the inability to detect the metabolite is unknown. However, these results are consistent with RNA and protein expression, where the intestinal mucosa was found to express both Cyp3a1 and Cyp3a2 at a much lower level than the liver. To date, there are no specific substrates that would differentiate the activity of Cyp3a1 and Cyp3a2. It is not known whether midazolam is also metabolized by rat Cyp3a9 and Cyp3a18. There are no commercial sources for antibodies or recombinant enzymes that would have enabled us to answer this question. The data presented here suggests that the 4-hydroxylation of midazolam in male rat liver reflects the ontogeny pattern of Cyp3a2 and not Cyp3a1. With the exception of the one day old age group, Cyp3a2 RNA and protein expression in the liver showed an overall pattern similar to activity (Figure 37). It is not clear whether a difference in RNA regulation (e.g. half life of the transcript) or protein translation is responsible for the difference in the ontogeny pattern. Since Cyp3a2 protein and Vmax determinations are based on microsomal protein isolation and concentration determination, any variations in these processes or biological variation in the microsomal protein in the day 1 group will affect the ontogeny pattern at this point, which might be another explanation for this difference.

Figure 37: Normalization of Cyp3a2 mRNA expression, Cyp3a2 protein expression and Cyp3a *in vitro* activity to the 112 day age group in male rat liver.



In the literature, Cyp3a activity development in the rat has been followed using 6-β-hydroxylation of steroids (Gonzalez, Song et al. 1986; Wright, Edwards et al. 1997; Johnson, Tanner et al. 2000; Kawai, Bandiera et al. 2000). The overall pattern showed an increase in activity with age in agreement with our results. Although the 6-β-hydroxylation of testosterone is specific to Cyp3a, testosterone is hydroxylated at a number of positions by a number of enzymes other than Cyp3a. Cyps 2a1, 2a2, 2b2, 2c11 and 2c13 are involved as well in testosterone metabolism in the rat liver (Arlotto, Trant et al. 1991). The major metabolites of testosterone by rat Cyp3a1 and Cyp3a2 are the 2-β hydroxy and the 6-β-hydroxytestosterone (Arlotto, Trant et al. 1991). In humans, testosterone is metabolized by Cyp3A into the 6-β-hydroxytsetosterone, 2-β-hydroxytestosterone and 16-α-hydroxy testosterone (Rendic and Di Carlo 1997).

Midazolam metabolism and not testosterone was used in the present study despite the fact that testosterone metabolsim has been traditionally used in the rat in *in vitro* ontogeny studies since testosterone metabolism is not specific to Cyp3a. Since the *in vitro* activity data was used to extrapolate to the *in vivo* clearance, the same substrate was used *in vitro* and *in vivo*. Only the 6-β-hydroxytestosterone metabolite needs to be followed to be able to characterize Cyp3a ontogeny *in vitro* and *in vivo*. This would be feasible *in vitro* where the reaction conditions could be manipulated to produce quantifiable levels of the metabolite. *In vivo* however, this might not be possible due to the limited amount of samples and due to the fact that testosterone is present endogenously. The clearance of testosterone is a composite of several hepatic pathways rather than a single one in contrast to midazolam. Using serum from neonatal animals adds the limitation of a small serum volume that would make it challenging in detecting and quantifying metabolites.

In the study by Wright et al (Wright, Edwards et al. 1997), 6-β-hydroxylation of androstenedione was found to increase from 3 weeks of age to 6 weeks of age in male rat liver microsomes. The activity was maintained at 9 weeks then dropped at 12 and 15 weeks. The authors pointed out that this pattern was not mirrored by Cyp3a2 protein development in males and hence other Cyp3a forms (e.g. Cyp3a18) might be responsible for the activity. Johnson et al (Johnson, Tanner et al. 2000) studied enterocytic and hepatic 6-hydroxylation of midazolam with age. Hepatic microsomes showed an increase

in activity from birth till adulthood. Enterocytic microsmal activity was low at birth, a surge was observed at weaning, then a plateau was observed up to 80 days if age and at 1 year. The overall intestinal activity marked by Vmax/Km was 50-fold lower than hepatic activity. Gonzalez et al (Gonzalez, Song et al. 1986) presented a study that had a similar pattern of development. The 6-β-hydroxylation of testosterone was absent at birth, increased at 1 and 2 weeks of age and continued increasing up to 12 weeks of age. Kawai et al (Kawai, Bandiera et al. 2000) examined the 2-β and 6- β-hydroxylation of testosterone in male rat liver. The pattern was similar for both metabolites where activity peaked at 34 days. The mRNA pattern of Cyp3a2, Cyp3a9 and Cyp3a18 was followed in the same rats but the activity did not correspond to the mRNA pattern of any of the enzymes. This pattern was different from our results and data presented in the literature.

2.3 Ontogeny Model Assumptions

2.3.1 Microsomal Protein

One of the ontogeny model assumptions is that microsomal protein normalized to liver weight remains constant with age. To test this assumption total microsomal protein was measured for each age group and normalized to liver weight (Figure 36). Microsomal protein was found not to be constant with age. It peaked at 21 days and then decreased afterwards. The inconsistency of microsomal protein during development in male rat liver is documented in the literature. The patterns reported were either increasing with age (Petermann and Hamilton 1958; Nakajima, Wang et al. 1992) or similar to the pattern observed in Figure 28 (Elbarbry and University of Saskatchewan. College of Graduate Studies and Research. 2006). The implication of this finding is that microsomal protein normalized to body weight is necessary to know for calculating the infant scaling factor from *in vitro* data to be able to extrapolate adult clearance to infant clearance. Hence, it would appear unwise to compare infant and adult *in vitro* activity measurements normalized to microsomal protein content alone.

Possible explanation of the variability seen in microsomal protein is the variability in liver weight due to glycogen content, the variability of the microsomal

fraction collected by centrifugation which might include other subcellular fractions in addition to microsomes.

2.3.2 Michaelis-Menten Affinity Constant (Km)

The ontogeny model proposed a constant Km of a substrate-enzyme system across developmemtal stages. To directly test this assumption, Michaelis-Menten profiles were constructed for the 4-hydroxylation of midazolm by male rat liver microsomes at different age groups (Figure 32). Table 4 shows Km values obtained by fitting each profile to a one-binding site non-linear model. Statistical analysis showed no significant difference between Km's for the different age groups. The Km's listed are the apparent Km's. It is assumed that only the free drug is available for enzymatic metabolism. Non-specific binding of midazolam to human and rat liver microsomes has been reported to be similar (Lu, Li et al. 2006). At constant protein amount and assuming that non-specific binding does not vary with midazolam concentrations, the true Km would be the product of apparent Km and the free fraction of drug. Assuming that non-specific binding per gram microsomal protein does not vary with age, then similar to the apparent Km, the true Km across age groups is non-significantly different. In contrast to our data, the Km CYP1A2-mediated methoxyresorufin-O-dealkylation was found to be significantly different at 5 and 7 days in male rat liver microsomes when compared to 112 day animals (Elbarbry and University of Saskatchewan. College of Graduate Studies and Research. 2006). The authors explained this discrepancy between age groups suggesting that other enzymes might be involved in the metabolism of methoxyresorufin at earlier stages of development and hence the Km of these age groups reflects the affinity to other enzymes in addition to Cyp1A2. They also commented that non-specific binding might vary with age due to changes in microsomal protein content (Elbarbry and University of Saskatchewan. College of Graduate Studies and Research. 2006). With midazolam, non-specific binding in rats and humans was similar (Lu, Li et al. 2006), despite microsomal protein differences that would make it less likely that non-specific binding varies with age in rats.

