

**Ontogeny of Rat CYP2E1 and CYP1A2: A Characterization and a  
Pharmacokinetic Model**

A Thesis

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## ABSTRACT

Infantile exposure to xenobiotics, e.g. from breastfeeding, poses a serious toxicity risk. Since the toxicokinetic mechanisms that principally determine exposure outcomes undergo a significant developmental maturation, infants may respond to exposures in a different way than adults. Hence, suitable model systems are required to provide risk relevant information in pediatric populations. This dissertation's primary goal was to provide a critical evaluation of two such model systems; first, a pharmacokinetic model that may predict an infant's capacity to eliminate toxicants by cytochrome P-450 (CYP) mechanisms and second, the developing rat as a model of human CYP2E1 and CYP1A2 ontogeny.

The first objective was to evaluate underlying assumptions of a pharmacokinetic model that describes the ontogeny of hepatic CYP activity using the rat. The study recognized some discrepancies with the stated assumptions. The impact of these discrepancies on the potential applicability of the model is discussed. As proof-of-concept, the observed data were fit to a model describing rat CYP2E1 and CYP1A2 ontogeny. A reasonable correlation ( $r = 0.75$ ) was observed between observed and predicted oral clearance values of a CYP2E1 substrate indicating the potential applicability of such a model in risk assessment.

The second objective was to conduct an extensive characterization of rat hepatic CYP2E1 and CYP1A2 ontogeny at mRNA, protein, activity and intrahepatic expression levels. The results were compared to available human data to determine the appropriateness of the rat for assessment of toxicokinetic mechanisms underlying age-dependent differences in susceptibility to toxicity. Similarities in age-dependent changes in mRNA, activity and zonal hepatic expression patterns were noted between the rat and human prior to weaning. Unlike human data, rats show good correlation between changes in CYP2E1 and CYP1A2 activity and transcript levels, but not with the immunoquantifiable protein. Recognizing such similarities and differences between rats and human regarding onset, rate and pattern of CYP ontogeny will

improve the accuracy of rat-to-human extrapolation of developmental toxicokinetic data.

Overall, the dissertation research provides mounting and supportive evidence for the use of such model systems in providing risk-relevant information in pediatric populations and to identify toxicokinetic mechanisms underlying age-dependent differences in susceptibility to toxicity.



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## LIST OF ABBREVIATIONS, SYMBOLS AND ACRONYMS

$\pm$	plus or minus
$^{\circ}\text{C}$	degree Celsius
AAG	alpha <sub>1</sub> -acid glycoprotein
ADME	Absorption, distribution, metabolism and elimination
AhR	aryl hydrocarbon receptor
AIC	akaike information criterion
AUC <sub>∞</sub>	area under the serum concentration-time curve
ARNT	aryl hydrocarbon receptor nuclear translocator
BW	body weight
BrW	brain weight
CAR	constitutive androstane receptor
CDNB	1-chloro-2,4 dinitrochlorobenzene
CI	confidence interval
Cl/F	oral clearance
Cl <sub>H</sub>	hepatic clearance
Cl <sub>int</sub>	intrinsic clearance
Cl <sub>S,Inf</sub>	infant systemic clearance
Cl <sub>S,Mat</sub>	maternal systemic clearance
C <sub>max</sub>	maximum serum concentration
C <sub>SS,Inf</sub>	infant steady state serum concentration
C <sub>SS,Mat</sub>	maternal steady state serum concentration
CYP	cytochrome P450
CZX	chlorzoxazone
DAB	3,3' diaminobenzidine hydrochloride
DME	drug metabolizing enzymes
Dose <sub>Inf</sub>	drug dose given to the infant
EDTA	ethylenediamine tetra acetate
EI <sub>(Conc)</sub>	exposure index related to maternal serum concentration
EI <sub>(Dose)</sub>	exposure index related to maternal dose
E <sub>H</sub>	hepatic extraction ratio
EPA	US environment protection agency
ER	endoplasmic reticulum
EROD	ethoxyresorufin dealkylase
F	bioavailability
FDA	US Food and Drug Administration
FFA	free fatty acids
4-NC	p-nitrocatechol
f <sub>u</sub>	fraction of the drug unbound in plasma
GH	growth hormone
GIT	gastrointestinal tract
GSH	glutathione

GST	glutathione-S-transferases
HAAs	heterocyclic aromatic amine
HC	Health Canada
HNF	hepatocyte nuclear factor
HPLC	High Pressure Liquid Chromatography
HSF	hepatic scaling factor
IHC	immunohistochemistry
ISF	infant scaling factor
ISF <sub>D</sub>	a fitted parameter for the model equation
IV	intravenous
k	first-order rate constant
K <sub>M</sub>	Michaelis-Menten constant
λ <sub>z</sub>	terminal elimination rate constant
LOD	limit of detection
LOQ	limit of quantitation
LW	liver weight
MC	3-methylcholanthrene
MeIQx	2-amino-3,8-dimethylimidazole[4.5]quinoxaline
mg	milligram(s)
min	minute(s)
μL	microliter(s)
mL	milliliter(s)
μm	micrometer(s)
mm	millimeter(s)
mM	millimole(s) per liter
MLP	maximum lifespan potential
MP	microsomal protein
MR	methoxyresorufin
MROD	methoxyresorufin-O-dealkylase
(M/S)	milk to serum ratio
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NAPQI	N-acetyl-p-benzoquinoneimine
NAT	N-acetyl transferases
NF	nuclear factor
nM	nanomole(s) per liter
NSAIDs	non-steroidal anti-inflammatory drugs
OSF	ontogeny scaling factor
PAGE	polyacrylamide gel electrophoresis
PAHs	polycyclic aromatic hydrocarbons
PAPS	3'-phosphoadenosine-5'-sulphophosphate
PBPK	physiologically-based pharmacokinetic
PBS	phosphate buffered saline
PBS <sub>t</sub>	phosphate buffered saline with 0.05 % tween 20
PD	postnatal day
pH	negative logarithm of hydrogen ions to the base 10
PK	pharmacokinetics



PNP	$\rho$ -nitrophenol
POD	phenacetin-O-dealkylase
PP	periportal
psi	pounds per square inch
PV	perivenous
PVDF	polyvinylidene difluoride
PXR	pregnane-X-receptor
QC	quality control
$Q_H$	hepatic blood flow
$r^2$	coefficient of determination
RHSF	relative hepatic scaling factor
RSD	relative standard deviation
RT-PCR	reverse transcription polymerase chain reaction
SD	standard deviation
SDS	sodium dodecyl sulfate
SULT	sulfotransferases
$\tau$	infant nursing interval
$t_{1/2}$	Elimination half life
TCDD	2,3,7,8 tetrachlorodibenzo-p-dioxin
$t_{max}$	time to reach maximum serum concentration
UDP	uridine 5'diphosphate
UGTs	uridine 5'diphosphate glucuronosyl-transferases
UMB	umbelliferone
UV	ultraviolet
$V_d$	volume of distribution
$V_{d,ss}$	apparent volume of distribution at steady-state
$V_{max}$	maximal enzyme activity
$V_{Milk}$	volume of milk ingested by infant

# **1. LITERATURE REVIEW**

## **I. INTRODUCTION**

Epidemiological research provides convincing evidence that breastfeeding is advantageous to the breast-fed infant, lactating mother and society. However, the ability of most drugs and chemicals to transfer from maternal plasma to breast milk puts the suckling infant under an uncertain exposure risk to harmful xenobiotics and raises concerns about breastfeeding safety. When a nursing mother is exposed to a xenobiotic(s), either from the environment or through drug therapy, a decision to discontinue the exposure (e.g. stop medication) or discontinue breast feeding is largely based on data regarding the ability of the drug to appear in breast milk, the amount transferred to the suckling infant and, more importantly, the ability of the suckling infant to handle such an exposure. Despite the growing information about the ability of chemicals to transfer into breast milk and the numerous methods developed to estimate the amount exposed to the suckling infant, very little is known regarding the ability of the infant to deal with the exposed dose. Following an external exposure dose, pharmacokinetic processes determine the concentration of a xenobiotic in the biophase. In many instances it is the capacity of the detoxification mechanisms to eliminate a xenobiotic that poses as the major underlying cause for a toxic outcome following an exposure.

Detoxification systems undergo significant maturation during postnatal development. In human paediatric populations, such age-related differences in elimination mechanisms are generally poorly understood. However, for a given dose, the rate and pattern of the ontogeny of elimination processes will alter the toxicological significance of a xenobiotic in an age-dependent manner and such developmental maturation will confound the extrapolation of toxicity data obtained in adults to the

developing infant. Although the recent establishment of the Children's PK Database (available at <http://www2.clarku.edu/faculty/dhattis>), a database presently under construction to identify differences in pharmacokinetics during development, facilitates extrapolation of risks between adults and infants, this database still falls short of providing sufficient information for the adequate extrapolation of toxicity data, particularly for environmental toxicants.

Interspecies extrapolation of toxicity studies performed with the developing animal is one potential approach proposed to elucidate the impact of the maturation of toxicokinetic processes on age-dependent differences in susceptibility to toxicity and to allow inclusion of risk-relevant information on the developing animal into risk assessment models. An animal model that can, at the very least, identify an underlying toxicokinetic mechanism for age-dependent differences in susceptibility to toxicity has great value in risk assessment. However, because of the interrelationship between genetic and environmental factors that influence the pattern and rate of xenobiotic elimination, a perfect animal model is unlikely to be found. Relevant differences will always exist between human and animal models and these differences will pose serious challenges for qualitative and quantitative predictions of human toxicological outcomes. Translating animal data to humans requires an understanding of how and where these systems are similar and different to evaluate their impact on extrapolation to human exposure risks.

This dissertation takes a systems level approach (i.e. evaluation of mRNA, protein and function) in the evaluation of two detoxification mechanisms, Cytochrome P450 (CYP) 2E1 and CYP1A2. This evaluation will provide critical descriptive information necessary to known information on human CYP ontogeny and to draw comparisons that determine the appropriateness of the developmental animal model system for assessment of toxicokinetic mechanisms underlying age-dependent differences in susceptibility to toxicity. Furthermore, this dissertation evaluates critical assumptions of a pharmacokinetic model that describes the ontogeny of CYP enzymes for its application as a risk assessment tool when infants are exposed to xenobiotics present in breast milk. Literature to date indicates that CYP enzymes in humans are regulated in a similar manner to rodents<sup>1</sup>. Therefore rodents are frequently used to provide initial information

about xenobiotic elimination in humans. Accordingly, a rat model system was used to evaluate the underlying assumptions of a pharmacokinetic ontogeny model of CYP enzyme-mediated clearance. Furthermore, rat and human CYP2E1 and CYP1A2 enzymes exhibit high homology and similar substrate profiles<sup>2-4</sup>. This could allow use of the rat as an appropriate model to study the developmental expression and function of human CYP2E1 and CYP1A2.

For the purpose of this literature review, the term infant encompasses ages from birth to 2 years and children from 2 years to 12 years of age in humans. However, the term neonate includes birth to postnatal day 21, weanling indicates postnatal days 21 to 28/35 and juvenile encompasses age from postnatal days 28/35 to 49/70 days for rat.

## **II. BACKGROUND**

### **1. Breastfeeding and Infant Exposure to Chemicals**

#### **1.1 Breastfeeding and its Benefits**

Extensive epidemiological research has identified diverse and compelling advantages of breastfeeding for infants, mothers, families, and societies and the use of human milk for infant feeding<sup>5</sup>. These advantages include health, social, environmental, and economic benefits<sup>5</sup>. Thus, current guidelines set out by international (i.e. World Health Organization and United Nations Children's Fund) and national (i.e. Canadian Pediatric Society, Health Canada and Dietitians of Canada) organizations recommended that infants be exclusively breastfed for at least the first six months of life, and continue breastfeeding with complementary foods for up to two years of age and beyond<sup>6-8</sup>. Surveys in Canada and United States report that approximately 70% of women initiate breastfeeding and 30% continue to breastfeed their infants to 6 months of age<sup>5</sup>.

The unique components of human milk make it superior for infant feeding, and differs markedly from all substitute feeding preparations<sup>9</sup>. The nutrients, enzymes, growth factors, hormones and immunological and anti-inflammatory properties of

human milk contribute to reductions in the incidence and severity of acute infectious diseases such as sepsis/meningitis<sup>10</sup>, otitis media<sup>11</sup>, respiratory tract infections<sup>12</sup>, gastroenteritis<sup>11</sup> and necrotizing enterocolitis<sup>13</sup>. As well, studies correlate breastfeeding with reduced incidences of sudden infant death syndrome<sup>14,15</sup>, Crohn's disease, insulin-dependent diabetes, lymphoma and allergic diseases<sup>13,15,16</sup> and reduced prevalence of childhood obesity<sup>17,18</sup>. Furthermore, breastfeeding may enhance performance on tests of cognitive developments and achievement of better academic scores<sup>19,20</sup>. Such health benefits may increase with duration and exclusivity of breastfeeding<sup>21</sup>.

Breastfeeding and lactation have many important benefits for nursing women. These benefits include decreased postpartum bleeding and more rapid uterine involution<sup>22</sup>, reduced risk of epithelial ovarian cancer<sup>23</sup> and breast cancer<sup>24,25</sup>, reduced risk of hip fracture in old age<sup>26,27</sup> and faster return to the pre-pregnancy weight<sup>28</sup>. The act of breastfeeding delays resumption of ovulation and increases child spacing<sup>29</sup>. Finally, breastfeeding is associated with increased maternal-infant bonding and maternal sense of worth<sup>5</sup>.

Breastfeeding has important social, economic and environmental benefits. These benefits include reduced health care costs due to reduced risk of infant illness and duration of hospitalization<sup>11,13</sup>. Also breastfeeding is associated with increased family saving due to the greater cost of formula feeding compared to breastfeeding cost<sup>11</sup> in addition to lower parental absence from work, and reduced environmental load for disposing formula cans and bottles<sup>5</sup>. Some countries, including Canada, have recognized the potential economical savings from breastfeeding and provide a subsidy to low income mothers for each month of breastfeeding<sup>11</sup>.

## **1.2 Infant Exposure to Drugs and Chemicals in Breast Milk**

Almost all maternally administered drugs and environmental chemicals appear in breast milk and find their way into the breast-fed infant<sup>30-33</sup>. Consequently, breast milk constitutes a major exposure route to chemicals for nursing infants. Governmental and regulatory agencies have become gravely concerned about the presence of chemicals in breast milk, particularly in light of recent surveys that suggest approximately 90% of

women take at least one medication, more than 20% take at least two or more medications in the first week after delivery and more than 5% of nursing mothers receive long-term drug therapy<sup>30,34</sup> (Table 1.1).

**Table 1.1-** Example of maternally-administered medications that may have possible effects on the nursing infant. Adapted from references (37-39)

<b>Drug</b>	<b>Reported or possible Effects on the Nursing Infant</b>
<b>1. Cytotoxic Drugs</b>	
Cyclosporine	Possible immune suppression, carcinogenesis
Doxorubicin	Possible immune suppression, carcinogenesis
Methotrexate	Possible immune suppression, carcinogenesis
<b>2. Drugs of Abuse</b>	
Amphetamine	Irritability, poor sleeping pattern
Cocaine	Intoxication, irritability, seizures, diarrhea
Heroin	Tremors, restlessness, poor feeding
<b>3. Cardiovascular Drugs</b>	
Acebutolol	Hypotension, bradycardia, tachypnea
Atenolol	Cyanosis, bradycardia
Amiodarone	Possible hypothyroidism
<b>4. Antipsychotics</b>	
Chlorpromazine	Drowsiness, decline in developmental scores, lethargy
Haloperidol	decline in developmental scores
<b>5. Antibiotics</b>	
Amoxicillin	None
Nalidixic acid	Hemolysis in infants with glucose-6-phosphate-dehydrogenase (G-6-PD) deficiency.
Nitrofurantoin	Hemolysis in infants with (G-6-PD) deficiency.
Tetracycline	Negligible absorption by infant
<b>6. Others</b>	
Phenobarbital	Sedation, infantile spasms
Sulfasalazine	Bloody diarrhea
Caffeine	Irritability, poor sleeping pattern, slower elimination

This table shows examples of these drugs that are usually taken by the nursing mother and could result in adverse effects to the developing infant.

These concerns have translated into recommendations to pharmaceutical companies to provide data about drug excretion in breast milk as a part of drug development and labeling<sup>35</sup>. Adherence to such recommendations will provide necessary and critical information about compounds newly introduced to the market. Nevertheless, the distribution of most other chemicals into breast milk and their actual milk levels remain largely unknown. Additionally, a recent FDA Task Force identified a need to advance research into mechanisms governing transfer of chemicals into milk and the factors influencing their milk levels<sup>36</sup>. Identification of such factors is critical in the determination of potential exposure doses and risk to the nursing infant<sup>16,31</sup>.

When maternal medication is necessary during lactation, breastfeeding parents and healthcare professionals are mainly concerned about infant exposure to the drug and how this may affect infant health in the long and short term<sup>16</sup>. Although many medications are considered compatible with breastfeeding<sup>37-39</sup>, recommendations are usually made to either discontinue breastfeeding or stop maternal therapy for fear of exposing an infant to drugs in mother's milk<sup>40</sup>. The American Academy of Pediatrics in its 2001 policy statement<sup>37</sup> reported lists of agents that transfer into breast milk and described their possible adverse effects on infants or on lactation. These lists should assist the physician in counseling a nursing mother regarding breastfeeding when the mother has a condition for which a drug is medically indicated.

Risk/benefit analyses facilitate decisions regarding breastfeeding during maternal medication. Such analyses must weigh the benefits (breastfeeding to infant and treatment of maternal illness) against the potential risks (infant exposure and withholding medication to the mother) to both the mother and the infant. Researchers have defined a number of different indices of exposure to provide assessments of risk (see section 13). Until recently, such exposure risk indices were based principally upon estimates of the amount of drug presented to the infant from breast milk<sup>41-43</sup>. Normally, drug dose presented to the infant from breast milk is compared to the normal paediatric dose rate, or to the maternal dose (relative to body weight) to assess the potential risk of

exposure to the infant<sup>43</sup>. However, even low level exposures may result in potential adverse effects when a nursing infant lacks the capacity to efficiently eliminate the exposed dose. Exposure risk indices, then, must make provision for an infant's capacity to eliminate compounds, i.e. these indices must incorporate infant systemic clearance as a critical factor defining exposure risk. This requirement presents a serious problem in risk assessment because we have limited knowledge of infant systemic clearance<sup>44-46</sup>.

### **1.3 Assessment of Infant Exposure Risk**

The literature reports numerous exposure indices used to predict and assess the potential risk of toxicity to the suckling infant following exposure to drugs from breast milk<sup>16,43,47-49</sup>. These indices consider duration of exposure (acute versus chronic) and include milk to serum ratio (M/S), infant dose relative to maternal dose, infant steady state serum concentration ( $C_{ss, \text{inf}}$ ), exposure index related to maternal dose ( $EI_{(\text{Dose})}$ ) and exposure index related to maternal serum concentration ( $EI_{(\text{Conc})}$ ).

Maternal dose and maternal systemic clearance determine the maternal plasma steady-state concentration which, in turn, acts as the driving force for drug concentration in mammary epithelial cells and ultimately, drug concentration in milk. The infant ingests a dose while nursing and the resulting serum concentration is a function of the pharmacokinetic processes absorption, distribution, biotransformation and elimination<sup>16</sup>. These processes undergo developmental changes as the infant grows<sup>50</sup>. A better risk assessment index will take into consideration all maternal and neonatal factors affecting the amount of drug presented to the suckling infant via milk and can predict the potential risk posed to the infant following exposure to this amount.