For the purpose of calculating the infant scaling factor from *in vitro* data, Km was considered constant with age and was not included in the calculation of the ontogeny scaling factor (OSF). This Km cancels out when dividing the infant intrinsic clearance by adult intrinsic clearance and OSF becomes the ratio of Vmax in the infant to that in the adult. If Km were different across age groups, the model would still be useful, however the OSF would be the ratio of intrinsic clearances in the neonate to that in the adult, where intrinsic clearance is the ratio of Vmax to Km.

2.3.3 Other Model Assumptions

In order to make the model most useful, another assumption that was not directly tested in this study is that the Vmax pattern would be similar from one substrate to the next for the same pathway. Hence, one could measure changes in activity with a probe substrate *in vitro* and this should be indicative of *in vivo* activity for any other substrate. This assumption was tested in the literature for Cyp1a2 and Cyp2e1 in the male rat liver; the ontogeny of 2 substrates for each enzyme was found to correlate (Elbarbry and University of Saskatchewan. College of Graduate Studies and Research. 2006). Testosterone 6-β hydroxylation has been studied in the literature as a marker for Cyp3a ontogeny. The general patterns described of an increase in activity with age was similar to our findings with midazolam, strongly suggesting that this model assumption holds true (Gonzalez, Song et al. 1986; Johnson, Tanner et al. 2000).

Two of the model assumptions are related to the *in vivo* characteristics of the substrate under study. First, the clearance of the drug is dictated by enzymatic metabolism in which uptake into the hepatocyte is not rate limiting (i.e., no uptake transporters involved). Midazolam as a Cyp3a substrate fulfills this assumption in that its lipophilicity is such that it readily passes through cell membranes. Hence Cyp3a metabolism, and not hepatic transporters, is responsible for midazolam clearance, so that changes in enzyme activity should be reflected in clearance. Second, the *in vitro* model is constructed to predict intrinsic clearance, which is largely influenced by changes in Vmax. Ideally, the drug would be a low extraction ratio drug and its systemic clearance following an intravenous dose would yield an estimate of intrinsic clearance with little or

no dependence on blood flow. However midazolam has an intermediate extraction ratio in the rat (Uhing, Beno et al. 2004), entailing the partial dependence of midazolam clearance on hepatic blood flow. As an alternative to the intravenous route of administration and to avoid the inclusion of hepatic blood flow estimate, oral clearance can be used *in vivo* as illustrated by Equation 30-Equation 34. However, this requires that intestinal bioavailability (F_G) is equal to 1. This assumption is strengthened both by our *in vitro* data, and data from the literature (Kotegawa, Laurijssens et al. 2002; Uhing, Beno et al. 2004; Kanazu, Okamura et al. 2005). Our *in vitro* data shows that mRNA, protein and *in vitro* activity of Cyp3a in the intestine was very much lower than in the liver. The *in vitro* activity in the intestine at 112 days was 100-fold less than that in the liver.

2.4 Model Prediction

Infant scaling factor (ISF) was calculated from *in vitro* data. *In vivo* oral clearance of midazolam was measured in age groups 7 days (neonate), 21 days (weanling) and 112 days (adult) to test the model prediction at two different developmental stages. Oral clearance of midazolam in 112 days (average weight 377g) was 64 L/hr/kg and similar to values reported in the literature. The reported oral clearance of midazolam in male rats (weight 125- 350g) in the literature ranged from 42 to 63 L/hr/kg (Kotegawa, Laurijssens et al. 2002; Strelevitz, Foti et al. 2006; Zhang, Tan et al. 2007). There are currently no midazolam *in vivo* studies in the literature in male rats as young as 7 or 21 day old. The predicted clearance for 7 and 21 day old rats was calculated according to Equations 36 and 37. Our criterion was that if the predicted clearance was 50-200% that of the measured *in vivo* clearance then the prediction was acceptable. Neither the 7 nor the 21 day predicted clearances fell in this range. The predicted clearance values werer 8-fold (predicted 29 L/hr/Kg, observed 3.71 L/hr/Kg) and 12-fold (predicted 62 L/hr/Kg, observed 5.2 L/hr/Kg) higher than those observed for 7 and 21 day age groups, respectively. Hence, the model was unable to predict *in vivo* oral clearance of midazolam using *in vitro* scaling factors.

The model assumes linear conditions where the unbound concentration (available for drug metabolism) is smaller than Km. There is evidence in the literature of dose dependent kinetics for midazolam in rats and humans (Bornemann, Min et al. 1985; Higashikawa, Murakami et al. 1999). In humans, a total dose of 30 mg was outside the linear range, where AUC increased disproportionately with the dose increase (Bornemann, Min et al. 1985). In rats, intestinal and hepatic first-pass metabolism appeared to be dose –dependent (Higashikawa, Murakami et al. 1999). These findings contradict one of the model assumptions.

When predicted clearance was calculated using Km, the predicted values better reflected the observed clearance. This might indicate the possibility of different Km values with age. One explanation might be the difference in lipid composition of microsomes in different age groups that affects the drug availability to the enzyme and thus affecting apparent Km.

A more viable explanation is that intestinal availability (F_G) in adults is much less than 1 contradicting an underlying assumption of oral clearance prediction. Although data from the literature (Kotegawa, Laurijssens et al. 2002; Uhing, Beno et al. 2004; Kanazu, Okamura et al. 2005) largely supported this assumption, there is some evidence to the contrary (Strelevitz, Foti et al. 2006). Our *in vitro* data shows that mRNA, protein and *in vitro* activity of Cyp3a in the intestine was very much lower than in the liver. The *in vitro* activity in the intestine at 112 days was 100-fold less than in the liver. However, scaling factors for the intestine are mostly lacking in terms of surface area, protein content and enzyme distribution along the whole organ. Such low activity might then be an indicator of significant intestinal metabolism if properly scaled. Together with the fact that no activity was detected in the 7 and 21 day old rat intestine, this might be an evidence of significant intestinal first pass effect in the adults and not in the pups which was not accounted for in the prediction causing the model to fail.

Our results do not discredit the model as a viable option for clearance prediction. Further experiments elucidating how Km and first pass metabolism vary or not with age along with dosing the rats with a lower dose of midazolam and using a different Cyp3a substrate would allow further evaluation of the model.

Chapter 7. Summary and Conclusions

Infant exposure to drugs in breast milk is determined by two important factors, the amount of drug ingested in the milk and the ability of the infant to clear the drug. There is a dearth of information about both determinants. Clinical trials set to explore either phenomenon suffer from ethical and logistical hindrances. Hence, models are needed to predict the appearance of drugs in milk and the clearance in the neonate. The aim of this dissertation was to examine models to predict milk to serum ratio of drugs in the mother and clearance in the neonate.

The models studied for the M/S prediction were the Atkinson and Begg model and the Fleishaker model. The Atkinson and Begg model is an empirical model that does not need any experimental determination of the parameters. It assumes passive diffusion of the drugs into milk. M/S is predicted by a multiple linear regression relationship of the physicochemical parameters affecting the diffusion of the unionized, unbound molecule into milk. The model was shown to be inadequate for predicting both basic and acidic drugs. The multiple regression relationship might need to be further examined with a larger set of drugs to improve the model prediction.

The Fleishaker model is a mechanistic model that relies on the *in vitro* determination of drug protein binding in milk and serum and the drug ratio in skim to whole milk. The model is better suited to predict whether the drugs passively diffused into milk or were actively transported, based on comparison with observed values. In its current form, the model is useful in understanding the mechanisms of drug transfer into milk when combined with observed values. Further understanding of the active transport processes involved in drug transfer into milk might make it feasible to add an active transport component to the model and improve its predictive ability.

The ontogeny of Cyp3a metabolism in the male rat was studied to understand the various aspects of development (mRNA, protein and activity) and to examine the assumptions of the clearance ontogeny model previously proposed by our laboratory. The mRNA expression development of Mdr1a and Mdr1b was followed as well, due to the importance of P-gp in clearance and the shared regulation and substrates of Cyp3a and P-gp. The patterns of ontogeny were different in the liver versus the intestine for

each gene. In general, the expression levels were lower in the intestine. Cyp3a1 and Mdr1a expression in the liver were high at birth and decreased subsequently. Cyp3a2 mRNA expression increased with age. Mdr1b expression in the liver was relatively low at all the age groups studied except for the 42 day old group, which was significantly higher. In the intestine, Cyp3a1 and Mdr1a mRNA expression increased with age. Mdr1b expression was the same for all the age groups except for the 42 day old rats, which had slightly lower expression. It was not possible to follow Cyp3a2 mRNA ontogeny in the intestine due to the very low expression. It appears that each gene is differentially regulated across tissues. However, Cyp3a1 and Mdr1a seem to have the same ontogeny pattern at the mRNA level within the same tissue. The 42 day old age group had a unique expression of Mdr1b in both the intestine and liver when compared to the rest of the age groups. Although there are some studies about the regulation of these genes in the rat liver and intestine, the actual triggers of these ontogeny patterns remain to be studied.

Cyp3a2 protein ontogeny in the male rat liver increased with age. In the intestine the expression was lower. Cyp3a1 protein was not followed due to the lack of a commercially available specific antibody.

Midazolam 4-hydroxylation by the male rat liver and intestinal microsomes was used as probe for *in vitro* Cyp3a activity. The intestinal activity, when detectable, was 100 fold lower than in the liver, in agreement with the mRNA and protein expression, which were lower in the intestine. The Vmax for 4-hydroxymidazolam formation increased with age in the male rat liver. Cyp3a2 mRNA expression, protein expression and Cyp3a *in vitro* activity in the male rat liver had the same ontogeny pattern when normalized to the adult (112 days) with the exception of the 1 day old where protein and activity were correlated and only RNA was low. Modeling of such a pattern might enable the prediction of activity from protein or mRNA levels.

The ontogeny model assumptions studied were whether microsomal protein does not change with age and if Km is constant for an enzyme-substrate system. The microsomal protein normalized to liver weight was found to change with age, necessitating that the scaling factors used to extrapolate *in vitro* activity to *in vivo*

clearance take microsomal protein into account. The Km was not different across the age groups for the 4-hydroxylation of midazolam by the male rat liver microsomes.

When applying the model prediction to the 7 and 21 day old age groups, the model did not predict the measured *in vivo* clearance of midazolam. The model grossly overpredicted clearance based on *in vitro* data. A possible reason for the difference between the predicted and observed values is that intestinal bioavailability in the intestine is much lower in the adults than in the pups, which was not accounted for in the model.

These results are inconclusive as to whether the model is useful in predicting *in vivo* clearance. Further studies are needed to test the model prediction of drugs cleared by Cyp3a in the male rat. *In vitro,* the use of hepatocytes rather than microsomes might better mimic *in vivo* conditions. *In vivo*, a different Cyp3a substrate and testing the model prediction in more age groups would be of great value.

Table 10: Atkinson and Begg calculations for acidic drugs

Drug	pka	Log D	M log Papp	M Papp	Bound in Plasma (%)	fu,s	fu,m	Mu/Pu (pH=7.2)	M/Pphase pH=7.2	K	Ln M/P' phase
Acyclovir	9.04	-1.76	-3.15	0.0007	15	0.85	0.96	0.99	0.84	0.99	-0.36
Amoxicillin	7.39	-2.43	-4.01	0.0001	18	0.82	0.96	0.81	0.66	1.00	-2.19
Captopril	3.70	-3.15	-4.94	0.0000	30	0.70	0.96	0.63	0.44	1.00	-4.47
Cefadroxil	7.22	-3.10	-4.88	0.0000	20	0.80	0.96	0.78	0.62	1.00	-2.60
Ciprofloxacin	8.75	-1.23	-2.47	0.0034	40	0.60	0.95	0.98	0.59	1.00	-0.20
Clonazepam	1.57	2.34	2.14	137.59	86	0.14	0.92	0.63	0.64	7.24	-6.41
Doxorubicin	8.20	1.22	0.69	4.94	76	0.24	0.93	0.95	0.28	1.25	-0.25
Enalapril	5.49	-0.98	-2.14	0.01	50	0.50	0.95	0.64	0.32	1.01	-4.18
Indomethacin	4.30	-0.01	-0.89	0.13	90	0.10	0.90	0.63	0.07	1.06	-3.22
Ketorolac	3.49	-0.79	-1.90	0.01	99	0.01	0.75	0.63	0.01	1.27	-1.76
Naproxen	4.20	0.07	-0.79	0.16	99	0.00	0.66	0.63	0.0028	1.46	-1.28
Oxazepam	11.60	2.31	2.10	125.86	99	0.01	0.78	1.00	0.08	6.89	-0.32
Piroxicam	5.07	-1.10	-2.30	0.01	99	0.02	0.80	0.63	0.01	1.20	-2.07

Table 11: Atkinson and Begg calculations for basic drugs

Drug	pka	Log D	M log Papp	M Papp	Bound in Plasma (%)	fu,s	fu, m	Mu/Pu (pH=7.2)	M/Pphase pH=7.2	K	Ln M/P' phase
Chlorpromazine	9.21	3.22	3.27	1878.45	98	0.02	0.82	1.58	2.70	85.70	-0.16
Clonidine	8.30	0.62	-0.08	0.83	20	0.80	0.96	1.52	1.26	1.03	0.80
Fluoxetine	9.62	1.83	1.48	30.25	94	0.06	0.88	1.58	0.23	2.45	-0.97
Fluoxetine	9.62	1.83	1.48	30.25	94	0.06	0.88	1.58	0.23	2.45	-0.97
Imipramine	9.40	2.40	2.22	164.44	90.1	0.10	0.90	1.58	1.32	8.46	0.10
Labetalol	7.40	1.24	0.72	5.24	50	0.50	0.95	1.29	0.80	1.24	0.10
Metoprolol	9.75	-0.16	-1.09	0.08	11	0.89	0.96	1.58	1.40	1.00	0.97
Quinidine	7.90	2.04	1.75	56.44	87	0.13	0.91	1.44	0.67	3.59	-0.30
Ranitidine	8.18	-0.29	-1.25	0.06	15	0.85	0.96	1.50	1.27	1.00	0.81
Terbutaline	8.72	-1.35	-2.62	0.00	20	0.80	0.96	1.56	1.24	1.00	0.84
Timolol	9.21	0.03	-0.84	0.14	60	0.40	0.95	1.58	0.64	1.02	0.26
Trimethoprim	7.12	0.74	0.07	1.19	37	0.63	0.95	1.20	0.80	1.05	0.06
Acebutolol	9.20	1.71	1.33	21.18	26	0.74	0.96	1.58	2.27	1.95	1.13
Quinine	8.50	2.11	1.84	69.49	93	0.07	0.89	1.54	0.45	4.20	-0.62
Nicotine	8.20	0.41	-0.35	0.45	4.9	0.95	0.96	1.50	1.45	1.01	0.92

Table 12: Predicted M/S (M/P’ phase) from the Atkinson and Begg model and observed M/S values from the literature.