#### **1.3.1 Duration of Exposure (Acute versus Chronic)**

Infant exposure risk to drugs present in the breast milk depends upon factors associated with the maternal dosage situation such as maternal dose, dosing frequency, duration of therapy and interval between dose administration and breastfeeding. Many literature reports suggest that adverse effects, if any, of acute and short term maternal



dosage regimens are mild and pose little risk to infants<sup>51-53</sup>. In such cases, times of breastfeeding and medication administration can be manipulated to minimize any possible adverse effect on the nursing infant according to the half life of the administered drug<sup>47</sup>. For drugs with short half lives, nursing mothers are usually advised to breastfeed immediately before taking medication. Breastfeeding while on medications with long half lives requires careful consideration of the relationship between the nursing time and when maternal concentrations are greatest. In such cases breastfeeding could be interrupted, or even suspended, for a short period of time so that the infant is not exposed to milk during times of peak maternal plasma levels. The chronic or long-term drug exposure to nursing infants via breast milk poses the greatest concern<sup>37</sup>. For example, adverse effects have been observed in nursing infants following administration of cardiac glycosides drugs to nursing mothers<sup>54</sup>. As well, opiates that are commonly used as postoperative analgesics adversely affect nursing infants after maternal administration in the first week after birth<sup>55,56</sup>.

As noted above, most recommendations are based on the amount of drug transferred to the infant and duration of exposure. Even with short term exposure, the immature elimination mechanisms in the developing infant could result in drug accumulation and an increased risk of toxicity. On the other hand, with chronic exposure of the nursing infant to certain chemicals in breast milk, their detoxification mechanisms can handle the dose and consequently, it would be safe to continue both breastfeeding and maternal medication without any discontinuation or interruption.

### **1.3.2 Milk to Serum (M/S) Ratio**

The ratio between drug concentration in milk and maternal serum is called the milk-to-serum ratio (M/S). This ratio is used in several exposure indices to estimate the maternal dose transferred to the infant via breast milk<sup>41,53,56,57</sup>. On its own, (M/S) ratio indicates the extent of drug distribution into milk and was frequently used as an index of exposure such that drugs with (M/S) ratios greater than one were avoided or used with caution during breastfeeding<sup>32,33,42</sup>. Accurate and useful assessments of (M/S) ratio are

made by assaying milk and serum concentrations following maternal drug administration to steady state or assaying the area under milk and serum concentration versus time curve after a single intravenous injection<sup>33,42</sup>. This value can be affected by many factors such as maternal pharmacokinetics, mammary physiology, physicochemical properties of the drug, variations in milk composition and pH, lactation period and maternal illness<sup>16,48</sup>. The value of (M/S) ratio only provides a measure of “input” without considering the multitude of additional factors that contribute to infant exposure risk (sections 1.3.3, 1.3.4 and 1.3.5)<sup>47</sup>.

### 1.3.3 Infant Dose as a Percentage of Absolute Maternal Dose

Drug dose given to the infant during a nursing interval ( $Dose_{Inf}$ ) via breast milk may be calculated using (M/S) ratio using Equation 1.1.

$$Dose\ rate_{Inf} = C_{ss, Mat} (M/S)(V_{Milk}/\tau) \quad (1.1)$$

Where  $C_{ss, Mat}$  is the average concentration at steady state in maternal serum,  $\tau$  is the infant nursing interval and  $V_{Milk} / \tau$  is the volume of milk ingested by an infant per nursing interval, approximately 150 mL/kg/day<sup>42,48</sup>. The dose of drug ingested by the infant can then be compared with the maternal dose as a body weight adjusted dose (mg/kg) as depicted in Equation 1.2.

$$\% \text{ Maternal dose} = \left( \frac{Dose_{Inf}}{Dose_{Mat}} \right) \times 100 \quad (1.2)$$

This index normalizes the infant dose received via the breast milk to maternal dose and considers an average dosing rate with chronic exposure. An arbitrary cut-off level of predicted infant exposure of less than 10% of the weight adjusted maternal dose has been recommended<sup>43</sup>. This arbitrary level is applied to drugs that have no known inherent toxicity and taken by a mother at a normal therapeutic dose. Levels higher than 10% indicate a higher exposure risk<sup>43</sup>.

At steady state, the minimum maternal concentration occurs just before the next oral dose. Assuming milk concentration parallels serum levels, nursing the infant just before

the next dose will minimize the relative infant exposure<sup>58</sup>. While this method incorporates more variables involved in infant exposure to drugs via breast milk, the index still does not accurately assess the amount of drug which actually enters the infant's bloodstream (i.e. the bioavailable dose), nor does it predict the capacity of the infant to handle such amount.

Another way to express infant dose to maternal dose is by evaluating an  $EI_{(Dose)}$  (an exposure index related to maternal dose). This exposure index views infant dose as a percentage of maternal dose. Its relative value is inversely related to the maternal systemic clearance but proportional to maternal bioavailability ( $F_{Mat}$ ),  $(M/S)$  ratio and milk consumption rate, Equation 1.3.

$$EI_{(Dose)} = \frac{Dose_{Inf}}{Dose_{Mat}} = \frac{F_{Mat}}{Cl_{S,Mat}} \left( \frac{M}{S} \right) \left( \frac{V_{Milk}}{\tau} \right) \quad (1.3)$$

As shown in Equation 1.3, estimation of  $EI_{(Dose)}$  would require estimation of maternal bioavailability and systemic clearance in addition to  $(M/S)$  ratio and milk consumption rate. Mean values for all of these parameters can be obtained from the literature.

#### 1.3.4 Infant Steady State Serum Concentration ( $C_{ss, Inf}$ )

$M/S$  values and infant dose as a percent of absolute maternal dose may incorrectly estimate the infant exposure risk as they do not take into consideration the contribution of infant pharmacokinetic (PK) factors in the infant response to a drug exposure. These PK parameters undergo continuous changes in the developing infant<sup>44,50</sup>. Therefore a particular dose (normalized to body weight) might result in different steady state concentrations ( $C_{ss, Inf}$ ) in the developing infant depending on the age. Pharmacological or toxicological effects in the infant, in most of the cases, correlate with drug concentration at the site of action<sup>47,50,59</sup>. Because of the difficulty in measuring drug concentration at the site of action, a proportional relationship between drug concentrations in the bloodstream and at the site of action is assumed, such that serum concentration can be used as a surrogate measure to concentration at the site of action.

Steady state serum concentration in an infant following any route of drug administration can be described by Equation 1.4 in which ( $C_{ss, Inf}$ ) is a function of infant bioavailability ( $F_{Inf}$ ), infant systemic clearance ( $Cl_{s, Inf}$ ) and dose ( $Dose_{inf}$ ).

$$C_{ss, Inf} = F_{Inf} \left( \frac{Dose\ rate, Inf}{Cl_{s, Inf}} \right) \quad (1.4)$$

When the infant is exposed to a drug in breast milk, Equation 1.4 is rewritten as:

$$C_{SS, Inf} = \frac{F_{Inf}}{Cl_{S, Inf}} \left[ C_{SS, Mat} (M/S)(V_{Milk}/\tau) \right] \quad (1.5)$$

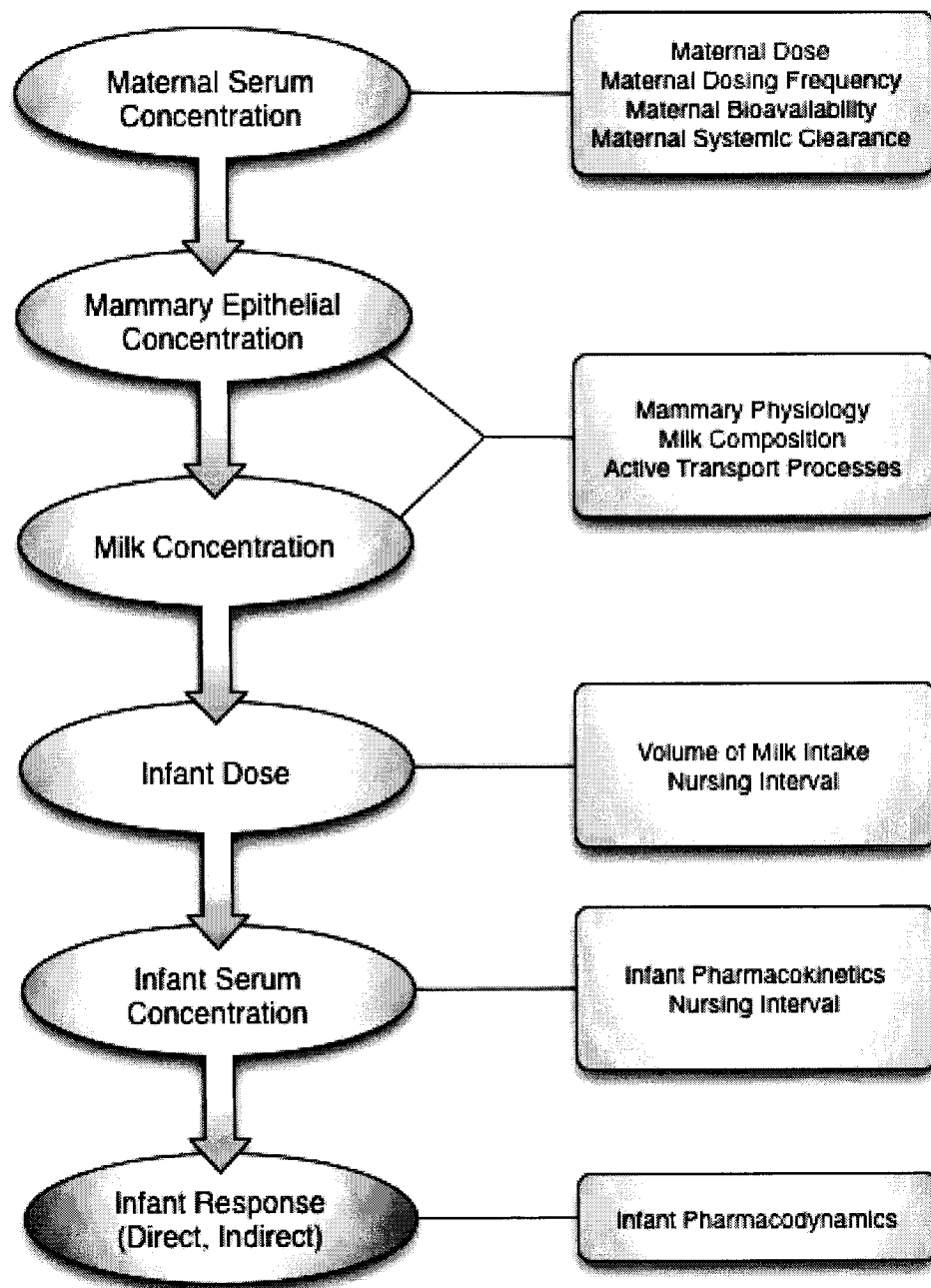
Assuming linear pharmacokinetics in the mother,  $C_{ss, Mat}$  can be described as a function of maternal bioavailability ( $F_{Mat}$ ), maternal dose ( $Dose_{Mat}$ ) and maternal systemic clearance ( $Cl_{s, Mat}$ ) as indicated in Equation 1.6.

$$C_{ss, Mat} = F_{Mat} \left( \frac{Dose\ rate, Mat}{Cl_{s, Mat}} \right) \quad (1.6)$$

Finally, by substituting the value of  $C_{ss, Mat}$  into Equation 1.5, Equation 1.7 can be derived.

$$C_{SS, Inf} = \frac{F_{Inf}}{Cl_{S, Inf}} \left[ \frac{F_{Mat} Dose_{Mat}}{Cl_{S, Mat}} (M/S)(V_{Milk}/\tau) \right] \quad (1.7)$$

Equation 1.7 provides insight into the factors that determine infant steady state serum concentration. Those factors can be divided into maternal factors (dose, bioavailability, systemic clearance and M/S ratio), infant factors (bioavailability and systemic clearance) and milk consumption rate (volume of milk and nursing interval). Figure 1.1 summarizes these factors resulting in the final infant response to a chemical present in breast milk.



**Figure 1.1-** Interrelationship between factors affecting infant exposure to drugs in breast milk and the resulting pharmacological/toxicological response.

Population estimates for maternal factors ( $F_{Mat}$ ,  $Dose_{Mat}$ ,  $Cl_{S,Mat}$ ) as well as milk consumption rate ( $V_{Milk}$  and  $\tau$ ) can be obtained from the literature<sup>42,48</sup>. (M/S) ratio can be predicted from various *in vitro* or *in vivo* models<sup>42,48,58</sup>. Consequently, infant bioavailability and infant systemic clearance remain the unknown determinants of  $C_{SS, Inf}$ . If a hundred percent infant bioavailability is assumed (the worst case-scenario for infant exposure),  $Cl_{S,Inf}$  remains the principal unknown determinant of  $C_{SS, Inf}$ . If the assumption that both the qualitative and quantitative nature of the pharmacodynamic response in the infant is the same as in adult holds true, then the resulting  $C_{SS, Inf}$  will produce the same pharmacological/toxicological outcome as observed in adults. Consequently,  $C_{SS, Inf}$  becomes an informative index of infant exposure.

### 1.3.5 Exposure Index Related to Maternal Concentration ( $EI_{(Conc)}$ )

A more recently elaborated exposure index relates the relevant exposure term  $C_{SS,Inf}$  to maternal steady state levels to give rise to  $(EI_{(Conc)})^{49}$ . Rearrangement of Equation 1.5 derives Equation 1.8, which presents the determinants of this exposure index. According to Equation 1.8, determinants of infant exposure risk are largely a function of infant PK (bioavailability and systemic clearance), the (M/S) value, and milk consumption rates.

$$EI_{(Conc)} = \frac{C_{SS,Inf}}{C_{SS,Mat}} = \frac{F_{Inf}}{Cl_{S,Inf}} \left( \frac{M}{S} \right) \left( \frac{V_{Milk}}{\tau} \right) \quad (1.8)$$

Although difficult to estimate, this exposure index is considered as the best estimate of infant exposure to chemicals present in breast milk<sup>16,47</sup>. As shown in Equation 1.8, estimation of  $(EI_{(Conc)})$  would require estimation of infant bioavailability and infant systemic clearance in addition to M/S ratio and milk consumption rate. Mean values for M/S and milk consumption rate can be obtained from the literature. Very little information is available regarding systemic clearance in infants due to logistic and ethical concerns<sup>60</sup>. Hence, understanding the mechanisms governing drug distribution into milk and developmental changes in infant PK, particularly systemic clearance, is very important for appropriate estimates of exposure level and the toxicological impact of this exposure.

## 2. Infant systemic Clearance as a Determinant of $C_{ss, Inf}$

The pharmacological/toxicological response to a drug often correlates well with the steady state serum concentration ( $C_{ss}$ ) of the drug. Because systemic clearance is a principal determinant of  $C_{ss}$  (see Equation 1.4), infant systemic clearance constitutes a critical PK parameter in the determination of the pharmacological/toxicological response to a drug from breast milk or from the environment<sup>44,47</sup>. Systemic clearance ( $Cl_S$ ) describes the efficiency of the elimination pathways to irreversibly remove drugs from the body. Although many elimination pathways contribute to the determination of  $Cl_S$ , infants eliminate drugs principally through hepatic ( $Cl_H$ ) and renal ( $Cl_R$ ) clearance mechanisms (Equation 1.9). This dissertation is concerned more about hepatic clearance and the role of developmental maturation of hepatic drug metabolizing enzymes in determining the capacity of the developing infant to eliminate drugs or chemicals following exposure from breast milk.

$$Cl_{S,Inf} = Cl_H + Cl_R \quad (1.9)$$

### 2.1 Hepatic Clearance ( $Cl_H$ )

Hepatic clearance is of critical importance due to the abundance of drug metabolizing enzymes (DME) in the liver. The liver has a unique anatomical position, as it is located between the gastrointestinal tract (GIT) and the general circulation and receives the majority of blood supply perfusing the GIT<sup>61</sup>. Due to these unique physiological and anatomical characteristics, the liver can reduce the fraction of the orally administered drug that reaches the systemic circulation. This phenomenon is commonly referred to as “first pass effect”.

Hepatic clearance of a drug is governed by three principal physiological parameters: hepatic blood flow, the extent of drug binding to plasma proteins or other blood constituents, and the inherent ability of hepatic metabolic enzymes to eliminate the drug (i.e. the hepatic intrinsic clearance)<sup>59</sup>. These parameters undergo rapid changes during

early infant life<sup>62,63</sup>. Such changes can cause dramatic influences on infant hepatic elimination capacity<sup>44,64</sup>. Several models have been developed to describe the interrelationship between those parameters and to predict the changes in hepatic clearance following changes in the above mentioned physiological determinants<sup>65</sup>. One of the most commonly used models is the ‘Well Stirred Model’, (Equation 1.10). This model is simple and can make qualitative predictions of the impact of changes in the determinants of hepatic clearance on various PK parameters<sup>65</sup>.

$$Cl_H = \frac{Q_H f_u Cl_{int}}{Q_H + f_u Cl_{int}} \quad (1.10)$$

Where,  $Q_H$  is the hepatic blood flow,  $f_u$  is the fraction of the drug unbound in plasma, and  $Cl_{int}$  is the hepatic intrinsic clearance. According to this model, hepatic clearance of drugs that are highly extracted by the liver (>80%) will be governed only by the rate of drug delivery to the liver (i.e.  $Q_H$ ), while changes in hepatic intrinsic clearance and the extent of drug binding have a little or no effect on  $Cl_H$ . Examples of such drugs are lidocaine<sup>66</sup>, propranolol<sup>67,68</sup> and propoxyphen<sup>65</sup>. On the other hand, drugs that have low hepatic extraction ( $E_H$ ) (<20%), hepatic clearance is limited by the degree of drug binding (hepatic uptake) and the intrinsic ability of the hepatic enzymes to metabolize the drug. Examples of these drugs are antipyrine<sup>69</sup> and warfarin<sup>65</sup>. Following oral administration of a high or low  $E_H$  drug, the apparent oral clearance is determined only by the extent of plasma protein binding and intrinsic clearance of the drug<sup>59</sup>.

### 2.1.1 Hepatic Blood Flow

Fetal liver receives its blood supply from three vessels; the umbilical vein (73%), portal vein (18%) and hepatic artery (9%)<sup>61,70-72</sup>. Birth results in a discontinuation of the supply of highly oxygenated blood from the umbilical vein and the liver becomes primarily dependent on poorly oxygenated blood from the portal vein (95%) and hepatic artery (5%)<sup>61,73,74</sup>. Studies in newborn lambs (at 2 hours and 10 hours after birth) and



neonatal lambs (7-10 days) showed that blood flow and oxygen delivery to the liver are significantly lower than those to the fetal liver<sup>73,74</sup>, but comparable to the adult liver<sup>75</sup>.

These dramatic changes in hepatic blood flow and the degree of oxygenation with birth may affect hepatic clearance of many drugs that show high hepatic extraction, where their clearance is hepatic blood flow-dependent. Using propranolol and lidocaine as high clearance drugs, their systemic clearance in neonatal sheep livers was greater in infants than fetuses, but not significantly lower than the adult clearance value<sup>67,68,76</sup>. Given the similar systemic clearance values of these high  $E_H$  drugs, comparable hepatic blood flow in neonates and adults could be expected. The problem of not having good data on developmental changes in  $Q_H$  in human is alleviated by the fact that immature or low metabolic capacity of the liver enzymes in neonates and developing infants decreases the hepatic extraction of drugs and make total clearance less hepatic blood flow-limited<sup>77,78</sup>. Accordingly, the immature hepatic drug metabolizing enzymes, not the hepatic blood flow, plays the major role in determining the capacity of infants to eliminate drugs by the liver. As mentioned above, changes in hepatic blood flow have no effect in the apparent clearance of orally administered drugs whether they have a low or high  $E_H$  values<sup>59</sup>.

### **2.1.2 Plasma Protein Binding**

Several studies have reported age-dependent differences in binding of drugs in human newborn and adult plasma<sup>79-81</sup>. These reports show significantly less binding to infant plasma compared to adult plasma. This may result in higher unbound drug fractions in the infant, which can affect drug distribution and elimination. Given that pharmacological and toxicological effects of many drugs are highly correlated to the unbound concentration<sup>59</sup>, less binding capacity in infants compared to adults, can increase their risk of toxicity following exposure to drugs from breast milk or from the environment.