Drug	M/P' phase (predicted)	M/S obs
Acyclovir	0.70	3.00
Amoxicillin	0.11	0.02
Captopril	0.01	0.01
Cefadroxil	0.07	0.01
Ciprofloxacin	0.82	1.60
Clonazepam	0.00	0.33
Doxorubicin	0.78	1.20
Enalapril	0.02	0.02
Indomethacin	0.04	0.37
Ketorolac	0.17	0.02
Naproxen	0.28	0.01
Oxazepam	0.72	0.10
Piroxicam	0.13	0.02
Chlorpromazine	0.86	2
Clonidine	2.22	2
Fluoxetine	0.38	0.38
Fluoxetine	0.38	0.68
Imipramine	1.10	1.6
Labetalol	1.11	1.5
Metoprolol	2.63	2.9
Quinidine	0.74	0.71
Ranitidine	2.24	3.8
Terbutaline	2.31	2
Timolol	1.29	0.8
Trimethoprim	1.06	1.25
Acebutolol	3.10	5.7
Quinine	0.54	0.31
Nicotine	2.51	2.92

References used for calculations: (Ardenne and Reitnauer 1975; Corrocher, Tedesco et al. 1975; Yokoyama, Kono et al. 1975; Dahl and Strandjord 1977; Eeg-Olofsson, Malmros et al. 1978; Spyker, Thomas et al. 1978; Wiles, Orr et al. 1978; Gugler, Kurten et al. 1979; Sovner and Orsulak 1979; Kafetzis, Siafas et al. 1981; Pickoff, Kessler et al. 1981; Boreus and de Chateau 1982; Ita, Singhvi et al. 1982; Lonnerholm and Lindstrom 1982; Fidler, Smith et al. 1983; Laskin 1983; Ostensen 1983; Kulas, Lunell et al. 1984; Lindberg, Boreus et al. 1984; Lindeberg, Sandstrom et al. 1984; Luck and Nau 1984; Dayer, Leemann et al. 1985; Egan, Costanza et al. 1985; Kearns, McConnell et al. 1985; Lunell, Kulas et al. 1985; McGourty, Silas et al. 1985; Sjovall, Alvan et al. 1986; Hartikainen-Sorri, Heikkinen et al. 1987; Lau, Emery et al. 1987; Duchin, McKinstry et al. 1988; Lowenthal, Matzek et al. 1988; Meyer, de Miranda et al. 1988; Ostensen, Matheson et al. 1988; Borgstrom, Nyberg et al. 1989; Wischnik, Manth et al. 1989; Dahlstrom, Lundell et al. 1990; Dostal, Weaver et al. 1990; Isenberg 1990; Redman, Kelly et al. 1990; Sallee and Pollock 1990; Donnelly and Macphee 1991; Hutabarat, Unadkat et al. 1991; Lebedevs, Wojnar-Horton et al. 1991; Rush, Snyder et al. 1991; Brocks and Jamali 1992; Verme, Ludden et al. 1992; Benowitz and Jacob 1993; Gladziwa and Klotz 1993; MacFadyen, Meredith et al. 1993; Oberbauer, Krivanek et al. 1993; Piscitelli, Rodvold et al. 1993; Altamura, Moro et al. 1994; Olkkola, Brunetto et al. 1994; Wells, Mortensen et al. 1994; Bork and Benes 1995; Foye, Lemke et al. 1995; McNamara, Meece et al. 1996; Venkatesan, Mathur et al. 1997; Yoshida, Smith et al. 1997; Yoshida, Smith et al. 1998; Yoshida, Smith et al. 1998; Kristensen, Ilett et al. 1999; Zhu, Jiang et al. 2002)

Table 13: Relative mRNA expression of Cyp3a1 in male Sprague-Dawley rat liver.

Age (days).Rat number	Mean
1.1	6.10
1.2	6.35
1.3	5.28
1.4	8.18
7.1	0.71
7.2	1.12
7.3	0.76
7.4	0.84
21.1	1.08
21.2	1.09
21.3	1.54
21.4	1.67
42.1	0.86
42.2	0.81
42.3	1.00
42.4	0.81
77.1	1.24
77.2	0.96
77.3	0.81
77.4	0.93
112.1	0.90
112.2	1.30
112.3	1.24
112.4	1.13

Table 14: Relative mRNA expression of Cyp3a2 in male Sprague-Dawley rat liver.

Age (days).Rat number	Mean
1.1	0.49
1.2	0.43
1.3	0.41
1.4	0.40
7.1	2.50
7.2	1.98
7.3	1.94
7.4	1.86
21.1	1.45
21.2	1.36
21.3	1.76
21.4	1.72
42.1	2.19
42.2	2.67
42.3	2.42
42.4	2.43
77.1	3.20
77.2	2.78
77.3	2.10
77.4	3.71
112.1	1.24
112.2	2.65
112.3	3.92
112.4	2.93

Table 15: Relative mRNA expression of Mdr1a in male Sprague-Dawley rat liver.

Age (days).Rat number	Mean
1.1	0.09
1.2	0.42
1.3	0.32
1.4	0.39
7.1	0.09
7.2	0.08
7.3	0.11
7.4	0.14
21.1	0.31
21.2	0.16
21.3	0.21
21.4	0.22
42.1	0.17
42.2	0.17
42.3	0.19
42.4	0.15
77.1	0.09
77.2	0.16
77.3	0.09
77.4	0.06
112.1	0.01
112.2	0.07
112.3	0.17
112.4	0.19

Table 16: Relative mRNA expression of Mdr1b in male Sprague-Dawley rat liver.

Age (days).Rat number	Mean
1.1	0.14
1.2	0.33
1.3	0.38
1.4	0.23
7.1	0.15
7.2	0.06
7.3	0.10
7.4	0.10
21.1	0.30
21.2	0.20
21.3	0.56
21.4	0.13
42.1	1.44
42.2	1.43
42.3	1.02
42.4	2.07
77.1	0.22
77.2	0.25
77.3	0.20
77.4	0.43
112.1	0.27
112.2	0.31
112.3	0.50
112.4	0.41

Table 17: Relative mRNA expression of villin in male Sprague-Dawley rat intestinal mucosa.

Age (days).Rat number	Mean
7.1	4.76
7.2	7.07
7.3	3.81
7.4	4.58
21.1	5.21
21.2	5.78
21.4	6.84
42.1	9.06
42.2	6.83
42.3	5.35
42.4	6.64
112.2	4.66
112.3	11.20
112.4	5.56

Table 18: Relative mRNA expression of Cyp3a1 in male Sprague-Dawley rat intestinal mucosa.

Age (days).Rat number	Mean
7.1	0.0003
7.2	0.0004
7.3	0.0003
7.4	0.0003
21.1	0.0004
21.2	0.0002
21.4	0.0005
42.1	0.0008
42.2	0.0003
42.3	0.0003
42.4	0.0003
112.2	0.0009
112.3	0.0009
112.4	0.0005

Table 19: Relative mRNA expression of Mdr1a in male Sprague-Dawley rat intestinal mucosa.

Age (days).Rat number	Mean
7.1	1.09
7.2	N/A
7.3	3.87
7.4	3.36
21.1	1.71
21.2	5.20
21.4	2.67
42.1	10.60
42.2	7.90
42.3	10.75
42.4	4.82
112.2	13.25
112.3	23.75
112.4	14.95

Table 20: Relative mRNA expression of Mdr1b in male Sprague-Dawley rat intestinal mucosa.

Age (days).Rat number	Mean
7.1	0.08
7.2	0.28
7.3	0.12
7.4	0.15
21.1	0.14
21.2	0.26
21.4	0.12
42.1	0.13
42.2	0.02
42.3	0.05
42.4	0.02
112.2	0.27
112.3	0.26
112.4	0.13

Table 21: Cyp3a2Protein expression male Sprague-Dawley rat liver microsomes determined by western blot (ng Cyp3a2 protein/5µg liver microsomal protein).

Age (days).Rat number	Mass
1.1	0.77
1.2	1.22
1.3	1.09
7.1	0.78
7.2	0.83
7.3	0.62
7.4	0.61
21.1	0.20
21.2	0.23
21.3	0.36
21.4	0.21
42.1	1.24
42.2	0.82
42.3	0.49
42.4	0.66
77.1	1.59
77.2	1.77
77.3	1.90
77.3	0.92
112.1	0.88
112.2	1.02
112.3	0.89
112.4	0.92

NONMEM ANALYSIS

NONMEM 7 day age group data format:

#ID	AMT	Time	DV	MDV
50	300	0	0	1
50	0	0.083	43.576	0
59	250	0	0	1
59	0	0.083	107.822	0
60	300	0	0	1
60	0	0.1	320.728	0
51	350	0	0	1
51	0	0.117	107.798	0
58	250	0	0	1
58	0	0.267	194.857	0
47	200	0	0	1
47	0	0.283	1201.77	0
48	250	0	0	1
48	0	0.283	775.369	0
49	200	0	0	1
49	0	0.283	1862.67	0
89	350	0	0	1
89	0	0.5	1082.81	0
90	300	0	0	1
90	0	0.5	2568.5	0
88	250	0	0	1
88	0	0.55	544.23	0
101	250	0	0	1
101	0	0.667	5404.19	0
100	250	0	0	1
100	0	0.683	3994.36	0
99	300	0	0	1

99	0	0.717	7244.29	0
91	200	0	0	1
91	0	1.033	1443.88	0
92	300	0	0	1
92	0	1.033	2357.29	0
103	300	0	0	1
103	0	1.05	1014.46	0
102	250	0	0	1
102	0	1.083	1165.95	0
94	250	0	0	1
94	0	1.5	2087.91	0
93	250	0	0	1
93	0	1.533	844.148	0
104	300	0	0	1
104	0	1.567	936.258	0
105	250	0	0	1
105	0	1.583	972.694	0
96	250	0	0	1
96	0	2.033	1885.65	0
95	200	0	0	1
95	0	2.05	1317.55	0
107	300	0	0	1
107	0	2.067	1104.67	0
106	300	0	0	1
106	0	2.083	3984.67	0
108	200	0	0	1
108	0	3.017	452.107	0
109	250	0	0	1
109	0	3.017	791.594	0
97	300	0	0	1
97	0	3.05	420.169	0

98	250	0	0	1
98	0	3.067	908.407	0
45	300	0	0	1
45	0	4.017	779.95	0
46	300	0	0	1
46	0	4.033	695.948	0
62	250	0	0	1
62	0	4.05	1531.76	0
61	250	0	0	1
61	0	4.083	744.928	0
54	300	0	0	1
54	0	6.017	158.64	0
53	300	0	0	1
53	0	6.05	49.248	0
63	250	0	0	1
63	0	6.05	455.925	0
52	200	0	0	1
52	0	6.067	55.322	0
55	250	0	0	1
55	0	8.033	14.375	0
64	300	0	0	1
64	0	8.033	97.297	0
65	300	0	0	1
65	0	8.033	552.739	0
56	250	0	0	1
56	0	8.05	27.504	0

NONMEM Control File for 7 day age group:

```
Created by MAGGIE ABBASSI
$PROBLEM POPULATION DATA ANALYSIS OF MIDAZOLAM
$INPUT  ID AMT TIME DV AGE1 AGE2 MDV ; FITTING OF
DESTRUCTIVE SAMPLING IN 7 DAY OLDS
                ; DATE CONTROL FILE CREATED 06-1-07
                ; INITIAL ESTIMATE OF CL AND KA OBTAINED FROM
AVERAGE INDIVIDUAL ANALYSIS
;-------------------------
;  UNITS              |
;  Time - hours       |
;  Dose - mcg         |
;  Cp  - ng/ml = mcg/L  |
;  Clearances/F - L/hour |
;  Volumes/F - L      |
;-------------------------
$DATA  MIDAZOLAM.7DAY.AGE.DATA.TXT IGNORE= #
$SUBROUTINES ADVAN2 TRANS2
$PK

    TVCL  =THETA(1)
    CL    = TVCL*EXP(ETA(1))
    TVV  = THETA(2)
    V   = TVV*EXP(ETA(2))
    CLRA  = 0
    TVKA=THETA(3)
    KA=THETA(3)

    S2    = V
    K     = CL  / V ;
```

```
    DUM   = 0
$ERROR IPRED= F
    Y     = IPRED*(1+ERR(1))
    IRES  = DV - F



$THETA
 (0, 0.0274,10) ;[CL]
 (0, 0.0945 ,10) ;[V]
 (0, 0.357, 1);[KA]

$OMEGA
 (0.1)
 (0.1)
$SIG
 0.3 FIX

$ESTIMATION METHOD=1 PRINT=1 MAXEVAL=9999 NOABORT
SIGDIGITS=3 POSTHOC
      MSFO=msfo.outputfile

$COVA
$TABLE ID  AMT  TIME  IPRED  IRES
    NOPRINT ONEHEADER FILE=table.txt
$TABLE ID  CL   V  KA
    FIRSTONLY NOPRINT ONEHEADER NOAPPEND FILE=etatable.txt
```

NONMEM output for 7 day age group:

ID	AMT	TIME	IPRE	IRES	Observed	PRED	RES	WRES
50	0.00	0.08	166.86	-123.29	43.58	357.66	-314.09	-0.68
59	0.00	0.08	170.14	-62.32	107.82	298.05	-190.23	-0.50
60	0.00	0.10	344.66	-23.93	320.73	423.96	-103.23	-0.19
51	0.00	0.12	282.46	-174.66	107.80	569.36	-461.57	-0.64
58	0.00	0.27	428.63	-233.78	194.86	804.31	-609.46	-0.65
47	0.00	0.28	1126.10	75.64	1201.80	671.71	530.06	0.68
48	0.00	0.28	790.62	-15.25	775.37	839.63	-64.26	-0.07
49	0.00	0.28	1742.20	120.48	1862.70	671.71	1191.00	1.53
89	0.00	0.50	1287.90	-205.11	1082.80	1691.00	-608.16	-0.35
90	0.00	0.50	2322.40	246.13	2568.50	1449.40	1119.10	0.75
88	0.00	0.55	823.38	-279.15	544.23	1267.50	-723.23	-0.57
101	0.00	0.67	4892.70	511.48	5404.20	1377.10	4027.10	3.10
100	0.00	0.68	3495.40	499.00	3994.40	1389.10	2605.20	2.00
99	0.00	0.72	6601.20	643.04	7244.30	1695.10	5549.20	3.55
91	0.00	1.03	1340.50	103.36	1443.90	1213.40	230.44	0.24
92	0.00	1.03	2121.60	235.65	2357.30	1820.20	537.12	0.37
103	0.00	1.05	1402.30	-387.83	1014.50	1821.30	-806.81	-0.55
102	0.00	1.08	1337.00	-171.01	1165.90	1518.40	-352.48	-0.29
94	0.00	1.50	1774.40	313.55	2087.90	1435.50	652.45	0.62
93	0.00	1.53	1202.30	-358.15	844.15	1423.70	-579.58	-0.56
104	0.00	1.57	1410.50	-474.23	936.26	1693.30	-757.06	-0.61
105	0.00	1.58	1235.40	-262.75	972.69	1405.00	-432.29	-0.42
96	0.00	2.03	1589.60	296.04	1885.70	1204.30	681.37	0.73
95	0.00	2.05	1151.40	166.18	1317.60	956.88	360.67	0.49
107	0.00	2.07	1267.30	-162.59	1104.70	1425.50	-320.81	-0.29
106	0.00	2.08	3352.90	631.75	3984.70	1416.20	2568.50	2.33
108	0.00	3.02	504.90	-52.80	452.11	604.03	-151.93	-0.25
109	0.00	3.02	780.56	11.04	791.59	755.04	36.55	0.05