Several factors may contribute to this age-related difference in plasma protein binding. These include differences in plasma protein concentration between infants and adults, differences in binding properties, and presence of endogenous compounds that

can compete with and displace the drug from the protein binding sites. Albumin and alpha<sub>1</sub>-acid glycoprotein (AAG) are the major plasma proteins responsible for binding of various acidic and basic drugs and endogenous compounds<sup>80,82,83</sup>. Concentration of plasma albumin and AAG are significantly lower at birth and increase progressively to reach the adult values at 6 months of age<sup>84</sup>.

Rather than differences in plasma protein concentrations, lower binding affinities of some acidic and basic drugs to albumin and AAG in newborn plasma compared to adult plasma<sup>80,81,85</sup> may account for the differences in drug binding<sup>79</sup>. Endogenous compounds such as bilirubin and free fatty acids (FFA) can compete with and displace drugs from plasma protein binding sites<sup>86-88</sup>. Higher circulating levels of bilirubin and FFA in infant plasma account for greater displacement of compounds relative to the adult<sup>79,82,84,86</sup>.

For high clearance drugs, their hepatic uptake and removal from blood is an efficient process and independent on the extent of drug binding. On the other hand, for low clearance drugs, only the unbound fraction becomes accessible to the elimination site. Hepatic clearance of those drugs will change in proportion to the unbound drug concentration. Since most compounds are low clearance compounds, McNamara and Alcorn<sup>89</sup> developed a model to predict the extent of protein binding in the infant in the absence of direct measurement of plasma protein binding based upon known binding characteristics in adults<sup>89</sup>.

### **2.1.3 Hepatic Biotransformation**

Many studies have reported the capacity of fetal and infant livers to metabolize xenobiotics<sup>90-94</sup>. Other studies demonstrate a prolonged half life in infants and children for drugs whose elimination is directly related to their rate of hepatic biotransformation<sup>64,95,96</sup>. This could result in drug accumulation and potential risk of toxicity in the exposed infants. While hepatic blood flow and other physiological determinants may affect the rate of drug elimination, the immature hepatic drug metabolizing enzymes is probably the predominant factor accounting for drug accumulation in infants. Understanding the ontogeny of enzyme(s) involved in the

biotransformation pathway(s) could allow a more accurate risk assessment and precise prediction of the toxicological outcome following exposure of the developing infant to foreign chemicals.

Hepatic drug biotransformation and elimination usually takes place in a two-step process and usually the two pathways, phase I and phase II metabolism, function in a sequential manner for drug elimination<sup>45,97</sup>. The first step, phase I, is principally mediated by cytochrome P450 (CYP) enzymes. These enzymes are located on the smooth endoplasmic reticulum and catalyze the biotransformation of both endogenous compounds and xenobiotics to more water-soluble derivatives<sup>98-100</sup>. Although CYP enzymes normally generate metabolites with diminished biological activity, there are numerous examples where these enzymes mediate the formation of reactive intermediates from chemically inert agents<sup>101-103</sup>. These reactive metabolites play a key step in initiating cytotoxicity and carcinogenicity<sup>98,104</sup>. The second step, phase II reactions, mediate the conjugation of exogenous and endogenous compounds (or reactive metabolites produced during phase I metabolism) with water-soluble endogenous molecules like uridine 5'diphosphate (UDP)-glucouronic acid, glutathione or sulfate<sup>46,105</sup>. The conjugated product is usually pharmacologically inactive and easily eliminated from the body via the bile or urine<sup>46</sup>.

When phase I reactions result in the production of reactive metabolites, the balance between phase I-induced bioactivation and phase II-mediated detoxification is a critical factor in determining the toxicological consequences of exposure to such an agent<sup>106,107</sup>. If the formation of reactive metabolite during phase I reaction exceeds the conjugation capacity of phase II enzymes, reactive metabolites accumulate in the cell and can bind to endogenous biomolecules like DNA and protein, inducing cellular damage<sup>105</sup>. Thus, the postnatal development of drug metabolizing enzymes (DME) activities, which catalyze the formation or detoxification of reactive intermediates, could have a profound effect on the ultimate toxicological outcome of exposure to xenobiotics.

### **2.1.3.1 Ontogeny of Hepatic CYP Enzymes**

The cytochrome P450 enzymes (CYP) are a group of heme-thiolate proteins that comprise a superfamily of related enzymes grouped into families and subfamilies based upon similarities in amino acid sequences<sup>2</sup>. These enzymes catalyze the oxidative metabolism of endogenous compounds such as steroids, fatty acids, vitamins, and prostaglandins as well as exogenous chemicals such as drugs, carcinogens and environmental toxins<sup>98,99,108</sup>. In addition, CYP enzymes play a major role in maintaining steady state levels of endogenous ligands involved in modulating gene transcription affecting homeostasis, growth and differentiation<sup>109</sup>. Xenobiotics are mainly metabolized by families CYP1, CYP2, CYP3 and CYP4<sup>100</sup>. To date, at least 57 CYP forms are responsible for the metabolism of substrates in human, and most of these are primarily expressed in the liver<sup>100</sup>.

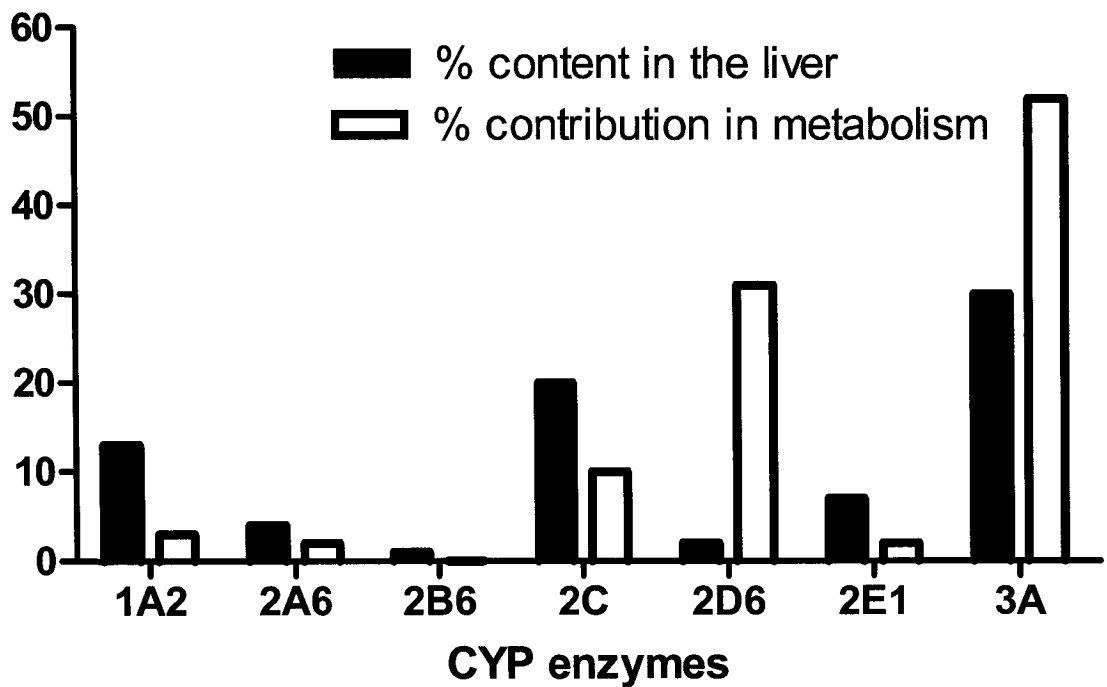
#### **2.1.3.1.1 Human CYP Enzymes**

CYP enzymes activity is very low during early stages of gestation and remains low until birth. After parturition many CYP enzymes undergo significant postnatal maturation<sup>110-113</sup>. The total CYP content in the human fetal liver is about one-third of the adult value, and increases after birth to reach the adult value during the first 10 years of life<sup>95,111</sup>. Cresteil<sup>105</sup> categorized CYP enzymes into three groups according to the onset of their activities:

- (a) Enzymes expressed in fetal livers (CYP3A7 and CYP4A1): These enzymes are mostly active on endogenous and some exogenous molecules. Their activities decline with postnatal maturation.
- (b) Enzymes expressed during the early neonatal period (CYP2E1 and CYP2D6): The activity of these enzymes are undetectable during gestation but surge immediately after birth.
- (c) Enzymes expressed later in neonatal development (CYP1A2, CYP2B, CYP2C and CYP3A4): The activity of these enzymes tend to surge a few

weeks after birth and have slower rates of development than early neonatal enzymes.

In adult human livers, CYP1A2, CYP2A6, CYP2C, CYP2D6, CYP2E1, and 3A protein account for approximately 70 % of the total hepatic CYP enzymes, with the CYP2C and CYP3A subfamilies being the major forms<sup>99,108,111</sup>. Reasonable levels of CYP1A2 (13%) and CYP2E1 (7%) are present, while CYP2A6, CYP2D6 and CYP2B6 represent less than 4%, 2% and 1%, respectively<sup>108,111</sup>. Figure 1.2 presents the levels of immunoquantifiable CYP proteins in adult human livers (expressed as a percentage of total CYP protein) and the percentage contribution of each CYP enzyme in the metabolism of drugs available in the market.



**Figure 1.2-** Average level of cytochrome P450 (CYP) enzyme content of adult human liver microsomes (recalculated from reference 108) and the estimation of the contribution of individual CYP enzymes in the metabolism of drugs currently available on the market (adapted from reference 100).

### 2.1.3.1.2 Rat CYP Enzymes

Few studies are reported regarding the ontogeny of CYP enzymes in rats. Total CYP content does not change much with age in male and female rats<sup>114</sup>, but when the results were expressed on a gram liver weight basis, 4- and 6- fold increase in the CYP content was detected at postnatal days 7 and 14, respectively, compared with day 1 of birth<sup>115</sup>. With the exception of CYP1A1, which is expressed during the early gestation, detectable expression of most of the other CYP enzymes occur at or near birth (CYP2B, CYP2C23, CYP3A) or immediately after birth (CYP2E1)<sup>1</sup>. The metabolism of marker substrates for CYP2B1 and CYP2E1 could not be measured in rat livers during early or late gestation<sup>115</sup>. After birth, CYP2B1 catalytic activity increased significantly to achieve activity level at postnatal day (PD) 4 comparable to the level observed in adult livers<sup>115</sup>. In contrast, postnatal activity of CYP2E1 increased linearly with age and comparable activity to that estimated in adult liver was measured at PD37<sup>1,115</sup>.

Rat hepatic CYP2C23 and CYP3A exhibit similar pattern of ontogenesis as CYP2E1 with expression reached its maximal value in early neonates and remained quite stable<sup>62,115</sup>. Other CYP enzymes are expressed after the first week of birth (CYP1A2, CYP2C6, 2C11, 2C12 and 4A10)<sup>1,114-116</sup>. While the catalytic activity of rat hepatic CYP1A2 was not detected in fetal livers, metabolism of its marker substrates increased at approximately linear rate during postnatal development with maximum levels achieved at PD35<sup>1,115</sup>. In contrast, the developmental profiles of CYP2C11 and CYP2C12 exhibit a characteristic rise at puberty (PD28)<sup>1</sup>.

An interesting study by Imaoka *et al* has shown sex-related differences in the developmental maturation of CYP expression in rats<sup>114</sup>. Activity of the male-specific form, CYP2C11 was induced developmentally and levels reached a plateau at 14-52 weeks of age followed by a marked decline at 104 weeks<sup>114</sup>. CYP2C11-mediated activities were not detected at any age in female rats. On the other hand, activity of the female-specific form, CYP2C12 increased with age in female rats<sup>114</sup>. However, this activity was very low or undetectable in male rats until juvenile age, but increased to a level similar to that of female rats at 104 weeks of age<sup>114</sup>.

Extensive studies have investigated the sex-related differences in drug metabolism in rats, a phenomenon commonly known as sexual dimorphism. Majority of these studies found out that growth hormone (GH) is the only endogenous factor known to maintain the expression of gender-specific or gender-dominant isoforms of CYP enzymes<sup>117</sup>. The sexually-dimorphic expression of CYP isoforms appear to be dictated mainly by the gender-dependent profiles of the circulating GH<sup>118,119</sup>. In male rats, GH secretion shows a strongly pulsatile pattern with episodic peak every 3-4 hours with generally undetectable levels between peaks<sup>117,118</sup>. In female rats, GH is continuously released with a pattern that is more frequent, irregular and with lower magnitude than males<sup>117,118</sup>.

The strongly pulsatile or episodic rhythm of GH secretion in male rats is responsible for the expression of male-specific (e.g. CYP2C11, CYP2C13, CYP2C22, CYP3A2) and male-dominant (e.g. CYP2B1, CYP2B2, CYP3A1) isoforms<sup>119</sup>. However, the continuous feminine pattern of GH secretion in female rats is responsible for expression of female-specific (e.g. CYP2C12) and female-dominant (e.g. CYP2C7, CYP2A1, CYP2E1)<sup>118</sup>.

Many studies have reported sexually dimorphic GH profiles in human<sup>120,121</sup> which results in sex differences in drug metabolism<sup>122</sup>. However, these sexually dimorphic effects of GH on human CYP expression are not as significant as those observed in rats and could be subtle and easily hidden by the extensive interindividual variations in human population<sup>108,122</sup>.

Due to their toxicological significance and major roles in detoxification of many important environmental chemicals and widely used pharmaceutical agents and their role in mediating cellular damage and carcinogenesis, the ontogeny of CYP2E1 and CYP1A2 in human and rats will be discussed in greater detail.

### 2.1.3.1.3 CYP2E1 Enzyme

The ethanol-oxidizing enzyme<sup>123</sup>, CYP2E1, is constitutively expressed in the liver and many other extrahepatic tissues including lung, brain and kidney<sup>100,124,125</sup>. CYP2E1 is highly inducible by treatment with ethanol, acetone, imidazole and other compounds, most of them being CYP2E1-substrates<sup>126-130</sup>. CYP2E1 is moderately abundant in adult human liver, where it constitutes approximately 7% of the total hepatic CYP enzymes<sup>108</sup>. Despite the relatively few drugs that are metabolized by CYP2E1, e.g. acetaminophen, chlorzoxazone and halogenated anaesthetics, this enzyme is involved in the bioactivation of many toxicologically important chemicals including ethanol, organic solvents and procarcinogens present in tobacco smoke to reactive intermediates that lead to hepatotoxicity and carcinogenicity (Table 1.2). Therefore, the postnatal development of CYP2E1, that mediates bioactivation or detoxification of toxicants, could significantly affect the toxicological outcome of xenobiotic exposure during development.

As shown in Table 1.2, CYP2E1 substrate profile includes endogenous compounds, which indicates an important physiological role for this enzyme. Studies have shown active utilization of acetone as a precursor by CYP2E1 in a gluconeogenic pathway during starvation and diabetic ketoacidosis<sup>123,126,131,132</sup>. Immediately after birth, the newborn experiences a temporary fasting situation that causes increases in plasma ketone bodies concentrations. This could explain the rapid surge of CYP2E1 protein following parturition<sup>133,134</sup>.

Ethanol and imidazole were found to induce rat CYP2E1 protein without affecting levels of mRNA. This suggests a posttranscriptional mechanism involving protein stabilization against degradation through ligand binding<sup>130</sup>. CYP2E1 is also induced through a transcriptional regulation mechanism under conditions of starvation and diabetic ketosis<sup>135-138</sup>.



**Table 1.2-** Examples of substrates metabolized/bioactivated by CYP2E1.

<b>CYP2E1 substrates</b>	<b>Toxicological effect</b>	<b>References</b>
<b>1. Halogenated anesthetics</b>		
1.1. Halothane	Hepatotoxicity	139,140
1.2. Isoflurane	Hepatotoxicity	140
1.3. Enflurane	Hepatotoxicity	140
<b>2. Alcohols</b>		
2.1. Ethanol	Hepatotoxicity	141-143
2.2. Methanol	Hepatotoxicity	144
2.3. Glycerol**	Hepatotoxicity	145,146
<b>3. Organic solvents</b>		
3.1. Styrene	Carcinogenicity	102,147
3.2. Benzene	Hepatotoxicity	148
3.3. Carbon tetrachloride	Hepatotoxicity	149
3.4. Chloroform	Hepatotoxicity	150
3.5. Trichloroethylene	Nephrotoxicity and lung cancer	151,152
<b>4. Nitrosamines</b>		
4.1. N,N dimethylnitrosamine	Hepatotoxicity and carcinogenicity	153
4.2. N,N diethylnitrosamine	Hepatotoxicity and carcinogenicity	144
<b>5. Drugs</b>		
5.1. Acetaminophen	Hepatotoxicity and nephrotoxicity	101,154
5.2. Chlorzoxazone*	N/A	155
5.3. Isoniazide	Hepatotoxicity	144
<b>6. Miscellaneous</b>		
6.1. Lauric acid**	N/A	100
6.2. Acetone**	N/A	132
6.3. p-nitrophenol*	N/A	156

N/A – No reported toxicity data

\*Probe substrates for CYP2E1

\*\*Endogenous substrates

Contradictory data are reported regarding the expression of CYP2E1 in human fetal liver. Several studies have failed to detect CYP2E1 mRNA by reverse transcription polymerase chain reaction (RT-PCR), protein using immunoblotting, or activity using  $\rho$ -nitrophenol oxidation in fetal liver<sup>111,133,157</sup>. Other studies reported very low levels of CYP2E1 mRNA, protein and activity in fetal liver samples at late gestational stages<sup>134,158,159</sup>. Postnatally, CYP2E1 protein and activity levels increase rapidly during the first 24 hours after birth without significant elevation of mRNA level<sup>133</sup>. At the age of one month, mRNA level rises gradually and in correlation with protein and activity levels. Adult CYP2E1 protein and activity levels are achieved at 1-10 years<sup>133</sup>.

As mentioned above, two mechanisms, transcriptional and posttranscriptional, have been proposed for the regulation of human hepatic CYP2E1<sup>160</sup>. The transcriptional mechanism primarily regulates CYP2E1 expression during the early postnatal period. Interestingly, transcriptional activation of *CYP2E1* gene after birth correlated with the demethylation of certain cytosine residues at the 5' end of the gene resulting in accumulation of RNA after one month of age<sup>133,134,160</sup>. CYP2E1 is also regulated by posttranscriptional mechanism through substrate-dependent stabilization as indicated above.

In rats, Carpenter *et al*<sup>161</sup> reported detectable levels of CYP2E1 protein and chlorzoxazone hydroxylase activity in fetal livers during late gestation. These levels were much less (16- fold) than levels from adult livers<sup>116</sup>. CYP2E1 mRNA is very low or undetectable in fetal livers during late gestation, but increase markedly within a few hours after birth<sup>1,114,115</sup>. Neonatal rats express small quantities of CYP2E1 protein despite larger quantities of mRNA<sup>114,115</sup>. Maximum CYP2E1 content and activity are observed at weaning (2-fold the adult value), followed by a gradual decline to reach the adult value at 14 weeks of age<sup>114</sup>.

#### 2.1.3.1.4 CYP1A2 Enzyme

CYP1A2 is a constitutive enzyme that is expressed principally in the liver<sup>108,111</sup>. Extrahepatic tissues such as lung and colon express low levels of CYP1A2<sup>162</sup>. In adult

human liver, CYP1A2 constitutes approximately 13 % of the total hepatic CYP content<sup>108</sup>. This enzyme catalyzes the metabolism of a wide variety of chemicals, some of which are potential inducers for CYP1A2 (Table 1.3). The list of substrates includes a variety of polycyclic aromatic hydrocarbons (PAHs), nitrosamines, and heterocyclic aromatic amine (HAAs). Several of these substrates are potential or suspected human and/or animal carcinogens. Several clinically used drugs such as phenacetin and endogenous compounds such as 17  $\beta$ -estradiol are also CYP1A2 substrates<sup>163</sup>. Therefore, and as CYP2E1, the ontogeny pattern of CYP1A2 could have a profound and age-dependent effect on the toxicological outcome of xenobiotic exposure.