97	0.00	3.05	615.67	-195.50	420.17	890.33	-470.16	-0.53
98	0.00	3.07	859.62	48.79	908.41	735.25	173.15	0.24
45	0.00	4.02	741.60	38.35	779.95	517.44	262.51	0.42
46	0.00	4.03	667.70	28.24	695.95	512.63	183.32	0.29
62	0.00	4.05	1425.00	106.76	1531.80	422.97	1108.80	2.15
61	0.00	4.08	701.78	43.15	744.93	414.87	330.06	0.65
54	0.00	6.02	158.00	0.64	158.64	153.56	5.08	0.02
53	0.00	6.05	89.19	-39.94	49.25	150.44	-101.19	-0.45
63	0.00	6.05	448.19	7.73	455.92	125.37	330.56	1.76
52	0.00	6.07	68.49	-13.17	55.32	99.24	-43.92	-0.30
55	0.00	8.03	22.19	-7.81	14.38	36.08	-21.71	-0.37
64	0.00	8.03	96.29	1.01	97.30	43.30	54.00	0.77
65	0.00	8.03	551.22	1.52	552.74	43.30	509.44	7.29
56	0.00	8.05	29.10	-1.60	27.50	35.69	-8.19	-0.14

NONMEM: Clearance (L/hr), V (L), Ka for 7 day age group

ID	CL	V	KA
45	0.05	0.05	0.64
46	0.05	0.05	0.64
47	0.05	0.02	0.64
48	0.05	0.04	0.64
49	0.04	0.01	0.64
50	0.05	0.09	0.64
51	0.05	0.09	0.64
52	0.06	0.03	0.64
53	0.06	0.03	0.64
54	0.05	0.04	0.64
55	0.06	0.03	0.64
56	0.06	0.04	0.64
58	0.05	0.08	0.64
59	0.05	0.07	0.64
60	0.05	0.05	0.64
61	0.04	0.06	0.64
62	0.02	0.05	0.64
63	0.04	0.08	0.64
64	0.05	0.06	0.64
65	0.03	0.11	0.64
88	0.05	0.07	0.64
89	0.05	0.06	0.64
90	0.05	0.02	0.64
91	0.05	0.03	0.64
92	0.05	0.03	0.64
93	0.06	0.05	0.64
94	0.04	0.03	0.64
95	0.04	0.04	0.64

96	0.04	0.04	0.64
97	0.06	0.03	0.64
98	0.05	0.05	0.64
99	0.02	0.01	0.64
100	0.03	0.01	0.64
101	0.02	0.01	0.64
102	0.06	0.05	0.64
103	0.06	0.07	0.64
104	0.06	0.05	0.64
105	0.06	0.05	0.64
106	0.02	0.02	0.64
107	0.06	0.04	0.64
108	0.06	0.04	0.64
109	0.05	0.04	0.64

NONMEM 21 day age group data format:

#ID	AMT	Time	DV	MDV
68	850	0	0	1
68	0	4.03	303.1	0
69	850	0	0	1
69	0	3.98	182.4	0
70	850	0	0	1
70	0	0.3	408.69	0
71	950	0	0	1
71	0	0.3	253.56	0
72	800	0	0	1
72	0	0.1	14.98	0
73	1050	0	0	1
73	0	0.08	576.23	0
74	850	0	0	1
74	0	0.52	1999.86	0
75	1000	0	0	1
75	0	0.52	1244.48	0
76	900	0	0	1
76	0	1.03	2266.43	0
77	1050	0	0	1
77	0	1	2801.07	0
78	950	0	0	1
78	0	1.53	1032.63	0
79	800	0	0	1
79	0	1.52	1340.98	0
80	800	0	0	1
80	0	2.03	2722.52	0
81	950	0	0	1
81	0	2.03	390.49	0

82	950	0	0	1
82	0	3.02	1033.14	0
83	950	0	0	1
83	0	3.05	1515.82	0
84	1000	0	0	1
84	0	6.08	11	0
85	850	0	0	1
85	0	6	17.31	0
86	850	0	0	1
86	0	8	7.71	0
87	1050	0	0	1
87	0	8	0.98	0
112	950	0	0	1
112	0	4.05	632.89	0
113	950	0	0	1
113	0	4.05	617.23	0
114	750	0	0	1
114	0	0.27	293.47	0
115	1100	0	0	1
115	0	0.25	867.06	0
116	1000	0	0	1
116	0	0.1	18.62	0
117	1050	0	0	1
117	0	0.08	37.8	0
118	1050	0	0	1
118	0	0.53	1033.25	0
119	950	0	0	1
119	0	0.55	1920.13	0
120	1050	0	0	1
120	0	1.02	2414.99	0
121	850	0	0	1

121	0	1.03	1115.75	0
122	700	0	0	1
122	0	1.3	738.61	0
123	1100	0	0	1
123	0	1.52	2265.75	0
124	950	0	0	1
124	0	1.57	2888.92	0
125	950	0	0	1
125	0	1.98	1353.6	0
126	800	0	0	1
126	0	2.02	987.94	0
127	600	0	0	1
127	0	3.05	355.97	0
128	750	0	0	1
128	0	3.1	1771.65	0
129	700	0	0	1
129	0	6.05	1.23	0
130	900	0	0	1
130	0	6.05	18.29	0
131	950	0	0	1
131	0	8	4.1	0
132	750	0	0	1
132	0	8	15.14	0

NONMEM Control File for 21 day age group:

```
; Created by MAGGIE ABBASSI
$PROBLEM POPULATION DATA ANALYSIS OF MIDAZOLAM
$INPUT  ID AMT TIME DV MDV ; FITTING OF DESTRUCTIVE SAMPLING IN 21 DAY OLDS
        ; DATE CONTROL FILE CREATED 06-1-07
        ; INITIAL ESTIMATE OF CL AND KA OBTAINED FROM winNonlin ANALYSIS
;-------------------------
;  UNITS              |
;  Time - hours       |
;  Dose - mcg         |
;  Cp  - ng/ml = mcg/L  |
;  Clearances/F - L/hour |
;  Volumes/F - L      |
;-------------------------
$DATA  MIDAZOLAM.21DAY2.DATA.TXT IGNORE= #
$SUBROUTINES ADVAN2 TRANS2
$PK

    TVCL  =THETA(1)
    CL    = TVCL*EXP(ETA(1))
    TVV  = THETA(2)
    V   = TVV*EXP(ETA(2))
    CLRA  = 0
    TVKA=THETA(3)
    KA=THETA(3)

    S2    = V
    K     = CL  / V ;
```

```
    DUM   = 0
$ERROR IPRED= F
    Y     = IPRED*(1+ERR(1))
    IRES  = DV - F



$THETA
 (0, 0.169,10) ;[CL]
 (0, 0.158 ,10) ;[V]
 (0, 0.357, 1);[KA]

$OMEGA
 (0.1)
 (0.1)
$SIG
 0.3 FIX

$ESTIMATION METHOD=1 PRINT=1 MAXEVAL=9999 NOABORT
SIGDIGITS=3 POSTHOC
      MSFO=msfo.outputfile

$COVA
$TABLE ID  AMT  TIME  IPRED  IRES
    NOPRINT ONEHEADER FILE=table.txt
$TABLE ID  CL   V  KA
    FIRSTONLY NOPRINT ONEHEADER NOAPPEND FILE=etatable.txt
```

NONMEM output for 21 day age group:

ID	TIME	IPRE	IRES	Observed	PRED	RES	WRES
73	0.08	420.45	155.78	576.23	375.09	201.14	0.88
117	0.08	319.80	-282.00	37.80	375.09	-337.29	-1.47
72	0.10	295.91	-280.93	14.98	349.41	-334.43	-1.57
116	0.10	369.88	-351.26	18.62	436.76	-418.14	-1.57
115	0.25	991.45	-124.39	867.06	1017.40	-150.36	-0.24
114	0.27	663.89	-370.42	293.47	732.81	-439.34	-0.99
70	0.30	816.44	-407.75	408.69	892.71	-484.02	-0.90
71	0.30	884.90	-631.34	253.56	997.73	-744.17	-1.24
74	0.52	1382.20	617.66	1999.90	1213.80	786.10	1.07
75	0.52	1394.80	-150.29	1244.50	1428.00	-183.47	-0.21
118	0.53	1427.40	-394.16	1033.30	1511.40	-478.18	-0.52
119	0.55	1497.20	422.97	1920.10	1388.10	531.99	0.63
77	1.00	2043.40	757.64	2801.10	1700.60	1100.40	1.00
120	1.02	1917.90	497.13	2415.00	1697.00	718.00	0.65
76	1.03	1709.90	556.54	2266.40	1452.80	813.63	0.86
121	1.03	1297.30	-181.51	1115.80	1372.10	-256.34	-0.29
122	1.30	943.30	-204.69	738.61	1061.00	-322.37	-0.44
79	1.52	1216.70	124.30	1341.00	1114.80	226.21	0.28
123	1.52	1872.20	393.52	2265.80	1532.80	732.94	0.65
78	1.53	1195.20	-162.57	1032.60	1318.00	-285.40	-0.30
124	1.57	2116.50	772.43	2888.90	1294.70	1594.20	1.66
125	1.98	1225.10	128.54	1353.60	1044.80	308.76	0.36
126	2.02	934.04	53.90	987.94	859.46	128.48	0.18
80	2.03	2158.60	563.91	2722.50	854.39	1868.10	2.63
81	2.03	711.22	-320.73	390.49	1014.60	-624.09	-0.74
82	3.02	946.53	86.61	1033.10	517.66	515.48	0.96
83	3.05	1388.80	127.04	1515.80	506.20	1009.60	1.90
127	3.05	346.77	9.20	355.97	319.71	36.26	0.11

128	3.10	1642.00	129.65	1771.70	384.93	1386.70	3.40
69	3.98	190.73	-8.33	182.40	218.35	-35.95	-0.13
68	4.03	290.93	12.17	303.10	209.62	93.48	0.36
112	4.05	607.56	25.33	632.89	230.48	402.41	1.40
113	4.05	592.24	24.99	617.23	230.48	386.75	1.34
85	6.00	23.31	-6.00	17.31	38.97	-21.66	-0.36
129	6.05	13.56	-12.33	1.23	30.71	-29.48	-0.61
130	6.05	24.00	-5.71	18.29	39.48	-21.19	-0.34
84	6.08	21.90	-10.90	11.00	42.72	-31.72	-0.47
86	8.00	7.63	0.08	7.71	6.51	1.20	0.10
87	8.00	3.63	-2.65	0.98	8.04	-7.06	-0.49
131	8.00	4.76	-0.66	4.10	7.27	-3.17	-0.24
132	8.00	14.99	0.15	15.14	5.74	9.40	0.92

NONMEM: Clearance (L/hr), V (L), Ka for 21 day age group:

ID	CL	V	KA
68	0.21	0.19	0.92684
69	0.26	0.19	0.92684
70	0.26	0.21	0.92684
71	0.27	0.22	0.92684
72	0.25	0.23	0.92684
73	0.24	0.17	0.92684
74	0.21	0.17	0.92684
75	0.25	0.19	0.92684
76	0.19	0.18	0.92684
77	0.19	0.18	0.92684
78	0.27	0.19	0.92684
79	0.22	0.19	0.92684
80	0.09	0.16	0.92684
81	0.33	0.19	0.92684
82	0.16	0.20	0.92684
83	0.12	0.19	0.92684
84	0.32	0.18	0.92684
85	0.30	0.18	0.92684
86	0.23	0.19	0.92684
87	0.33	0.18	0.92684
112	0.15	0.20	0.92684
113	0.15	0.20	0.92684
114	0.26	0.21	0.92684
115	0.25	0.20	0.92684
116	0.25	0.23	0.92684
117	0.25	0.22	0.92684
118	0.27	0.20	0.92684
119	0.22	0.18	0.92684

120	0.20	0.18	0.92684
121	0.27	0.19	0.92684
122	0.28	0.19	0.92684
123	0.19	0.19	0.92684
124	0.13	0.17	0.92684
125	0.21	0.19	0.92684
126	0.23	0.19	0.92684
127	0.23	0.19	0.92684
128	0.08	0.18	0.92684
129	0.34	0.18	0.92684
130	0.30	0.18	0.92684
131	0.28	0.19	0.92684
132	0.19	0.20	0.92684

NONMEM 112 day age group data format:

#ID	AMT	Time	DV	MDV
2	7500	0	0	1
2	0	0.166667	168.2494	0
3	7000	0	0	1
3	0	0.333333	194.935	0
4	7000	0	0	1
4	0	0.5	230.8778	0
5	7500	0	0	1
5	0	1	193.6514	0
6	7500	0	0	1
6	0	1.5	22.08481	0
7	7500	0	0	1
7	0	2	48.30726	0
8	7500	0	0	1
8	0	3	2.12	0
9	7500	0	0	1
9	0	4	5.674165	0
10	7500	0	0	1
10	0	6	20.82152	0
12	7500	0	0	1
12	0	0.166667	98.10196	0
13	8500	0	0	1
13	0	0.333333	36.23911	0
14	8500	0	0	1
14	0	0.5	254.0732	0
15	7500	0	0	1
15	0	1	68.97136	0
16	7500	0	0	1
16	0	1.5	204.6975	0