While one study reported detectable levels of CYP1A2 mRNA in human fetal livers few days prior to parturition<sup>1</sup>, many studies failed to detect any fetal CYP1A2 mRNA at any gestational age<sup>157,164</sup>. Most reports indicate the absence of CYP1A2 protein expression in human fetal livers. Very low protein levels are detected during the early postnatal period, but undergo a gradual increase in expression in infants' ages 1-3 months. The protein level attains about 50 % of the adult value at age 1-10 years<sup>108,111,158,165</sup>. The development of CYP1A2 activity parallels the increase in protein expression<sup>111,165,166,91</sup>. Generally, CYP1A2 shows delayed ontogeny compared with other CYP enzymes<sup>165</sup>.

Studies have shown that the constitutive expression of human CYP1A2 is regulated by a liver-enriched transcription factor, HNF-1<sup>167</sup> and an upstream stimulatory factor<sup>168</sup>. Expression of CYP1A2 in extrahepatic tissues, e.g. olfactory tissues is regulated by nuclear factor, NF-1<sup>169</sup>. Treatment of rodents with high concentrations of the inducer 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD) or 3-methylcholanthrene (MC) increases hepatic mRNA expression and detection of low mRNA levels in lung, kidney and intestine<sup>170</sup>. This increase in mRNA level is partially due to transcriptional activation and mainly due to posttranscriptional mRNA stabilization<sup>98</sup>. Several studies have confirmed the role of Aryl hydrocarbon (*Ah*) receptors in governing induction of *CYP1A2* gene expression<sup>170,171</sup>.

**Table 1.3-** Examples of substrates metabolized/bioactivated by CYP1A2.

CYP1A2 Substrates	Toxicological effect	References
<b>1. Polycyclic aromatic hydrocarbons</b>		
1.1. Benzo[a]Pyrene**	Carcinogenicity	172
1.2. 3-Nitrobenzanthrene	Genotoxicity	173
1.3. Benzo[a]phenanthrene	Carcinogenicity	103
<b>2. Heterocyclic amines</b>		
2.1. 2-Acetylaminofluorene	Carcinogenicity and genotoxicity	174
2.2. MeIQx <sup>†</sup>	Hepatocarcinogenesis	175
<b>3. Nitrosamines</b>		
3.1. N,N dimethylnitrosamine	Carcinogenicity	155
3.2. N,N diethylnitrosamine	Carcinogenicity	155
3.3. N-nitrosodibutylamine	Carcinogenicity	155
<b>4. Drugs</b>		
4.1. Phenacetin*	Hepatotoxicity and carcinogenicity	176,177
4.2. Acetaminophen	Hepatotoxicity and carcinogenicity	154,174
4.3. Propranolol	N/A§	178
4.4. Caffeine	N/A	91
4.5. Imipramine	N/A	165
<b>5. Endogenous compounds</b>		
5.1. 17 $\beta$ -estradiol	N/A	163
5.2. Retinol	N/A	179
5.3. Arachidonic acid	N/A	179
5.4. Estrogen	N/A	163
<b>6. Miscellaneous</b>		
6.1. Methoxyresorufin*	N/A	180
6.2. Ethoxyresorufin <sup>†</sup>	N/A	181,182
6.3. Benzyoxyresorufin	N/A	180

<sup>†</sup>MeIQx – 2-amino-3, 8-dimethylimidazole [4.5] quinoxaline

§ N/A - No reported toxicity data

\* Probe substrates for CYP1A2 in rats

\*\* Inducer for CYP1A2

<sup>†</sup> Probe substrate for CYP1A2 in human

In rats, the constitutive levels of CYP1A2 throughout fetal development could not be detected at protein or activity levels<sup>1,114,115</sup>. CYP1A2 mRNA is detected as early as a few days prior to parturition<sup>114,115</sup>. The protein and activity levels are very low in neonatal rats but a marked surge occurs immediately before weaning (5-fold the adult values) followed by a sharp decline to reach the adult value at 12 weeks of age<sup>183</sup>.

### **2.1.3.2 Ontogeny of Phase II Enzymes**

Phase II drug metabolizing enzymes play an important role in metabolic inactivation or detoxification. These enzymes are capable of converting phase I metabolites or the parent substrates into a more polar and water-soluble product. Phase II metabolizing enzymes accomplish this role by catalyzing the conjugation of their substrate to a small molecular weight and polar endogenous molecule, like UDP-glucouronic acid, inorganic sulfate, glutathione, or acetyl coenzyme A<sup>97</sup>. The resulting conjugation product is usually more water-soluble and can be readily excreted in urine or bile. As well, the conjugation product is inactive and non-toxic, with few exceptions<sup>184</sup>. Therefore, phase II conjugation reactions are considered, in most cases, as effective detoxification pathways. Some endogenous molecules, e.g. bilirubin, steroids and catecholamines, are also substrates for Phase II enzymes<sup>97</sup>. This could reflect the physiological and biochemical roles of these enzymes in affecting cellular growth and differentiation. Generally, Phase II enzymes expression undergoes tremendous developmental changes.

#### **2.1.3.2.1 Glucuronide conjugation**

Glucuronide conjugation reactions are mediated by UDP glucuronosyl-transferases (UGTs). These enzymes catalyze the conjugation of glucuronic acid to several endogenous and exogenous substrates<sup>185</sup>. Endogenous substrates include bilirubin, fatty acids, estradiols and thyroxine<sup>186</sup>, while exogenous compounds include numerous clinically used drugs, e.g. acetaminophen, NSAIDs and opioids. In general, glucuronidation capacity is low in infants, which can result in drug accumulation and higher risk of toxicity<sup>185</sup>. An example of this is the gray baby syndrome associated with markedly reduced capacity for chloramphenicol glucuronidation in infants<sup>187</sup>. The

catalytic activity of UGTs towards bilirubin was found to be less than 1% of the adult values during fetal and early postnatal periods, and the activity increases to reach adult values by 3 months of age, after which it remains constant<sup>188</sup>. Morphine, a UGT enzyme substrate<sup>189</sup> exhibits very low plasma clearance in infants when compared with older children<sup>190,191</sup>. Immunoreactivity studies with polyclonal antibodies indicate that enzyme activity develops in parallel with the immunodetectable protein levels<sup>189,192</sup>. In rats, conjugation reactions catalyzed by UGTs are measurable during the first 10 days of gestation but remain low during fetal and perinatal period<sup>115,193</sup>. After birth, activity increases to reach a maximum value at three weeks followed by a decline to 50% the maximum value at four weeks<sup>115</sup>.

#### **2.1.3.2.2 Sulfate conjugation**

Sulfate conjugation is a major pathway in metabolism and elimination of endogenous and exogenous compounds<sup>194</sup>. Sulfation is catalyzed by a family of cytosolic proteins called sulfotransferases (SULT). These enzymes catalyze the conjugation of inorganic sulfate derived from the active donor molecule 3'-phosphoadenosine-5'-sulphophosphate, with a hydroxyl or phenolic functional groups<sup>194,195</sup>. Sulfation normally results in the reduction of biological activity and increase in water solubility relative to parent molecule. Therefore, a sulfate conjugate may be excreted from the body in urine and/or bile, or undergoes bioactivation by sulfatase enzymes<sup>196</sup>. The ontogeny of SULT enzymes is inadequately described as most of the studies were done using substrates that may or may not be specific to SULT isozymes<sup>46</sup>. A study by Richard *et al*<sup>197</sup> demonstrates high expression of SULT protein and activity in fetal and neonatal livers. The activity and protein levels increased with age so that the level measured in adult livers were approximately 5-fold that observed in fetal livers<sup>198</sup>. Generally, sulfate conjugation is an efficient pathway in fetus, newborn, and infant compared with other phase II reactions<sup>46,199</sup>.

#### **2.1.3.2.3 N-acetylation**

N-acetyl transferases (NAT) catalyze the addition of an acetyl moiety to xenobiotics or endogenous compounds that carry a primary aromatic amino or hydrazine functional

group<sup>200,201</sup>. Acetylation of *p*-amino benzoic acid, a selective substrate for NAT, is detected in fetal hepatic and extrahepatic tissues<sup>202</sup>. The acetylation activity in adult liver tissues is more than 3-fold the fetal activity<sup>202</sup>. Studies in infants and children show the capacity of NAT enzymes to acetylate several NAT substrates. This activity was almost half the activity detected in adult<sup>203,204</sup>. A study in mice showed expression of NAT mRNA in fetal liver that increases with age after birth in a non-linear fashion<sup>205</sup>. Infant livers were able to acetylate several NAT's substrates with activity that increases with age<sup>205</sup>.

#### **2.1.3.2.4 Glutathione-S-Transferases**

The glutathione-S-transferases (GST) represent a group of detoxification enzymes that are expressed in most tissues, particularly the liver and kidney<sup>206</sup>. These enzymes catalyze the conjugation of reduced glutathione (GSH) with an electrophilic center in a wide variety of xenobiotics<sup>184,207,208</sup>. Therefore, GST enzymes protect vital cellular nucleophilic constituents against electrophilic reactive species and prevent cellular damage<sup>209</sup>. In addition, GST enzymes act as an intracellular carrier protein for the transport of a wide variety of endogenous hydrophobic molecules, including bilirubin, heme and hormones<sup>208</sup>. Due to their toxicological significance and critical role in providing protection against cellular damage, genotoxicity and carcinogenesis, glutathione-S-transferases (GST) will be discussed in detail in this dissertation.

Given their important detoxification role in protecting against cellular damage and carcinogenesis, changes in the developmental expression of GST enzymes could have a profound toxicological impact on developing infants exposed to xenobiotics. Extensive studies in human and rodents livers have shown that GST enzymes are homodimers or heterodimers of subunits that have been grouped into seven classes based upon primary structure, catalytic properties and N-terminal amino acid sequences, namely alpha, mu, pi, sigma, theta, zeta and omega<sup>207</sup>. Dimerization of the subunit members of the same class gives rise to a significant number of isozymes (some examples are shown in Table 1.4). These isozymes show different, and sometimes, overlapping substrate specificity.

**Table 1.4-** Human GST isozymes (adapted from reference 207).

Class/family	Isozymes (examples)
Alpha	GSTA1-1, GSTA1-2, GSTA2-2, GSTA3-3, GSTA4-4
Mu	GSTM1-1, GSTM1-2, GSTM2-2, GSTM2-3, GSTM3-3, GSTM4-4, GSTM5-5
Pi	GSTP1-1
Sigma	GSTS1-1
Theta	GSTT1-1, GSTT2-2
Zeta	GSTZ1-1
Omega	GSTO1-1, GSTO 2-2

The expression of GST enzymes is complex and exhibits tissue, sex and age dependent patterns<sup>208</sup>. Studies for measuring the ontogeny of GST activity in humans were done using 1-chloro-2,4 dinitrochlorobenzene (CDNB) as a general electrophilic substrate<sup>210-215</sup>. These results indicate the ability of the fetus to detoxify possible reactive molecules that are generated intracellularly or cross the placenta. Although these studies suffer from a lack of specificity for GST isozymes, they demonstrate GST activity in the fetal livers at early gestational ages. Recently, with the utilization of highly specific antibodies, data regarding the developmental expression and cellular distribution of some GST isozymes have become available. The expression of Mu isozymes was detected as early as 10 weeks of gestation and did not change significantly until birth<sup>216</sup>. After birth, the expression level increased to reach the adult value at infant age of 42-85 weeks<sup>216,217</sup>. The expression of Pi isozymes was detected in the fetal liver at 8 week gestational age and undergoes downregulation until birth, where the expression is either very weak or undetected<sup>211,216,218</sup>. After 6 months of life, this isozyme is completely undetected in infant and adult livers<sup>217,219</sup>. The expression of alpha class isozymes was detected in fetal livers after 10 weeks of gestation<sup>216,220</sup>. The expression levels increase by age after birth, where they become the predominant isozymes in infant and adult liver<sup>220</sup>. Adult levels of expression were achieved at about 50 weeks of postnatal age<sup>211,216</sup>.



In rats, the activity of GST enzymes is detected as early as 10 days of gestation and increases up to 5-fold during the fetal hepatoorganogenesis period<sup>115,193</sup>. After birth, the activity increases more than 2-fold, followed by a linear increase with age<sup>115,200,210</sup>. After 10 months of age, GST activity remains constant<sup>115</sup>.

### 3. Relevance of CYP Enzyme Ontogeny to Developmental Toxicity

Physiological and biochemical processes that govern pharmacokinetics (absorption, distribution, metabolism and elimination) of administered/exposed dose undergo significant and dynamic changes during developmental maturation<sup>50,221</sup>. These pharmacokinetic (PK) differences between developmental ages result in differences in therapeutic/toxic outcomes that can not be expected by simple adult-to-infant extrapolation based on body weight or body surface area adjustment factors<sup>222-224</sup>. To remove uncertainties in such extrapolations, the internal dosimetry in various developmental ages of childhood has to be considered in relation to adults.

Since metabolism (mediated mainly by hepatic CYP enzymes) is a principal determinant of plasma concentration of chemicals, a thorough understanding of the ontogeny of these hepatic enzymes is required for establishing accurate and potential exposure risk assessment measures, especially in pediatric populations. For example, knowing that CYP1A2 enzyme is immature in full-term neonate may suggest that this population is under low risk of cancer due to CYP1A2-mediated bioactivation compared to adult. However, chemicals that are eliminated by CYP1A2 pathway may accumulate in neonates and enhance risk of toxicity compared to adults.

Given these significant bioactivation and detoxification roles of CYP enzymes, the susceptibility of the developing infant to a toxicant can be critically influenced, both qualitatively and quantitatively, by the developmental maturation (ontogeny) of CYP enzymes<sup>222</sup>. However, most of the available information regarding CYP bioactivation potential, induction and inhibition properties, effect of xenobiotics and pathophysiological states on their regulation and expression are based on *in vitro* and *in vivo* studies performed in adult life stage<sup>225-227</sup>.

Developmental toxicology studies suggest that exposure to xenobiotics can affect the developing child in ways often different than adults and can lead to profound and permanent effects if the insult occurs during a sensitive developmental period (critical window)<sup>222</sup>. However, due to several logistic and ethical issues, very few toxicological studies are available in pediatric populations<sup>228</sup>. Also, the few studies conducted in animal models (mostly rodents) do not assess toxicity at the most critical developmental ages. Furthermore, assessments are solely based on the maternal exposures without consideration of the knowledge of the developmental window of susceptibility (i.e. the onset of pharmacodynamic processes that lead to toxicity) and the internal dosimetry in the neonate during that developmental window<sup>229</sup>.

To overcome the ethical considerations that preclude the use of children in toxicological assessments, suitable animal models should be used to define a toxicokinetic basis for age-dependent differences in toxicity<sup>228</sup>. The appropriate animal model of developmental toxicology should exhibit sufficient similarities to human to ensure appropriate risk assessments. To relate susceptibility to injury by CYP enzyme-mediated xenobiotic metabolism to the time course of maturational changes in the xenobiotics metabolism, however, requires a detailed understanding of hepatic CYP enzyme ontogeny in an appropriate animal model.

#### **4. Methods Used to Study the Ontogeny of Hepatic Drug Metabolizing Enzymes (DME)**

The ontogeny of hepatic DME is generally studied by two principal approaches. The first approach is an *in vivo* study of the age-related changes in the elimination of a probe substrate for the enzyme being investigated. The second approach is an *in vitro* description of the age-related changes in expression of mRNA levels (using specific nucleic acid probes), protein contents (using specific monoclonal or polyclonal antibodies), and enzyme activity (using specific probe substrates).

#### 4.1 *In Vivo* Approach

In this approach, the ontogeny of enzyme activity is assessed *in vivo* by monitoring the systemic clearance of an *in vivo* metabolic probe substrate. For example, diazepam and midazolam have been frequently used to determine the ontogeny of CYP2C and CYP3A activities, respectively<sup>93,94</sup>. Some methods depend on monitoring the metabolic changes in some endogenous probe substrate. For example, age-related changes in urinary 6 $\beta$  hydroxyl cortisol/cortisol ratio can be used to assess the ontogeny of CYP3A in human<sup>90,92</sup>. The *in vivo* approach has many limitations:

1. The ethical, moral and technical boundaries of drug research in pediatrics<sup>60</sup>. Regulatory agencies have started to encourage the pharmaceutical industry to establish safety in children.
2. Most of the *in vivo methods* are invasive and require multiple blood and /or urine collections. However, non-invasive techniques are becoming available. For example, breath tests using erythromycin or caffeine to assess the ontogeny of CYP3A or CYP1A2, respectively<sup>230,231</sup>.
3. In many situations, the elimination of the *in vivo* probe substrate is mediated by elimination pathways other than hepatic mechanisms. For example, diazepam and caffeine undergo renal elimination in addition to hepatic biotransformation<sup>91,93</sup>. Therefore, specificity of the probe substrates have to be considered carefully before interpretation of ontogeny studies using an *in vivo* approach.
4. This approach can not be used to assess the hepatic expression of DME during the gestational stage.

#### 4.2 *In Vitro* Approach

This approach depends on the use of human liver tissues obtained immediately after death. Rapid collection of these tissues after death and their storage at -80 °C without frequent freeze-thaw cycles are very critical to decrease the rate of enzymatic degradation and mRNA loss<sup>232</sup>. Liver tissues are used to prepare hepatic microsomes,

cytosols or hepatocytes, which are used to assess the ontogeny of various phase I and phase II metabolic enzymes on the three systems biology levels. Taking into consideration the ontogeny of hepatic DME at mRNA, protein and activity levels can help in identifying the consequences of exposure to endogenous and exogenous factors on the normal ontogeny pattern. Such exposures during critical periods of development could induce permanent alterations in enzyme activity (i.e. genetic imprinting) that tend to be irreversible<sup>223,233</sup>. As well, activity levels of hepatic DME determine the bioactivation potential and the elimination kinetics of numerous toxicants. Characterization of the maturation profiles of these enzymes can facilitate predictions of xenobiotic disposition and toxicological impact during development<sup>77,234,235</sup>. Finally, incorporating molecular techniques into traditional toxicity studies can provide potential markers for the early detection of toxicity<sup>236</sup>.

#### **4.2.1 mRNA Expression Level**

This approach involves estimation of the corresponding mRNA using specific nucleic acid probes. There are four methods commonly used to assess mRNA expression; northern blotting, RNase protection assay, *in situ* hybridization and real time reverse transcription polymerase chain reaction (RT-PCR). The latter is the most sensitive and flexible for mRNA quantitation<sup>237-240</sup>. Improved detection sensitivity allows the characterization of tissue and cell-specific distribution of genes where expression is limited, e.g. in fetal and neonatal livers.

#### **4.2.2 Protein Expression Level**

Due to the increased availability of monoclonal and polyclonal antibodies, immunoblotting (western blotting) became widely used to determine age-related changes in expression of different hepatic phase I and phase II enzyme<sup>94,105,108,111,157,241,242</sup>.

### 4.2.3 Catalytic Enzyme Activity

This approach depends on incubating a probe substrate with age-dependent hepatic microsomes or cytosols under appropriate conditions, followed by monitoring metabolite formation rates that corresponds to the metabolic pathway under investigation. With such approach, the probe substrate should be specifically metabolized by the enzyme of interest. As well, the analytical method has to be sensitive enough to detect low metabolite levels in cases of low enzyme activity.

## 5. Developmental Changes in Zonal Expression of CYP Enzymes

Hepatic DME exhibit a prominent zonation across the hepatic acinus, the functional unit of the liver<sup>243</sup>. The zonal expression and induction of DME are related to the vascular architecture of the liver<sup>244</sup>. In the adult, CYP enzymes exhibit high expression and inducibility in hepatocytes of the perivenous region<sup>243,245,246</sup> which increases the vulnerability of this zone to toxicity due to accumulation of CYP-mediated generation of reactive intermediates. Phase II conjugation enzymes, in particular GST and UGT, also exhibit high expression levels in the perivenous region<sup>247,248</sup>. However, the gradient of expression from periportal to perivenous hepatocytes is less steep when compared to CYP enzymes<sup>245,249</sup>. Therefore, cells in the perivenous region may not have the detoxification capacity to compensate for the high rate of reactive intermediates formation, especially after exposure to CYP inducers. Another factor increasing the susceptibility of this region to toxicity is the minimal expression of glutathione peroxidase. This enzyme is responsible for the elimination of peroxides and its expression is focally concentrated in the periportal zone<sup>247</sup>.

Ratanasavanh *et al*<sup>243</sup> showed age-dependent changes in the hepatic zonal expression pattern of several CYP enzymes. In the fetal and perinatal period, the liver exhibits a homogenous distribution of CYP enzymes, with some preference towards the perivenous zone for several enzymes. During postnatal development, a progressive heterogenous distribution of CYP enzymes is observed with more expression in the perivenous cells,

and for some CYP enzymes the expression becomes completely absent in the periportal hepatocytes<sup>243</sup>.

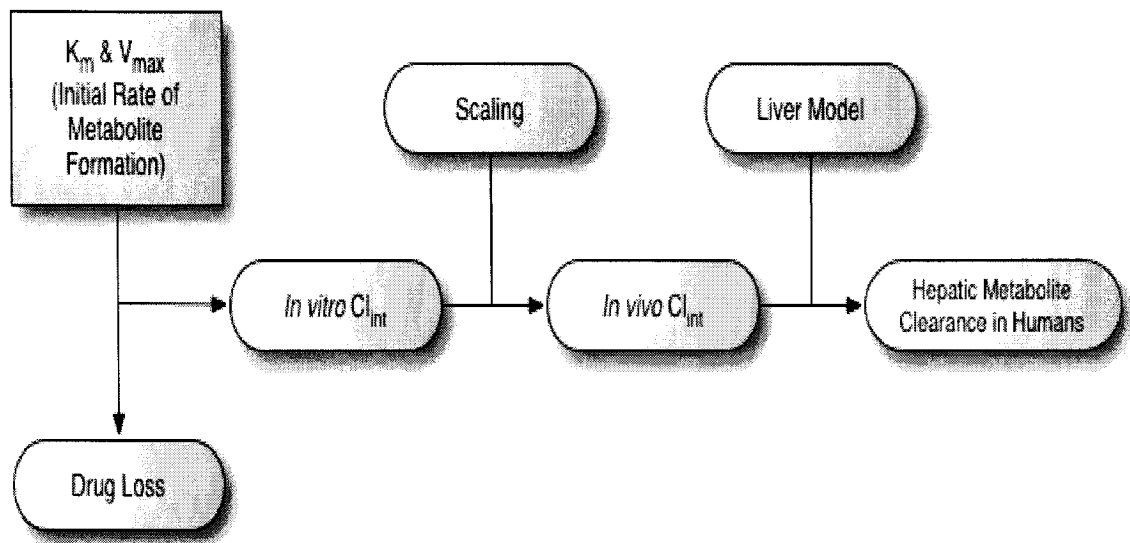
In rat livers, distribution of some CYP enzymes remains homogeneous from birth to 5 days after birth<sup>1</sup>. However, by 15 days of age, CYP expression becomes localized in the perivenous hepatocytes.

## 6. Approaches to Predict Hepatic Clearance

Several approaches have been applied for the prediction of hepatic drug clearance in humans. These approaches are based upon *in vitro* data in human and animals, and/or *in vivo* pre-clinical data. *In vitro* metabolism studies provide estimates of the Michaelis-Menten kinetic parameters and, hence, an estimate of *in vitro* intrinsic clearance ( $Cl_{int}$ ). This  $Cl_{int}$  estimate, then, is extrapolated to the whole body by the use of hepatic scaling factors, which is subsequently incorporated into a model of hepatic clearance ( $Cl_H$ ) (i.e. the Well-Stirred Model)<sup>225</sup>. Predictions based on *in vivo* pre-clinical data can be performed by using allometric scaling techniques<sup>250</sup>. An alternative approach is the use of physiologically-based pharmacokinetic (PBPK) models that have been developed based upon physiological principles and measured physiological parameters<sup>234</sup>.

### 6.1 Prediction from *In Vitro* Data

The basis of the extrapolation from *in vitro* data to *in vivo* situations involves the parameter, intrinsic clearance ( $Cl_{int}$ ), which is a pure measure of inherent capacity of hepatic enzymes to eliminate a drug in absence of other physiological determinants of hepatic clearance such as hepatic blood flow and drug binding within blood matrix<sup>225</sup>. *In vitro*  $Cl_{int}$  can be obtained from metabolic studies using hepatocytes, liver slices, and hepatic microsomes<sup>251,252</sup>. The strategy of *in vitro-in vivo* extrapolation has been fully explained<sup>225,227,253</sup> and is illustrated in Figure 1.3.



**Figure 1.3-** Strategy for extrapolation of *in vitro* intrinsic clearance to *in vivo* hepatic metabolic clearance (modified from reference 225).

The first stage of the strategy is to calculate *in vitro*  $Cl_{int}$  from the ratio of the maximum velocity of the metabolic reaction ( $V_{max}$ ) to the Michaelis-Menten constant ( $K_M$ ) determined from the initial rate of metabolic formation or drug loss as a function of substrate concentration<sup>250</sup>. The second stage involves scaling of the *in vitro* intrinsic clearance estimate to an *in vivo*  $Cl_{int}$ . The hepatic scaling factor will depend on the *in vitro* system initially used. The last stage of the strategy includes the application of a liver model that allows the use of  $Cl_{int}$  to estimate the hepatic clearance of a specific pathway(s). The use of a liver model accounts for the influence of non-enzymatic factors (e.g. hepatic blood flow and plasma/blood protein binding of a drug) in determining the *in vivo* clearance process.

## 6.2 Allometric Scaling

Allometric scaling is based upon an observable relationship between body weight and a physiological parameter, e.g. hepatic or renal blood flow<sup>254</sup>. A similar approach can be applied to pharmacokinetic processes where the allometric scaling method considers the relationship between a physiological parameter and a PK parameter, e.g.

body weight and volume of distribution ( $V_d$ ). Allometric scaling can be applied to correlate human pharmacokinetics with pharmacokinetics in other species<sup>227</sup>.

Numerous physical and physiological functions vary according to some mathematical function of body weight (BW), and appear reasonably uniform across a wide range of animal species. This can allow prediction of PK parameters in one species based on values from other mammalian species<sup>250,255</sup>. In addition to cross-species scaling, allometric scaling can be used to extrapolate within humans, which can be beneficial in adjusting drug dosages in infants and predicting their susceptibility to drug toxicity<sup>256</sup>.

The allometric equation allows prediction of PK parameters from the product of an allometric coefficient and the body weight to a power function represented by Equation 1.11.

$$Y = a.(BW)^b \quad (1.11)$$

where Y is the biological characteristic to be predicted (e.g. clearance), BW is the body weight,  $a$  is the allometric coefficient and  $b$  is the allometric exponent.

Although some success has been achieved using this empirical equation<sup>256</sup>, many predictions are rather poor especially for compounds that undergo hepatic metabolism<sup>250,255</sup>. Boxenbaum<sup>231,255</sup> suggested the incorporation of empirical correction factors such as maximum lifespan potential (MLP) and brain weight (BrW), as correction factors to predict systemic clearance for compounds that undergo oxidative metabolism, Equations 1.12 and 1.13.

$$Cl. MLP = a. (BW)^b \quad (1.12)$$

$$Cl. BrW = a. (BW)^b \quad (1.13)$$

Although these correction factors improve predictions, allometric scaling generally fails to provide accurate quantitative predictions of metabolic clearance in adult or pediatric patients<sup>255</sup>. This is largely due to species difference in rates of drug metabolism and differences in drug metabolism pattern (phase I and phase II).

For high clearance compounds,  $Cl_H$  is limited by  $Q_H$ , which scales allometrically across different species<sup>254</sup>. Typically, allometric models provide reasonable predictions for high clearance drugs. For drugs with low or intermediate clearance values, a more



accurate prediction of  $Cl_H$  could be achieved using allometric scaling that combines *in vitro* hepatocytes and *in vivo* clearance data in animal species with *in vitro* hepatocytes data in humans<sup>250</sup> according to Equation 1.14.

$$Cl_H = Cl_{\text{animal (in vivo)}} \left( \frac{Cl_{\text{human (hepatocytes)}}}{Cl_{\text{animal (hepatocytes)}}} \right) \quad (1.14)$$

Many factors contribute to the uncertainty associated with allometric predictions of hepatic clearance in humans. Such factors include inter-individual variability, extrahepatic metabolism, involvement of an active transport process and inactivation of the metabolic enzymes during processing or storage of the liver samples<sup>235,250,255</sup>.

### 6.3 Physiologically-Based Pharmacokinetic (PBPK) Models

PBPK modeling provides a more scientifically credible extrapolation across species and across different routes and classes of exposure and, therefore, can provide more accurate risk assessments<sup>78,234</sup>. Pharmacokinetic approaches that are used to describe the relationship between the exposure and concentration-time profile are, in many cases, limited and provide little relationship to the physiological processes involved. PBPK, being based on physiological and physicochemical properties of a chemical, provides a more meaningful approach to understand the consequences of an exposure to such a chemical<sup>234</sup> and facilitates prediction of events in humans from animal data<sup>78</sup>.

Physiological parameters used to build a PBPK model include, organ size, structure, blood flow and functions. Chemical parameters include binding with blood constituents, binding to tissues, membrane permeability and sensitivity to enzymatic reactions<sup>251</sup>. However, generation and validation of a PBPK model is resource and time intensive, and it would be neither possible nor practical to generate PBPK models for all chemicals<sup>78,257</sup>.

## 7. Development of a Pharmacokinetic Model of Hepatic CYP-Mediated Elimination

A novel mathematical pharmacokinetic model that describes the postnatal maturation of hepatic human CYP-mediated elimination has been described<sup>77</sup> (See Appendix I for details of the model). This model was developed based on an *in vitro/in vivo* extrapolation of CYP enzyme activity data for CYP-specific probe substrates in age-dependent human fetal and pediatric hepatic microsomes<sup>225,227,251</sup>. The model can be used to predict age- and enzyme-specific infant scaling factors (ISF) that directly correlates adult clearance values with the ability of the infant to eliminate drugs by the same metabolic pathway (j), as shown in Equations 1.15 and 1.16.

$$Cl_{H(j,t)}^{\text{infant}} = ISF_{H(j,t)} Cl_{\text{systemic}}^{\text{adult}} \quad (1.15)$$

$$ISF_{(j,t)} = ISF_{(j,0)} + ISF_D (1 - e^{-kt}) \quad (1.16)$$

Where  $ISF(j,t)$  is the predicted hepatic CYP enzyme-specific infant scaling activity *in vivo* at age,  $t$  (day);  $ISF_{(j,0)}$  is the scaled *in vivo* CYP enzyme activity present at birth;  $ISF_D$  is a fitted parameter; and  $k$  is the first-order rate constant describing the rate of increase in CYP enzyme pathway efficiency ( $\text{day}^{-1}$ ). Comparing the predicted values of clearance by the model with those published in the literature demonstrated that the model gave reasonable predictions for clearance mediated by some CYP pathways; however, poor predictions were shown for other pathways<sup>77</sup>.

Development of a robust and predictive model of CYP-mediated clearance will require extensive hepatic microsomal activity data and an evaluation of the underlying model assumptions. The critical assumptions that were used to build the general model of CYP enzyme ontogeny include<sup>77</sup>:

1. Hepatic microsomal protein (MP) content (mg per g liver weight) is constant throughout postnatal developmental. Therefore an adult MP value (52.5 mg per g liver)<sup>258</sup> was assumed for all infants age groups.
2. Substrate affinity of the CYP enzyme (expressed as the Michaelis-Menten constant,  $K_M$ ) is constant and not a function of developmental age.
3. Age-dependent differences in metabolic clearances reflect exclusively the

differences in the amount of functional enzymes ( $V_{\max}$ ).

4. Catalytic CYP activity is proportional to the functional CYP protein level.
5. The drug has a low hepatic extraction ratio value.
6. Different substrates for the same CYP enzyme should exhibit similar pattern of age-dependent changes in  $V_{\max}$  values.
7. No significant contribution from uptake and/or efflux transporters in the intrinsic clearance.
8. The elimination pathway is known and similar between infants and adults.

If these assumptions are valid then the observed differences in  $Cl_{\text{int}}$  between infants and adults are due to the degree of functional maturation of the particular CYP enzyme or the amount of functional CYP enzyme (i.e.  $V_{\max}$ ).

## 8. Use of Rats as an Animal Model in Studying CYP Ontogeny

As mentioned above, hepatic drug metabolizing enzymes (DME) undergo a significant and dynamic maturation during development. Therefore, simple extrapolation of adult ontogeny data to infants could result in under- or over-predictions of the exposure risk. Development of an accurate toxicological risk assessment tool to predict the potential risk of toxicity in the developing infant following exposure to xenobiotics requires a full understanding of the developmental maturation and factors affecting activities of the DME (e.g. CYP enzymes) that are involved in bioactivation of xenobiotics. Ethical and technical limitations restrict the use of human fetal and infant tissues in drug metabolism studies<sup>60</sup>. Therefore, a suitable animal model system has to be used followed by appropriate extrapolation to human.

Due to the sufficient similarities between rats and human regarding developmental physiology (e.g. liver development), enzymology and molecular mechanisms affecting drug metabolism<sup>1,259</sup>, the rat model system has been used frequently to provide initial information about drug metabolism in human<sup>1,116,260-262</sup>. Many CYP genes, particularly *CYP1A* and *CYP2E1* subfamilies show remarkable conservation between human and rats<sup>2</sup>. These genes are regulated in diverse ways including transcriptional

and posttranscriptional mechanisms<sup>160</sup>. Most of the hepatic CYP genes are under control of liver-enriched transcriptional factors, which themselves undergo certain developmental changes<sup>160,167,169</sup>. Comparison of DNA sequences of both human and rat *CYP2E1* gene revealed a high degree of nucleotide similarities (80-94%) within 150 bp of the RNA polymerase start point<sup>4,263,264</sup>. This could indicate the presence of a well-conserved regulatory region that might bind transcription factors. As well, Hepatocyte Nuclear Factor-1 $\alpha$  (HNF-1  $\alpha$ ) is the primary factor responsible for hepatic expression of *CYP1A2* and *CYP2E1* genes in both rat and human<sup>160,169</sup>. Developmental activation of the genes encoding liver-enriched transcriptional factors determines when a particular CYP enzyme will be expressed. Physiological/hormonal or developmentally-programmed factors that control these genes are still not fully understood both in human and rat<sup>160</sup>.

Expression of CYP genes in human is regulated in a similar manner to rodents<sup>1,160,259</sup>. For example, the induction of *CYP1A* gene in human and rats occurs via the aryl hydrocarbon receptor (AhR), which associates with the AhR nuclear translocator (ARNT) in response to many polycyclic aromatic hydrocarbons (PAHs) such as Benzo[a]Pyrene and 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD)<sup>170</sup>. Similarly, the constitutive androstane receptor (CAR) and pregnane-X-receptor (PXR), both heterodimerize with the retinoid X receptor (RXR)<sup>265-267</sup>. This complex binds to a *cis*-acting sequence in the promoter region of *CYP2B* or *CYP3A* genes leading to induction of the gene expression by xenobiotics such as phenobarbital-like compounds (*CYP2B*) or dexamethasone/ rifampin-type compounds (*CYP3A*) in both human and rats<sup>265,267</sup>.

Due to such close similarity between human and rats regarding CYP basic enzymology, biochemistry and regulation, rat will be used as an animal model to evaluate the underlying assumptions of the PK model of hepatic CYP-mediated clearance (section 8). As well, rat is a commonly used model for toxicological and nutrition studies, in human disease states and in pre-clinical studies. Consequently, the ontogeny of hepatic CYP2E1 and CYP1A2 will be characterized at the hepatic mRNA, protein, activity level and immunolocalization in the liver. The significance of these studies will be twofold: the first is to assess the appropriateness of the rat as a

potential model system. Second, studying the ontogeny on a systems level can provide insight into enzyme regulation and potential sites of perturbation due to various endogenous and exogenous factors during development.

## **9. Relevance of the Project to Paediatric Health and Developmental Toxicology**

The developing infant is exposed to xenobiotics through breastfeeding, drug therapy and from the surrounding environment<sup>233</sup>. The available information regarding the developmental maturation of physiological and biochemical determinants of drug pharmacokinetics (PK) or toxicokinetics (TK) is very limited<sup>250</sup>. Consequently, most of the approaches used to design dosage regimens or assess risk of toxicity following exposures of pediatric populations to xenobiotics are based upon dosages or toxicity information from adult populations<sup>221,233</sup>.

Accurate estimation of the exposure outcome in infants necessitates a full characterization of the developmental maturation (ontogeny) of physiological processes that determine the potential ability of the developing child to handle such exposures. One of the most critical determinants of an exposure outcome is the activity of the xenobiotic elimination mechanisms, especially cytochrome P450 (CYP) enzymes<sup>222</sup>. These enzymes catalyze xenobiotic elimination, but may also bioactivate xenobiotics leading to the generation of reactive intermediates that can induce cellular damage and cancer<sup>98,100</sup>. Because these enzymes undergo significant and dynamic maturation during fetal and postnatal maturation<sup>110,268</sup>, the developing infant is expected to respond in a different way than the adult to the same exposure dose (normalized to body weight)<sup>229,233</sup>. Accordingly, simple adult-to-infant extrapolation of toxicity data can lead to an underestimation (or sometimes overestimation) of the toxicological risk to xenobiotic exposure<sup>221,224</sup>. The consequences of this inaccurate risk assessment can sometimes be very serious, e.g. infant toxicity from drugs in breast milk or discontinuation of either breastfeeding or nursing maternal therapy.

Being critical in determining the internal dosimetry of the exposure dose, the degree of maturation of these CYP enzymes at a particular developmental stage will determine the pharmacological/toxicological outcome to this exposure at that particular

developmental stage<sup>224</sup>. Unfortunately, limited information is available regarding the ontogeny of CYP enzymes in pediatric populations.

As an initial step towards improving risk assessment in paediatric populations, a general ontogeny model was developed to predict age-specific CYP enzyme activity<sup>77</sup>. This model could be a promising risk assessment tool if it has the ability to predict the capacity of an infant to eliminate specific CYP substrates. One of the purposes of this dissertation is to evaluate the underlying assumptions of this model and identify all model parameters needed to account for the developmental patterns of the various CYP enzymes. Furthermore, as a proof-of-concept, the potential ability of the model to predict *in vivo* clearance of CYP substrates will be assessed in a rat model.

Another important purpose of this dissertation is to extensively characterize the ontogeny of CYP enzymes in a rat animal model at the mRNA, protein, activity and intrahepatic expression levels. Although interspecies differences exist in the rate and pattern of CYP enzyme maturation, characterization of CYP ontogeny in rats and a comparison with known human CYP ontogeny may identify a model system to investigate underlying toxicokinetic mechanisms for age-dependent differences in susceptibility to toxicity. The outcome of this study will have a significant impact in risk assessment in paediatric populations as it will allow risk estimation during critical developmental windows where the toxic effects would not be observable in adults<sup>229,233</sup>.

A third objective of this study was to characterize the ontogeny pattern of the important phase II enzymes, glutathione-S-transferases (GST)<sup>207,268</sup>. GST enzymes play a key role in the detoxification of reactive metabolites generated by CYP-mediated reactions<sup>207</sup>. A parallel characterization of CYP and phase II enzyme activity during maturation is very significant in estimating the developmental changes in the bioactivation/detoxification balance and the exposure outcome to the developing infant.