17	8500	0	0	1
17	0	2	33.68613	0
18	7500	0	0	1
18	0	3	7.790713	0
19	7500	0	0	1
19	0	4	5.176053	0
20	7000	0	0	1
20	0	6	26.65926	0
21	7500	0	0	1
21	0	2	158.3851	0
23	7500	0	0	1
23	0	0.166667	90.69091	0
24	7500	0	0	1
24	0	0.333333	192.5114	0
25	7500	0	0	1
25	0	0.5	284.6366	0
26	7500	0	0	1
26	0	1	244.9198	0
27	7500	0	0	1
27	0	1.5	33.24913	0
28	7000	0	0	1
28	0	2	69.69412	0
29	7000	0	0	1
29	0	3	13.77647	0
30	7000	0	0	1
30	0	4	3.36	0
31	7000	0	0	1
31	0	6	12.36589	0
33	7500	0	0	1
33	0	0.166667	49.51896	0
34	7500	0	0	1

34	0	0.333333	722.4294	0
35	8000	0	0	1
35	0	0.5	353.8323	0
36	7500	0	0	1
36	0	1	51.95315	0
37	7500	0	0	1
37	0	1.5	91.89146	0
38	7500	0	0	1
38	0	2	18.99279	0
39	7500	0	0	1
39	0	3	9.252923	0
40	8000	0	0	1
40	0	4	5.08	0
41	7650	0	0	1
41	0	6	25.40474	0

NONMEM Control File for 112 day age group:

```
; Created by MAGGIE ABBASSI
$PROBLEM POPULATION DATA ANALYSIS OF MIDAZOLAM
$INPUT  ID AMT TIME DV MDV ; FITTING OF DESTRUCTIVE SAMPLING IN 112 DAY OLDS
        ; DATE CONTROL FILE CREATED 06-1-07
        ; INITIAL ESTIMATE OF CL AND KA OBTAINED FROM winNonlin ANALYSIS
;-------------------------
;  UNITS              |
;  Time - hours       |
;  Dose - mcg         |
;  Cp  - ng/ml = mcg/L  |
;  Clearances/F - L/hour |
;  Volumes/F - L      |
;-------------------------
$DATA  MIDAZOLAM.112DAY2.DATA.TXT IGNORE= #
$SUBROUTINES ADVAN2 TRANS2
$PK

    TVCL  =THETA(1)
    CL    = TVCL*EXP(ETA(1))
    TVV  = THETA(2)
    V   = TVV*EXP(ETA(2))
    CLRA  = 0
    TVKA=THETA(3)
    KA=THETA(3)

    S2    = V
    K     = CL  / V ;
```

```
    DUM   = 0
$ERROR IPRED= F
    Y     = IPRED*(1+ERR(1))
    IRES  = DV - F



$THETA
 (0, 17.57,100) ;[CL]
 (0, 28.47 ,100) ;[V]
 (0, 0.357, 10);[KA]

$OMEGA
 (0.1)
 (0.1)
$SIG
 0.3 FIX

$ESTIMATION METHOD=1 PRINT=1 MAXEVAL=9999 NOABORT
SIGDIGITS=3 POSTHOC
      MSFO=msfo.outputfile

$COVA
$TABLE ID  AMT  TIME  IPRED  IRES
    NOPRINT ONEHEADER FILE=table.txt
$TABLE ID  CL   V  KA
    FIRSTONLY NOPRINT ONEHEADER NOAPPEND FILE=etatable.txt
```

NONMEM output for 112 day age group:

ID	TIME	IPRE	IRES	DV	PRED	RES	WRES
2	0.17	152.91	15.34	168.25	129.72	38.53	0.36
12	0.17	113.80	-15.69	98.10	129.72	-31.62	-0.30
23	0.17	110.46	-19.77	90.69	129.72	-39.03	-0.37
33	0.17	94.40	-44.89	49.52	129.72	-80.20	-0.76
3	0.33	180.80	14.13	194.93	165.63	29.30	0.23
13	0.33	138.23	-101.99	36.24	201.13	-164.89	-1.07
24	0.33	185.05	7.46	192.51	177.46	15.05	0.11
34	0.33	623.42	99.01	722.43	177.46	544.97	4.00
4	0.50	200.03	30.85	230.88	172.99	57.89	0.46
14	0.50	229.99	24.08	254.07	210.06	44.01	0.29
25	0.50	234.05	50.58	284.64	185.35	99.29	0.74
35	0.50	279.17	74.67	353.83	197.71	156.13	1.09
5	1.00	158.32	35.34	193.65	137.77	55.88	0.61
15	1.00	118.63	-49.65	68.97	137.77	-68.80	-0.75
26	1.00	182.66	62.27	244.92	137.77	107.15	1.17
36	1.00	114.56	-62.61	51.95	137.77	-85.82	-0.94
6	1.50	51.03	-28.95	22.09	86.32	-64.23	-0.95
16	1.50	156.76	47.94	204.70	86.32	118.38	1.75
27	1.50	57.16	-23.91	33.25	86.32	-53.07	-0.78
37	1.50	89.16	2.73	91.89	86.32	5.58	0.08
7	2.00	49.36	-1.05	48.31	52.20	-3.89	-0.07
17	2.00	40.82	-7.13	33.69	59.16	-25.47	-0.41
21	2.00	133.94	24.45	158.39	52.20	106.19	1.93
28	2.00	63.85	5.85	69.69	48.72	20.98	0.41
38	2.00	28.91	-9.92	18.99	52.20	-33.21	-0.60
8	3.00	5.75	-3.63	2.12	18.74	-16.62	-0.50
18	3.00	9.45	-1.66	7.79	18.74	-10.95	-0.33
29	3.00	14.18	-0.41	13.78	17.49	-3.71	-0.12

39	3.00	10.58	-1.33	9.25	18.74	-9.49	-0.29
9	4.00	5.73	-0.05	5.67	6.71	-1.03	-0.06
19	4.00	5.26	-0.09	5.18	6.71	-1.53	-0.09
30	4.00	3.58	-0.22	3.36	6.26	-2.90	-0.18
40	4.00	5.21	-0.13	5.08	7.15	-2.07	-0.11
10	6.00	20.81	0.01	20.82	0.86	19.96	5.57
20	6.00	26.65	0.01	26.66	0.80	25.86	7.74
31	6.00	12.36	0.01	12.37	0.80	11.56	3.46
41	6.00	25.39	0.01	25.41	0.88	24.53	6.72

NONMEM: Clearance (L/kg), V (L), Ka for 112 day age group:

ID	CL	V	KA
2	25.41	20.83	3.86
3	25.32	22.45	3.86
4	24.40	20.60	3.86
5	22.21	22.09	3.86
6	33.21	19.34	3.86
7	26.28	24.45	3.86
8	30.31	19.15	3.86
9	26.17	24.25	3.86
10	15.92	42.78	3.86
12	25.83	28.82	3.86
13	26.51	38.87	3.86
14	24.93	22.15	3.86
15	30.28	27.73	3.86
16	14.22	20.02	3.86
17	29.07	21.67	3.86
18	28.62	21.18	3.86
19	26.42	23.87	3.86
20	13.78	44.45	3.86
21	11.93	21.92	3.86
23	25.86	29.76	3.86
24	25.52	23.74	3.86
25	23.43	18.33	3.86
26	19.18	19.32	3.86
27	32.00	20.48	3.86
28	22.14	27.18	3.86
29	26.69	23.66	3.86
30	27.31	22.59	3.86
31	18.29	39.19	3.86

33	25.98	35.24	3.86
34	13.77	5.33	3.86
35	22.03	15.85	3.86
36	31.56	28.02	3.86
37	25.01	25.18	3.86
38	30.62	20.05	3.86
39	28.20	21.72	3.86
40	26.64	23.55	3.86
41	14.76	43.91	3.86

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