Overall the main aim of this research project is to improve exposure risk assessment in paediatric populations through the potential identification of an animal model for extrapolation of developmental toxicological data to humans and the development of a PK model that describes CYP enzyme ontogeny to provide a predictive model for risk assessment.

## 2. PURPOSE OF PROJECT

The overall purpose of this dissertation research is to characterize the developmental maturation of two toxicologically relevant CYP enzymes, CYP2E1 and CYP1A2, and general GST activity in the rat and to evaluate the assumptions of a pharmacokinetic model that describes the postnatal maturation of these CYPs and the model's ability to predict *in vivo* elimination capacity. To achieve this purpose, the research addresses the following objectives and hypotheses:

### Objective 1

To evaluate the underlying assumptions of the general PK model describing the postnatal maturation of hepatic cytochrome P450 enzyme activity using a male Sprague-Dawley rat model system.

*Hypothesis:* Age-dependent increases in intrinsic clearance are attributed only to changes in  $V_{\max}$  (the Michaelis-Menten parameter that is proportional to the quantity of metabolically active enzyme/mg microsomal protein).

#### *Specific aims*

- a. Development and validation of HPLC assays for probe substrates and metabolites of CYP2E1 and CYP1A2 to serve as markers for the maturation of CYP enzyme activity.
- b. Assessment of the age-dependent changes in microsomal protein content, liver weight and body weight from late gestation to adulthood in rats.
- c. Characterization of the Michaelis-Menten parameters ( $K_M$  and  $V_{\max}$ ) of CYP-specific substrates in age-dependent rat hepatic microsomes. This will allow the assessment of the age-dependent changes in intrinsic clearance of specific CYP2E1 and CYP1A2 probe substrates.

- d. Characterization of the developmental changes in  $V_{\max}$  using a second probe substrate to test whether enzyme activity assessed from one substrate will accurately predict the level of activity for all substrates of the same enzyme.
- e. Determination of age-dependent changes in CYP2E1 and CYP1A2 protein levels using immunoblotting technique followed by comparison with the developmental changes in enzyme activity ( $V_{\max}$ ). This will test if there is proportional relationship between enzyme activity and protein level during development.

**Objective 2:** To develop a PK model describing the ontogeny of hepatic CYP2E1 and CYP1A2 activity in a rat model system.

*Specific aims:*

- a. Estimation of the age-dependent changes in infant scaling factors (ISF) using the calculated values of HSF and  $V_{\max}$  at postnatal and adult ages in rats.
- b. Use of a PK modeling software to model age-dependent changes in ISF.
- c. Assessment of the ability of CYP2E1 model to predict the oral clearance of chlorzoxazone, a CYP2E1 probe substrate, in rats.

**Objective 3:** To characterize the ontogeny of rat hepatic CYP2E1 and CYP1A2 at mRNA, protein, activity and intrahepatic expression levels. Comparison of the expression data with known information about the ontogeny of these enzymes in human livers would provide evidence for the use of the rat as a model for developmental toxicokinetic studies.

*Hypothesis:* Age-dependent changes in mRNA, protein expression, activity and hepatic zonal expression patterns in rat are similar to developmental patterns in human livers reported in the literature.

*Specific aims*

- a. Evaluation of developmental changes in rat hepatic CYP2E1 and CYP1A2 mRNA expression using real-time RT-PCR.
- b. Evaluation of rat hepatic CYP2E1 and CYP1A2 protein expression in age-dependent rat hepatic microsomes using specific antibodies and immunoblotting techniques.



- c. Evaluation of rat hepatic CYP2E1 and CYP1A2 maximum enzyme activity ( $V_{\max}$ ) in age-dependent rat hepatic microsomes using saturating concentrations of specific probe substrates.
- d. Characterization of the age-dependent changes in hepatic zonal expression of CYP2E1 and CYP1A2 enzymes in frozen liver sections using immunohistochemical analysis.

**Objective 4:** To evaluate the metabolic balance between hepatic CYP2E1 or CYP1A2 and GST during development.

*Hypothesis:* The ontogeny of hepatic GST activity parallels the activity of hepatic CYP2E1 and CYP1A2 enzymes.

*Specific aims*

- a. Assessment of the ontogeny of GST activity in age-dependent rat hepatic cytosols using a general probe substrate.
- b. Comparison of the ontogeny of hepatic CYP2E1 and CYP1A2 with GST activity.

### 3. MATERIALS AND METHODS

#### 3.1 Materials

##### 3.1.1 Animals

Sprague-Dawley rats were obtained from Charles River Canada (St. Constant, PQ). Eight pregnant rats were received at day 14 of pregnancy and were allowed to acclimatize for one week. At day 21 of pregnancy, four pregnant rats were anesthetized under isoflurane and the fetuses were collected. The remaining pregnant rats were allowed to give birth and one male pup from each dam was collected at postnatal ages 1, 3, 5, 7 and 10 days. Male Sprague-Dawley rats at ages 1, 2, 3, 5, 8, 11 and 15 weeks (n =4) were obtained from Charles River Canada (St. Constant, Quebec) and allowed an acclimatization period of one week. Rats were housed under controlled temperature ( $22 \pm 2^{\circ}\text{C}$ ), and were maintained on a 12 hour light:dark cycle (0700 to 1900 hours). All rats received standard laboratory rat chow and water *ad libitum* throughout the acclimatization period. At the appropriate age, total body weights were recorded. Subsequently, rats were lightly anaesthetized (isoflurane), killed by guillotine, and their livers rapidly removed and rinsed in ice-cold 0.9% NaCl. Wet liver weights were recorded and the liver tissues were divided so that mRNA and protein expression levels, enzyme activity, and immunohistochemistry (IHC) could be simultaneously studied. Liver tissues to be used for microsomal preparation and IHC were flash frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until use. Liver samples for mRNA expression analysis were stored in RNAlater™ (Sigma-Aldrich, Oakville, ON) solution at  $-20^{\circ}\text{C}$  until RNA extraction.

All procedures were conducted in accordance with the Canadian Council of Animal Care guidelines for the care and use of laboratory animals and were approved by the Animal Care and Supply Committee of the University of Saskatchewan.

### 3.1.2 Chemicals and Reagents

Chlorzoxazone (CZX), 6-hydroxychlorzoxazone (6-OH CZX), umbelliferone (UMB), *p*-nitrophenol (PNP), *p*-nitrocatechol (4NC), salicylamide, phenacetin, acetaminophen, caffeine, resorufin, methoxyresorufin (MR) and all chemicals used for microsomal preparation, determination of microsomal protein content and enzyme assays were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Acetonitrile and methanol were high pressure liquid chromatography (HPLC) grade obtained from EMD chemicals (Durham, NC). Rat CYP2E1 and CYP1A2 supersomes were obtained from BD Bioscience (Woburn, MA, USA). Prestained SDS-PAGE standards, peroxidase-conjugated antibody (Goat anti-rabbit IgG HRP conjugate), Immun-Star HRP kit, Bio-Rad Trans-Blot cell and polyvinylidene difluoride (PVDF) membranes (0.2  $\mu$ m) were purchased from Bio-Rad Laboratories (Hercules, CA). Rabbit anti-Human/Rat CYP2E1 and sheep anti-rat CYP1A2 polyclonal antibodies and rabbit anti-sheep IgG HRP conjugate antibody were obtained from Chemicon International (Temecula, CA). RNeasy Midi columns for RNA extraction and QuantiTect RT-PCR kit were obtained from Qiagen (Mississauga, ON). Biotinylated goat anti-rabbit IgG H&L was purchased from Abcam (Hornaby, ON). All other chemicals used were analytical grade.

## 3.2 Methods

### 3.2.1 Preparation of Hepatic Microsomes and Cytosols

Hepatic microsomes were prepared as described previously<sup>269</sup>. Briefly, 0.5 g liver samples were accurately weighed and homogenized in 2 mL ice-cold homogenization buffer (50 mM Tris buffer, 150 mM KCl, 0.1 mM dithiothreitol, 1 mM Ethylenediamine tetraacetate (EDTA), 20% glycerol and 0.1 mM phenylmethylsulfonylfluoride) using a Polytron homogenizer. The homogenate was centrifuged at  $9000 \times g$  for 30 min at 4°C in a Beckman L8-55 Ultracentrifuge (Palo Alto, CA). The supernatant was carefully transferred to clean ultracentrifuge tubes and centrifuged at  $100\,000 \times g$  for 30 min at 4°C. The supernatant (the cytosol) was removed and transferred to labeled tubes and

stored at -80°C until use. The pellet was washed in 2 mL of ice-cold 150 mM KCl and centrifuged again at  $100\,000 \times g$  for 30 min at 4°C. The pellet was resuspended in 2 mL of ice-cold 0.25 M sucrose solution and aliquots were stored in cryogenic microcentrifuge tubes (400  $\mu$ L) at -80°C until use.

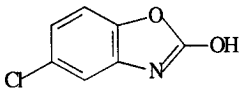
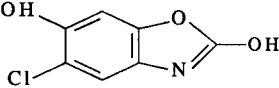
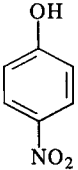
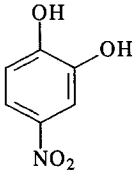
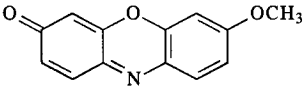
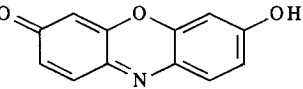
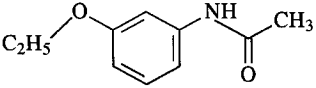
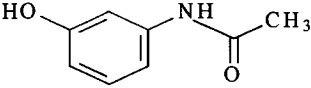
### 3.2.2 Determination of Microsomal and Cytosolic Protein Content

Protein concentrations of microsomal and cytosolic fractions were determined in triplicate by the method of Lowry *et al*<sup>270</sup> using bovine serum albumin as a calibration standard. Absorbance was measured at 750 nm on an Agilent 8453E UV-visible spectrophotometer using Chemstation software (Palo Alto, CA). Cytochrome P450 content was estimated by absorbance differences between 450 and 490 nm based on the method of Omura and Sato<sup>271</sup>.

### 3.2.3 Microsomal Incubations with CYP2E1 and CYP1A2 Probe Substrates

Preliminary experiments in 50-day old male rat hepatic microsomes were conducted to determine the optimum incubation conditions (incubation time and microsomal protein content) to give linear metabolite formation kinetics for CYP2E1-mediated hydroxylation of CZX and PNP and CYP1A2-mediated methoxyresorufin-O-dealkylation (MROD) and phenacetin-O-dealkylation (POD) activities. Michaelis-Menten parameters,  $K_M$  and  $V_{max}$ , for CYP2E1 and CYP1A2 were determined by measuring formation velocities of 6 OH-CZX and resorufin at substrate concentrations of 0 to 1000  $\mu$ M and 0 to 1000 nM, respectively. Metabolite formation velocities for PNP and phenacetin were measured at saturating probe substrate concentrations of 500 and 150  $\mu$ M respectively, in age-specific rat hepatic microsomes to provide estimates of  $V_{max}$ . Table 3.1 demonstrates enzymatic reactions mediated by CYP2E1 and CYP1A2 enzymes, their substrates and the major metabolites formed by these reactions.

**Table 3.1** – Selected enzymatic reactions mediated by CYP2E1 and CYP1A2, their selective substrates, formed metabolites and analytical methods used for measuring enzyme activity.

Enzyme	Reaction	Substrate	Metabolite	Method of quantitation
CYP2E1	Hydroxylation	chlorzoxazone	6-hydroxychlorzoxazone	HPLC-UV detection
				
CYP1A2	Dealkylation	p-nitrophenol	4-nitrocatechol	HPLC-UV detection
				
CYP1A2	Dealkylation	methoxyresorufin	resorufin	HPLC-fluorescence detection
				
CYP1A2	Dealkylation	phenacetin	acetaminophen	HPLC-UV detection
				

### 3.2.3.1 CZX hydroxylation activity

Microsomal incubation mixtures consisted of CZX (0-1000  $\mu\text{M}$ ), 0.4 mg/mL liver microsomal protein, 2 mM  $\text{MgCl}_2$ , 1 mM NADPH, and 50 mM phosphate buffer, pH 7.4, in a final volume of 0.5 mL. Incubations were carried out at 37°C in uncapped 12×75 mm glass culture tubes in a shaking water bath. The reaction was terminated by addition of 50  $\mu\text{L}$  ice-cold phosphoric acid (50 %, v/v in water) followed by the addition of 50  $\mu\text{L}$  of the internal standard solution (0.078 mM umbelliferone). The mixtures were vortex mixed for 20 s. After centrifugation at 12 000  $\times g$  in an Eppendorf microcentrifuge (Model 5417 C, Brinkman instruments, Westbury, NY) for 20 min, 30  $\mu\text{L}$  of the supernatant was injected directly onto the analytical column for immediate HPLC analysis of 6-OH CZX.

### 3.2.3.2 PNP hydroxylation activity

Microsomal incubation mixtures consisted of PNP (500  $\mu\text{M}$ ), 0.4 mg/mL liver microsomal protein, 1 mM ascorbic acid, 2 mM  $\text{MgCl}_2$ , 1 mM NADPH, and 50 mM phosphate buffer, pH 6.8, in a final volume of 0.5 mL. After a preincubation period of 1 min at 37°C, the reaction was started by addition of NADPH and incubated at 37°C in glass culture tubes for 30 min in a shaking water bath. The reaction was terminated by addition of 50  $\mu\text{L}$  ice-cold phosphoric acid (50 %, v/v in water) then 50  $\mu\text{L}$  of the internal standard solution (salicylamide, 6 $\mu\text{g}/\text{mL}$ ) was added. The mixtures were vortex mixed for 20 s. After centrifugation at 12 000  $\times g$  for 20 min, 20  $\mu\text{L}$  of the supernatant was injected directly onto the analytical column for immediate HPLC analysis of 4NC.

### 3.2.3.3 Methoxyresorufin-O-dealkylation (MROD) activity

Incubation mixtures contained 50 mM potassium phosphate buffer (pH 7.4), 2 mM  $\text{MgCl}_2$ , 0.08 mg/mL liver microsomal protein, methoxyresorufin (0-1000 nM) and 1 mM NADPH in a final volume of 0.5 mL. Methoxyresorufin was dissolved in methanol

(final concentration <1%). After pre-incubation for 1 minute, the reaction was started by the addition of NADPH. The mixture was incubated at 25°C for 8 minutes with shaking. The reaction was stopped by placing the tubes on ice and adding 0.5 mL ice-cold methanol with vortexing. After cooling on ice for 15 minutes, the mixture was centrifuged at 6000 × g for 20 minutes. The supernatant was filtered with a membrane filter of 0.45 µm pore size. Fifty µL of the filtrate was analyzed by HPLC for resorufin.

#### *3.2.3.4 Phenacetin-O-dealkylation (POD) activity*

Incubation mixtures contained 50 mM potassium phosphate buffer (pH 7.4), 4 mM MgCl<sub>2</sub>, 0.4 mg/mL rat liver microsomal protein, phenacetin (150 µM) and 1 mM NADPH in a final volume of 0.5 mL. Phenacetin, dissolved in methanol, was added to the incubation tubes and evaporated at 45°C with the vacuum concentrator. The incubation mixture, except for microsomes and NADPH was redissolved by sonication for 1 minute. The mixture, including microsomes and NADPH, was incubated at 37°C for 60 minutes with shaking. The reaction was stopped by placing the tubes on ice and adding 100 µL cold acetonitrile followed by the addition of 50 µL internal standard solution (Caffeine, 5 µg/mL in methanol). The mixture was centrifuged at 10 000 × g for 5 minutes, and the supernatant was evaporated at 45°C for 15 minutes with a vacuum concentrator. Fifty µL of the remaining sample was analyzed by HPLC for acetaminophen.

#### *3.2.4 HPLC Analyses*

Metabolite formation velocities in rat hepatic microsomal preparations were monitored using validated HPLC methods. The HPLC system consisted of Waters Model 600 solvent delivery system, Model 486 variable UV-VIS detector or a model 2475 multiwavelength fluorescence detector, Model 717 Plus autosampler and a Millennium data module (Millipore-Waters, Milford, MA, USA). All chromatographic separations were carried out on a reversed phase C<sub>18</sub> column (Supelcosil<sup>®</sup> 150 × 4.6 mm I.D., 5 µm particle size) maintained at 25°C.

HPLC method validation procedures were performed according to FDA guidelines to evaluate the suitability of the method for the quantitative determination of the analyte(s) of interest in age-specific rat hepatic microsomes. Specificity was tested by analysis of four different rat hepatic microsomal preparations supplemented only with internal standard to ensure the absence of endogenous compounds with the same retention times as the metabolite of interest. The linearity of the method was evaluated by processing a 5-10 point calibration standards spiked into blank hepatic microsomal suspensions on different days. The peak height ratios between metabolite and the internal standard were plotted against the nominal concentration of the metabolite. A linear least-squares regression analysis was conducted to determine slope, intercept and coefficient of determination ( $r^2$ ) to demonstrate linearity of the method. The accuracy and precision of the proposed method were determined by analysis of the quality control (QC) samples. The intra-day accuracy and precision were assessed from the results of six replicate analyses of QC samples (low, medium and high) on a single assay day. The inter-day accuracy and precision were determined from the same QC samples analyzed on 3-6 consecutive days. Precision is expressed as % relative standard deviation (RSD), while accuracy (%) is expressed as [(calculated amount/predicted amount) x100]. The limit of detection (LOD) was defined as the lowest detectable concentration, taking into consideration a signal-to-noise ratio of 3. Limit of quantification (LOQ) was determined at the lowest concentration at which the precision, expressed as % RSD, is less than 20% and accuracy, expressed as relative difference between the measured and true value, is less than 20%.

#### *3.2.4.1 CZX hydroxylation activity*

Chlorzoxazone 6-hydroxylation to 6 hydroxychlorzoxazone (6-OH CZX) was assayed by a modification of a previously reported method<sup>272</sup>. UV absorbance was monitored at 295 nm ( $\lambda_{\text{max}}$  for 6-OH CZX). CZX, 6-OH CZX, and umbelliferone (internal standard) were eluted under isocratic conditions using a mobile phase composed of acetonitrile and 0.25% acetic acid (20:80) delivered at 1.0 mL/min. Calibration curves were constructed from known concentrations of 6-OH CZX working



solutions and 50  $\mu\text{L}$  of the internal standard solution (0.078 mM umbelliferone) added to heat-inactivated (55°C for 5 min) rat hepatic microsomes and diluted with 50 mM phosphate buffer (pH 7.4) to achieve calibration standards of 0.31-40  $\mu\text{M}$  6-OH CZX in a total volume of 0.5 mL. Three quality control (QC) samples at 0.625  $\mu\text{M}$  (low), 1.25  $\mu\text{M}$  (medium), and 5  $\mu\text{M}$  (high) were prepared independent of those used for the calibration curves. The mixtures were vortex mixed for 20 s. After centrifugation at 12 000  $\times g$  in an Eppendorf microcentrifuge (Model 5417 C, Brinkmann instruments, Westbury, NY, USA) for 20 min, 30  $\mu\text{L}$  of the supernatant was injected directly onto the analytical column for immediate HPLC analysis. Concentration of the metabolite was calculated by interpolation from the linear calibration curve using measured metabolite to internal standard peak height ratios. The metabolite formation rates were calculated in units of nmol 6-OH CZX formed per minute per mg microsomal protein.

#### 3.2.4.2 PNP hydroxylation activity

*p*-Nitrophenol (PNP) hydroxylation to *p*-nitrocatechol (4NC) was assayed as reported previously<sup>273</sup>. UV absorbance was monitored at 250 nm ( $\lambda_{\text{max}}$  for 4NC). PNP, 4NC, and salicylamide (internal standard) were eluted under isocratic conditions using a mobile phase composed of 22% acetonitrile, 0.1% trifluoroacetic acid and 0.5% triethylamine delivered at 1.0 mL/min. Known amounts of 4NC working solutions were added to heat-inactivated (55°C for 5 min) rat hepatic microsomes and diluted with 50 mM phosphate buffer (pH 6.8) to achieve calibration standards of 0.1-40  $\mu\text{M}$  4NC in a total volume of 0.5 mL. Three quality control (QC) samples at 0.5  $\mu\text{M}$  (low), 5  $\mu\text{M}$  (medium), and 20  $\mu\text{M}$  (high) were prepared independent of those used for the calibration curves. These QC samples were prepared on the day of analysis in the same way as calibration standards. To 500  $\mu\text{L}$  calibration standards, QC samples, or microsomal incubation mixtures, 50  $\mu\text{L}$  ice-cold phosphoric acid (50 %, v/v in water) and 50  $\mu\text{L}$  of salicylamide solution (6  $\mu\text{g}/\text{mL}$  in methanol) were added. The mixtures were vortex mixed for 20 s. After centrifugation at 12 000  $\times g$  in an Eppendorf microcentrifuge (Model 5417 C, Brinkmann instruments, Westbury, NY, USA) for 20 min, 20  $\mu\text{L}$  of the supernatant was injected directly onto the analytical column for immediate HPLC

analysis of 4NC. The metabolite formation rates were calculated in units of nmol 4NC formed per minute per mg microsomal protein.

#### *3.2.4.3 Methoxyresorufin-O-dealkylation (MROD) activity*

MROD activity was assayed by an HPLC method with fluorescence detection as reported previously<sup>274</sup>. The excitation and emission wavelengths were fixed at 560 and 585 nm, respectively. The mobile phase consisted of 20 mM phosphate buffer (pH 6.8), methanol and acetonitrile at a ratio of 50:45:5, delivered at 0.8 mL/min. Known amounts of resorufin working solutions were added to heat-inactivated (55°C for 5 min) rat hepatic microsomes and diluted with 50 mM phosphate buffer (pH 7.4)-methanol (50:50) to achieve calibration standards of 0.04-2 nM. Three quality control (QC) samples at 0.10 nM (low), 0.40 nM (medium), and 1.0 nM (high) were prepared independent of those used for the calibration curves. These QC samples were prepared on the day of analysis in the same way as calibration standards. To 500  $\mu$ L calibration standards, QC samples, or microsomal incubation mixtures, 50  $\mu$ L ice-cold methanol was added. The mixtures were vortex mixed for 20 s. After centrifugation at 6 000  $\times$  g in an Eppendorf microcentrifuge (Model 5417 C, Brinkmann instruments, Westbury, NY, USA) for 20 min, the supernatant was filtered with a membrane filter of 0.45  $\mu$ m pore size. Fifty  $\mu$ L of the filtrate was analyzed by HPLC for resorufin. The metabolite formation rates were calculated in units of pmol resorufin formed per minute per mg microsomal protein.

#### *3.2.4.4 Phenacetin-O-dealkylation (POD) activity*

Phenacetin-O-dealkylation (POD) to acetaminophen activity was monitored by an HPLC method as reported before<sup>177</sup> using caffeine as an internal standard. UV absorbance was monitored at 254 nm ( $\lambda_{\max}$  for acetaminophen). The mobile phase consisted of 50 mM potassium dihydrogen phosphate and acetonitrile at a ratio of 85:15 (v/v (%)) and delivered at 1.0 mL/min. Acetaminophen was quantified based on calibration curves constructed from a series of standards containing varying known

amounts of acetaminophen (0.2-20  $\mu\text{M}$ ) and the internal standard (caffeine 5  $\mu\text{g/mL}$ ) in heat-inactivated rat hepatic microsomes and 50 mM phosphate buffer (pH 7.4). Three quality control (QC) samples at 0.5  $\mu\text{M}$  (low), 2  $\mu\text{M}$  (medium), and 10  $\mu\text{M}$  (high) were prepared independent of those used for the calibration curves. These QC samples were prepared on the day of analysis in the same way as calibration standards. The rate velocities were calculated in units of nmol acetaminophen formed per minute per mg microsomal protein.

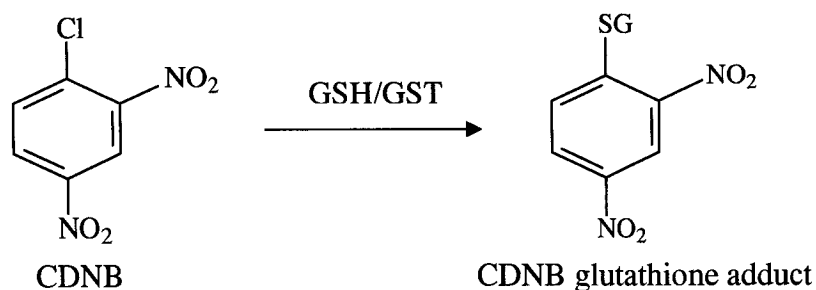
### 3.2.5 Measurement of General Glutathione-S-Transferases (GST) Activity

Ontogeny of GST activity was monitored by spectrophotometric analysis of glutathione conjugation of 1-chloro-2,4-dinitrobenzene (CDNB), a general GST probe substrate<sup>275</sup> as illustrated in Figure 3.1. This method determines the activity of numerous GST isoforms with the exception of GST- $\tau$ <sup>276</sup>. A complete assay mixture without the enzyme was used as a control. Assays were conducted in a thermostated cell compartment at 25°C containing 0.1 M phosphate buffer at pH 6.5, GSH (2.5 mM), 1-chloro-2, 4-dinitrochlorobenzene (1.6 mM), and cytosol from different age groups. The reaction was initiated by the addition of age-dependent cytosolic fraction and the GST activity was determined by monitoring the change in absorbance at 340 nm for both control and sample over a 5-minute period. GST activity was calculated according to equations 3.1 and 3.2.

$$\text{GST activity (nmol/min)} = \frac{\Delta\text{Abs/min (sample)} - \Delta\text{Abs/min (control)}}{9.6} \times \text{DF} \quad (3.1)$$

$$\text{Cytosolic GST activity (nmol/min/mg)} = \frac{\text{GST activity}}{\text{mg protein}} \quad (3.2)$$

Where, 9.6 (nmol/cm) is the molar extinction of CDNB and DF is the dilution factor for rat liver cytosol.



**Figure 3.1-** Glutathione-S-Transferases (GST)-mediated conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with glutathione (GSH)

### 3.2.6 Age-Dependent Scaling Factors and Model Development

Hepatic scaling factors for a particular age ( $HSF_{(t)}$ ) were estimated from the product of microsomal protein yield ( $MP_{(t)}$ ) (mg microsomal protein per gram liver) and liver weight (g) ( $LW_{(t)}$ ) normalized to body weight (g) ( $BW_{(t)}$ ) as a function of developmental age,  $t$ , according to Equation 3.3.

$$HSF_{(t)} = MP_{(t)} \times \frac{LW_{(t)}}{BW_{(t)}} \quad (3.3)$$

Normalization of HSF of a particular developmental age to HSF in adult produced age-specific relative hepatic scaling factor ( $RHSF_{(t)}$ ) that relates the abundance of microsomal protein content per body weight in infant to the adult, Equation 3.4.

$$RHSF_{(t)} = \frac{HSF_{(t)}}{HSF_{(adult)}} \quad (3.4)$$

As well, age-specific ontogeny scaling factor ( $OSF_{(t)}$ ) were determined by normalizing functional enzyme activity ( $V_{max(t)}$ ) observed at a particular age ( $t$ ) to that

observed in adult, Equation 3.5. Accordingly, OSF represents the elimination capacity of a specific enzyme in the infant relative to the adult.

$$\text{OSF}_{(t)} = \frac{V_{\max (t)}}{V_{\max (\text{adult})}} \quad (3.5)$$

The product of these scaling factors,  $\text{RHSF}_{(t)}$  and  $\text{OSF}_{(t)}$ , is the age-specific infant scaling factor ( $\text{ISF}_{(t)}$ ) that represents the ontogeny of an individual functional enzyme normalized to a body size, Equation 3.6, and correlates adult *in vivo* clearance due to an individual CYP enzyme to the capacity of the infant at age (t) to eliminate drugs by the same metabolic pathway, Equation 1.15.

$$\text{ISF}_{(t)} = \text{RHSF}_{(t)} \text{OSF}_{(t)} \quad (3.6)$$

$$\text{Cl}_{\text{H}(j,t)}^{\text{infant}} = \text{ISF}_{(j,t)} \text{Cl}_{\text{H}(j,t)}^{\text{adult}} \quad (1.15)$$

### 3.2.7 Protein Expression Analyses

Microsomal proteins were separated on a sodium dodecyl sulfate gel containing 10% polyacrylamide (SDS-PAGE) by the method of Laemmli *et al*<sup>277</sup>. Heat denatured microsomes (10.5  $\mu\text{g}$  for CYP2E1 and 0.4  $\mu\text{g}$  for CYP1A2), rat CYP2E1 purified protein (20, 50 & 100 pmol) or rat CYP1A2 purified protein (20, 80, 200 and 500 pmol) were loaded onto the gel and the electrophoresis was carried out at 125 volts for 1.5 to 2 hr. Proteins were transferred onto a PVDF membrane with a Bio-Rad Trans-Blot cell at 25 volts for 50 minutes. The membrane was blocked with phosphate-buffered saline (PBS) containing 0.05% tween 20 (PBSt). Subsequently, the membrane was blocked with PBSt containing 5% milk incubated overnight at 4°C. Membrane was then incubated with Rabbit anti-Human/Rat CYP2E1 polyclonal antibody prepared in the blocking solution (1:750) for 3 hr at 20°C or incubated with sheep anti-rat CYP1A2 polyclonal antibody prepared in PBS solution (1:1000 dilution) overnight at 4°C. After washing 3 times with PBSt and once with PBS, the

membrane was incubated with peroxidase-conjugated antibody prepared in the blocking solution (1:3000) for 2 hr at 20°C. After 3 washes with PBSt and one wash with PBS, the peroxide conjugate was detected with the Immun-Star HRP kit. The blot was scanned with an LKB laser densitometer (Amersham Pharmacia Biotech, Piscataway, NJ) to estimate the amount of reacting protein. Reference samples were used to calibrate determinations and results were expressed as absorbance unit/ $\mu\text{g}$  sample protein.

### 3.2.8 mRNA Expression Analyses

#### 3.2.8.1 RNA isolation

RNeasy Midi RNA isolation kits (Qiagen) were used to isolate total RNA following the manufacturer's protocol. Liver tissue (250 mg) was homogenized in buffer RLT and centrifuged ( $4500 \times g$ ) to remove cellular debris. Following centrifugation, the lipid layer was removed and the supernatant transferred to a clean 15 mL polypropylene tube and one volume of 70% ethanol was added. The sample was mixed vigorously and then applied to the Midi column. The column was washed with a series of buffers following the manufacturer's protocol. Total RNA was eluted from the Midi column with 250  $\mu\text{L}$  RNase-free water. Total RNA concentration was determined by the measurement of absorbance at 260 nm with a UV/VIS spectrophotometer (#8453E, Agilent Technologies, Palo Alto, CA) (Equation 3.7). Total RNA purity was verified by measuring the ratio of  $A_{260}/A_{280}$  of a 1:100 dilutions (RNA: 10mM TrisCl pH 7.5). Ratio between 1.9 and 2.1 indicates good purity.

$$[\text{RNA}] = 40 \mu\text{g/ml} \times A_{260} \times \text{Dilution factor} \quad (3.7)$$

#### 3.2.8.2 Primer design

Gene sequences used for primer design were obtained from NCBI Genebank ([www.ncbi.nih.gov](http://www.ncbi.nih.gov)). Primer sequences (Table 3.2) were generated with assistance from

the *Primer3* software ([www.broad.mit.edu/cgi-bin/primer/primer3](http://www.broad.mit.edu/cgi-bin/primer/primer3)) to determine the expression levels of *CYP2E1* and *CYP1A2* genes.

**Table 3.2** – Primer sequences and amplicon sizes for real-time RT-PCR analysis.

Gene	Sequence ID	Left Primer (Sense)	Right Primer (Antisense)	Amplicon Size (bp)
<i>CYP2E1</i>	NM 031543	tccaacctacccatgaagc	ccaacacacacacgctttcc	221
<i>CYP1A2</i>	NM 012541	gggtgttgagaggcacaagg	cactagggcctgcttgatgg	221
<i>β-actin</i>	NM 031144	agcgtggctacagcttcacc	tgccacaggattccataccc	237

### 3.2.8.3 Real-Time RT-PCR

Real time RT-PCR was carried out with the SmartCycler® (Cepheid, Sunnyvale, CA) in one step using Qiagen QuantiTect RT-PCR kit according to the manufacturer’s protocol with slight modifications. Briefly, reactions were performed in a final volume of 25  $\mu$ L containing aliquots of 40 ng of total RNA and primers at a final concentration of 1  $\mu$ M. The RT-PCR protocol (Table 3.3) consisted of 1 cycle at 50°C for 30 min, followed by 95°C for 15 min. Three-step thermocycling was performed for 40 cycles: denaturation at 94°C for 15 s, annealing at 55 or 58°C for 30 s and extension at 72°C for 30 s. Standard curves were constructed by serial dilution of total RNA in RNase-free water with linear mRNA expression ranging from 0.05-500 ng.

**Table 3.3** – General real-time RT-PCR protocol

Step	Time	Temperature (°C)
Reverse Transcription	30 min	50
PCR Initial activation step	15 min	95
Denaturation	15 sec	94
Annealing	30 sec	varied
Extension	30 sec	72

The standard curves were generated by plotting crossing point (Ct) values (the PCR cycle number required for fluorescence intensity to exceed an arbitrary threshold in the exponential phase of the amplification) versus log of the amount of

total RNA added to the reaction and the amount of CYP2E1 or CYP1A2 RNA in all age-related liver samples were interpolated from the standard curve as arbitrary units. Fold changes in mRNA expression for all ages were calculated by comparing the mean ratio (mRNA expression / total RNA) at each age group to the adult ratio. Absence of genomic DNA contamination in the RNA samples was confirmed by the use of RT-negative RNA samples. Specific PCR products were confirmed with 2% agarose gel stained with ethidium bromide where the amplified PCR products showed a single band at the expected amplicon size.

During the optimization process, annealing temperature was selected based upon the highest primer efficiency (E) according to equation 3.8.

$$\text{Slope} = \frac{-1}{\log E} \quad (3.8)$$

These optimization experiments also were performed to ensure that the efficiency of the target and the internal control gene ( $\beta$ -actin) was approximately equal and slopes fell within a range of -2.7 to -3.6. For primer and assay optimization, the slope was measured from a three-point calibration curve using serial dilutions of control RNA (adult female Wistar rat) with final total RNA concentrations of 50 ng, 5 ng and 0.5 ng. Results of age-dependent changes in mRNA expression will be expressed as mean  $\pm$ SD of the ratio CYP2E1 RNA/total RNA as a ratio to the adult value.

### 3.2.9 Immunohistochemical Analyses

Fresh liver tissues were carefully embedded in OCT compound (Sakura Finetek, Torrance, CA) in plastic mold, taking care not to trap air bubbles surrounding the tissue. Tissue was allowed to freeze by setting the mold on top of liquid nitrogen until 70-80% of the block turns white, the block was subsequently put on top of dry ice for 15-20 min and stored at -80°C. Frozen blocks were allowed to equilibrate in the cryostat chamber for about 5 minutes before cutting the sections. Sections (8  $\mu$ m) were obtained at temperatures between -18 to -20°C. The sections were fixed in buffered formalin (4%



formaldehyde in PBS) for 3 min at room temperature. The sections were then incubated for 15 min (CYP2E1) or 30 min (CYP1A2) in PBS containing 0.9% v/v hydrogen peroxide to block any endogenous peroxidase activity. All subsequent operations were carried out in a humidified chamber, washing the sections with PBS (1x) between each incubation. For blocking nonspecific binding, sections were incubated in 5% goat serum and 1% bovine serum albumin in PBS for 20 min (CYP2E1) or for 3 hrs (CYP1A2). Sections were incubated for 4 hr with rabbit anti-human/rat CYP2E1 polyclonal antibody (1:250 in the blocking solution) or with sheep anti-rat CYP1A2 polyclonal antibody (1:100 in the blocking solution) at room temperature. Negative control samples (without adding the primary antibody) were applied to test the specificity of the antibody used. After washing with PBS, slides were incubated for 40 min with biotinylated anti-rabbit IgG (1:200 in PBS). This was followed by incubation with avidin-biotinylated horseradish peroxidase H reagent (*Vectastain ABC kit; Vector Laboratories, Burlingame, CA*) for 75 min in a dark room. After a 5 min wash with phosphate buffer, the reaction was detected using 3, 3' diaminobenzidine (DAB) as a chromogen [0.06% w/v DAB containing 0.0034% v/v H<sub>2</sub>O<sub>2</sub> in phosphate buffer (1x)].

### *3.2.10 Oral Clearance Study with Chlorzoxazone*

#### *3.2.10.1 Animals*

Four pregnant Sprague-Dawley rats were obtained from Charles River Canada (St. Constant, PQ). They were allowed to give birth and one male pup from each dam was collected at postnatal ages 3, 7, 10, 14 and 21 days. Male Sprague-Dawley rats at ages 8 and 15 weeks (n =4) were obtained from Charles River Canada and allowed an acclimatization period of one week. Rat husbandry was similar to conditions indicated in Section 3.1.1 and the study was approved by the Animal Care and Supply Committee of the University of Saskatchewan.

### 3.2.10.2 Study Design

Four neonates per age per sacrifice time were dosed by oral gavage with CZX dissolved in 0.1M NaOH: 0.9% saline (30:70) at a dose of 30 mg/kg body weight. Different CZX stock solutions were prepared so that the oral dosed volume ranged from 0.1-0.15 ml according to the pup's body weight. Disposable 1.0 mL syringes with a suitable stainless steel feeding needles (Popper & Sons. Inc., New Hyde Park, NY) were used for dosing neonates, juvenile and adult animals (Table 3.4). The concentration and homogeneity of the dosing solution were assessed by HPLC analysis of CZX.

**Table 3.4** – Sizes of feeding and blood sampling needles used in CZX *in vivo* oral clearance study

Age (days)	Feeding Needle size	Sampling Needle size
	Gauge × length (inches)	Gauge × length (inches)
3	24 × 1	27 × 0.75
7-14	22 × 1.5	27 × 0.75
21-28	20 × 3	22 × 1
63-112	18 × 3	Jugular cannulas

The pups were removed from their mothers 4-5 hours before and returned 1-2 hours after CZX oral administration. During that time, pups were maintained in a warm environment by putting them on suitable heating pads. Blood samples were collected from neonates at predose and 5, 10, 20, 30, 45, 60, 90, 120, 240 and 360 minutes postdosing. For blood collection, a destructive sampling schedule (four neonates/age/sacrifice time) was employed due to the limited blood volume of the neonatal pups. Neonates were anesthetized with isoflurane and blood samples were collected via cardiac puncture using suitable hypodermic needles (Tyco Healthcare Group, Mansfield, MA), (Table 3.4). Neonates were immediately decapitated after blood collection.

For the 63 and 112 days old rats, animals were allowed a one-week acclimatization period. One day prior to oral dosing, the rats underwent surgical implantation of vascular cannulas (Silastic tubing, I.D. × O.D. (0.64 × 1.19 mm), Dow Corning

Corporation, Midland, MI) into the right jugular vein under isoflurane anesthesia. For access, cannulas were passed under the skin and fixed between the shoulder blades. At the end of the surgery, cannulas were instilled with heparinized saline (1 USP heparin unit/mL saline) until blood collection. Rats were fasted for 4-5 hours prior to oral dosing, because fasting for a longer time could induce CYP2E1 activity<sup>137,278</sup>. Prior to CZX administration, a zero time blood sample (250  $\mu$ L) was collected from the jugular cannula, followed by infusing equal volume of sterile saline back into the jugular cannula. After CZX oral administration, blood samples were collected by the same manner at 5, 10, 20, 30, 45, 60, 90, 120, 240, 300 and 360 minutes postdosing. After the last blood sample, rats were anesthetized and euthanized by isoflurane overdose and exsanguination. Blood samples were allowed to clot for 30 minutes, centrifuged at 6 000  $\times$  g and serum was transferred to a clean 1.5 mL eppendorf microcentrifuge tube and stored at -20°C until HPLC analysis of CZX.

#### 3.2.10.3 HPLC Assay of CZX

The concentration of CZX in serum samples were determined by a modification of a reported HPLC method<sup>279</sup>. Briefly, a 100  $\mu$ L aliquot of 0.2M acetate buffer (pH 4.7) and 100  $\mu$ L aliquot of internal standard (UMB, 10  $\mu$ M) were added to a 75  $\mu$ L aliquot of serum sample, QC sample or CZX standard solutions in uncapped 12 $\times$ 75 mm glass culture tubes. After manual mixing for 10 minutes, a 1.0 mL aliquot of tert-butyl methyl ether was added and the mixture was shaken for 10 minutes and then centrifuged at 3 000  $\times$  g for 10 minutes. The upper organic layer was transferred to a clean tube and evaporated at 37°C under nitrogen. The residue was reconstituted in a 100  $\mu$ L mobile phase and a 30  $\mu$ L aliquot was injected into the HPLC column. The mobile phase (acetonitrile and 0.25% acetic acid (20:80)), was delivered at 1.0 mL/min. UV absorbance was monitored at 287 nm. The method was validated for the determination of CZX in rat serum samples as described in section 3.2.4.

### 3.2.11 Kinetics and Statistical Analyses

All data within the same age was reported as mean  $\pm$ SD. Metabolite formation velocity as a function of substrate concentration was fit to a one-enzyme site Michaelis-Menten equation. The parameters,  $V_{\max}$  and  $K_M$ , were estimated by an iterative nonlinear least squares regression analysis using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA). Significant differences in total microsomal protein content, CYP protein content, and  $V_{\max}$  and  $K_M$  estimates, ontogeny of protein and mRNA expression between the different age groups were assessed with one-way Analysis of Variance with Tukey's post-hoc test for pair-wise multiple comparisons using GraphPad Prism. For CZX  $K_M$  estimates, a Wilcoxon signed rank test was used for the pooled data. The level of significance was set at  $P < 0.05$ . The observed  $ISF_{(t)}$  values were fit to various models describing the developmental maturation of CYP enzyme with WinNonLin 5.1 (Pharsight Corporation, Mountain View, CA, USA). Criteria determining the goodness of fit and suitability of the model included visual inspection of the regression line, the randomness of the residual plots, the accuracy of the predicted to the extrapolated estimates, the Aikaike information criterion and the coefficient of variation of the parameter estimates. For the in vivo oral clearance study, PK parameters including area under the serum concentration-time curve ( $AUC_{\infty}$ ), maximum serum concentration ( $C_{\max}$ ), time to reach  $C_{\max}$  ( $t_{\max}$ ), terminal elimination rate constant ( $\lambda_z$ ), apparent volume of distribution at steady-state ( $Vd_{ss}$ ) and oral clearance ( $Cl/F$ ) were estimated using a non-compartmental analysis (WinNonLin 5.1). Elimination half life ( $t_{1/2}$ ) at each age was obtained from the ratio  $0.693/\lambda_z$ .

## RESULTS

### 4.1 Evaluation of the Assumptions of an Ontogeny Model of CYP Enzymes

#### 4.1.1 Age-Dependent Changes in Hepatic Scaling Factor (HSF)

To estimate the HSF at each age, wet liver weight (g), total body weight (g) and hepatic microsomal protein content (mg/g liver) were determined for each animal (n=8) at each age group.

##### 4.1.1.1 Total Body and Liver Weights

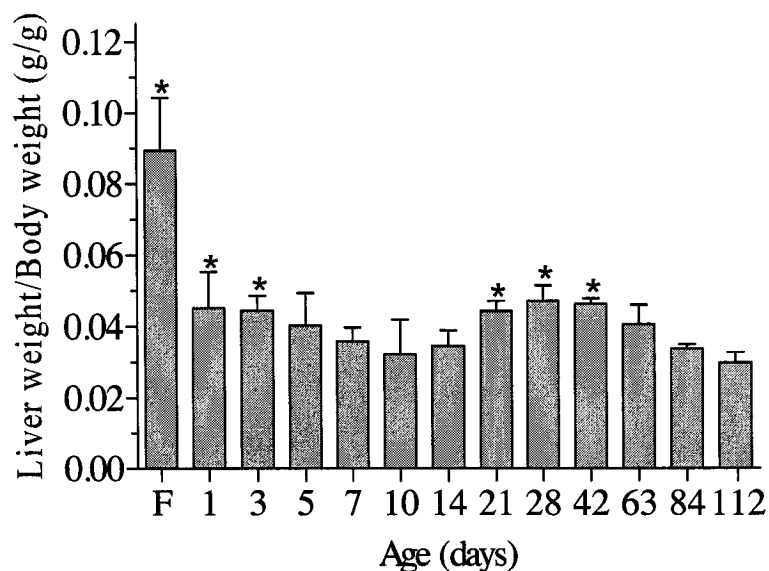
Table 4.1 lists total body and wet liver weights in male Sprague-Dawley rats at fetal (gestation day 20), neonatal, juvenile and adult ages.

**Table 4.1** – Age-dependent changes in mean ( $\pm$  SD) body and liver weights for male Sprague-Dawley rats (n=8).

Age (days)	Total Body Weight (g)		Liver Weight (g)	
	Mean	SD	Mean	SD
F <sup>a</sup>	3.64 <sup>b</sup>	1.3	0.34 <sup>b</sup>	0.17
1	7.09 <sup>b</sup>	0.5	0.32 <sup>b</sup>	0.077
3	9.40 <sup>b</sup>	0.9	0.42 <sup>b</sup>	0.070
5	12.9 <sup>b</sup>	1.4	0.52 <sup>b</sup>	0.119
7	17.4 <sup>b</sup>	1.4	0.63 <sup>b</sup>	0.096
10	26.2 <sup>b</sup>	4.5	0.86 <sup>b</sup>	0.34
14	32.7 <sup>b</sup>	3.3	1.13 <sup>b</sup>	0.24
21	59.4 <sup>b</sup>	5.2	2.62 <sup>b</sup>	0.24
28	105 <sup>b</sup>	6.7	4.93 <sup>b</sup>	0.54
42	203 <sup>b</sup>	12.9	9.39 <sup>b</sup>	0.75
63	399 <sup>b</sup>	32.7	16.2	2.6
84	411 <sup>b,c</sup>	11.0	13.8 <sup>c</sup>	0.51
112	507	29.2	15.0	1.4

<sup>a</sup> Fetuses were collected at 20 day gestation from 3 dams and the fetal livers from individual dams were pooled, <sup>b</sup> Statistically significant from adult (112 days), <sup>c</sup> n=4.

As expected, mean total body and liver weights increased with postnatal age, with the most prominent increases occurring during puberty (42-63 days). The variation in total body and liver weights observed within and between age groups can be attributed to normal interindividual variation in the rate and pattern of body and organ growths. Normalization of wet liver weight to total body weight revealed relatively constant values with postnatal maturation (Figure 4.1).

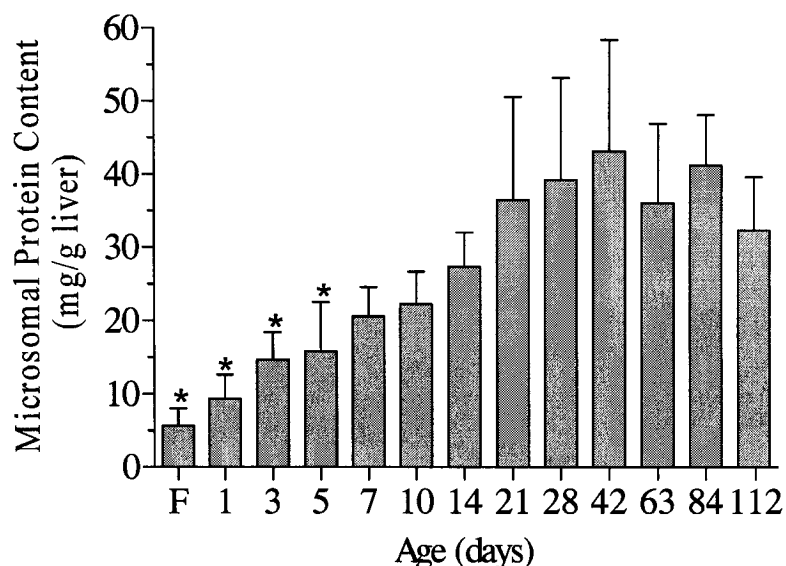


**Figure 4.1** –Wet liver weight (g) normalized to total body weight (g) in male Sprague-Dawley rats (n=8, except at 84 days where n=4). Results are expressed as mean  $\pm$ SD. Asterisks indicate statistical difference from adult (112 days),  $P < 0.05$ . F =gestation day 20.

#### 4.1.1.2 Hepatic Microsomal Protein Content

To evaluate whether microsomal protein (MP) content remains constant with developmental age (the first major assumption of our ontogeny model of CYP enzyme activity) and to calculate age-dependent HSF values, hepatic microsomal protein content for each age group (n =8) was measured by the method of Lowry *et al*<sup>270</sup>. The results shown in Figure 4.2 indicate that average MP (mg/gram liver weight) was low at birth (18 % of the adult value,  $P < 0.05$ ) and increased steadily to reach a maximum value (133

% of the adult value,  $P > 0.05$ ) at postnatal day (PD) 42, followed by gradual, but not statistically significant, decline towards the adult value. The only significant difference from the adult MP level was observed in hepatic microsomes from fetuses and postnatal days younger than 7 days (Figure 4.2).



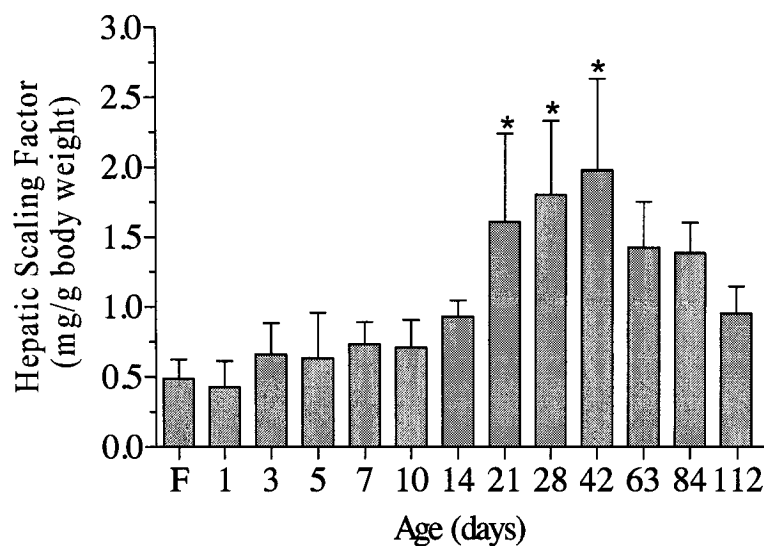
**Figure 4.2** – Mean ( $\pm$  SD) hepatic microsomal protein content in male Sprague-Dawley rats ( $n=8$ , except for PD 84 where  $n=4$ ). Asterisks indicate statistical difference from adult (112 days),  $P < 0.05$ . F = gestation day 20.

#### 4.1.1.3 Hepatic Scaling Factor (HSF)

Results of liver weights, total body weights and hepatic microsomal protein content at each developmental age were used to calculate age-dependent changes in HSF values (Figure 4.3) according to equation (3.3). Relative to adult, there was no significant difference in HSF values between 20 day gestation and 14 day age groups (about 50-90% of the adult value,  $P > 0.05$ ). By 21 days of age, the HSF value had increased more than threefold and did not change further at 28 or 42 days of age (about 150-200% of the adult value,  $P < 0.05$ ), but decreased gradually from puberty (PD 42-63) group to the adult age group.

Upon close examination of the data the pattern observed in the mean HSF with age (Figure 4.3) mirrored the developmental pattern of microsomal protein content per

gram liver (Figure 4.2), since the ratio of liver weight to total body weight remained relatively constant with postnatal development.

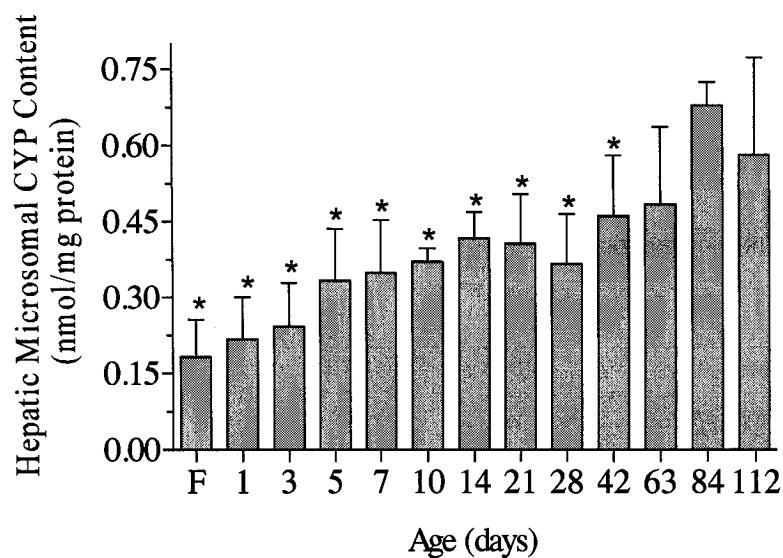


**Figure 4.3** – Mean ( $\pm$  SD) hepatic scaling factor (mg/g body weight) in male Sprague-Dawley rats ( $n=8$ , except for PD 84 where  $n=4$ ). HSF was calculated using Equation 3.3. Asterisks indicate statistical difference from adult (112 days),  $P < 0.05$ . F = gestation day 20.

#### 4.1.1.4 Age-Dependent Changes in Total CYP protein Content

Total CYP protein content was determined in age-specific rat hepatic microsomes by carbon monoxide difference spectroscopy<sup>271</sup>. As shown in Figure 4.4, total CYP content was fairly low in fetal microsomes (less than 25% the adult level,  $P < 0.05$ ) and increased slightly between postnatal days 1 and 28. There was no significant difference between hepatic microsomal CYP content at puberty (PD63) and adult ages.





**Figure 4.4** – Mean ( $\pm$  SD) total hepatic microsomal CYP content (nmol/mg microsomal protein) in male Sprague-Dawley rats ( $n=8$ , except for PD 84 where  $n=4$ ). CYP content was determined by the method of Omura and Sato<sup>271</sup>. Asterisks indicate statistical difference from adult (112 days),  $P < 0.05$ . F = gestation day 20.

#### 4.1.2 Enzyme Kinetic Studies in Rat Hepatic Microsomes

To evaluate the age-dependent changes in  $K_M$  and  $V_{max}$  values, enzyme kinetic studies in rat hepatic microsomes were conducted using probe substrates specific for CYP2E1 and CYP1A2 enzymes, namely chlorzoxazone (CZX) and methoxyresorufin (MR), respectively.  $K_M$  and  $V_{max}$  values were determined by measuring metabolite formation velocities of CZX and MR at substrate concentrations of 0 to 1000  $\mu\text{M}$  and 0 to 1000 nM, respectively. As well,  $V_{max}$  values were estimated using a second probe substrate,  $\rho$ -nitrophenol (PNP) for CYP2E1 and phenacetin for CYP1A2.

##### 4.1.2.1 Assessment of the Optimum Incubation Conditions

Preliminary experiments using hepatic microsomes from 50-day old male Sprague-Dawley rats were conducted to determine the optimum incubation conditions regarding incubation time and microsomal protein content to give linear metabolite formation kinetics for CYP2E1-mediated CZX and PNP hydroxylation and CYP1A2-mediated

dealkylation of MR and phenacetin. Results of these experiments are indicated in Table 4.2. These conditions were used in subsequent enzyme kinetic experiments.

**Table 4.2** –Results of preliminary experiments in male rat hepatic microsomes to assess the optimum microsomal incubation conditions to ensure linear metabolite formation kinetics.

	Optimum incubation conditions	
	Incubation time (min)	Protein content (mg/mL)
1. CZX hydroxylation	30	0.4
2. PNP hydroxylation	30	0.4
3. MR O-dealkylation	8	0.08
4. Phenacetin O-dealkylation	60	0.4

#### 4.1.2.2 Validation of the HPLC Methods

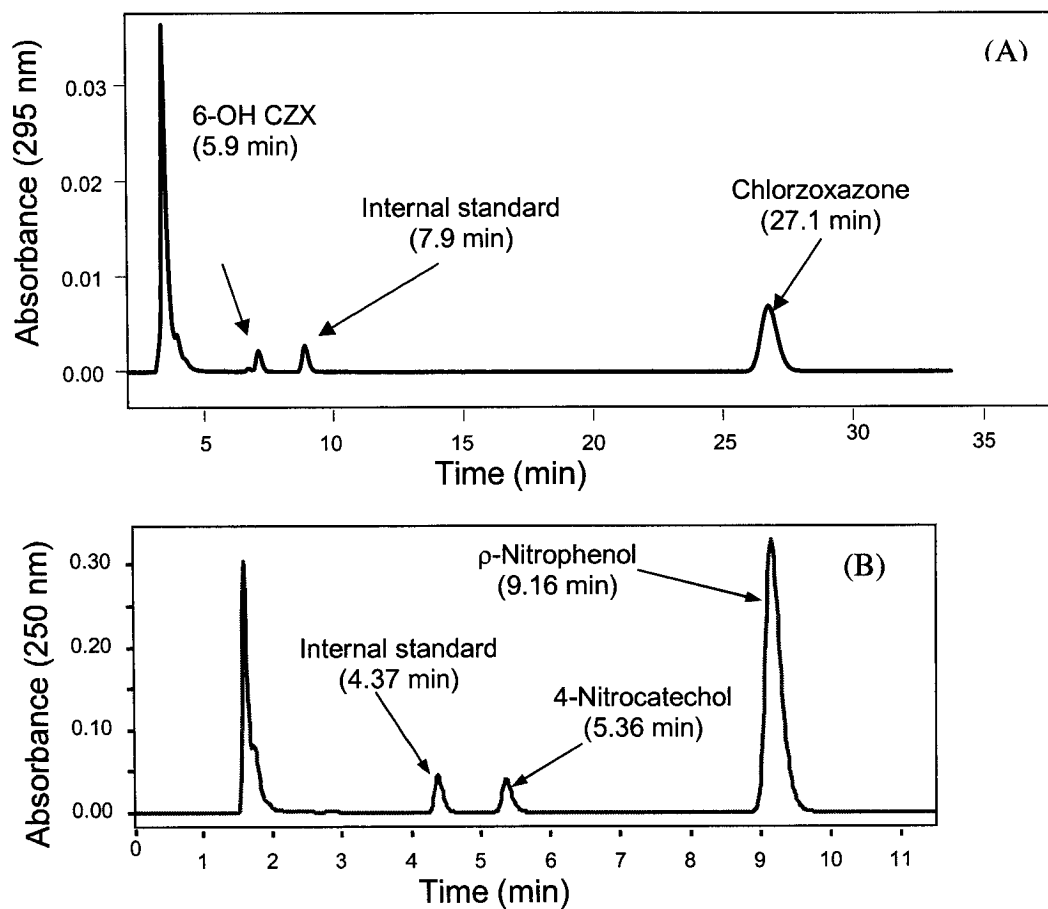
To monitor metabolite formation rate in age-dependent hepatic microsomes, validated HPLC methods were used. Validation was performed according to the FDA guidelines (section 3.2.4). Table 4.3 demonstrates the basic validation parameters for all the HPLC methods used for evaluation of CYP2E1 and CYP1A2 enzyme kinetics and CZX oral clearance.

**Table 4.3** –Method validation parameters for the HPLC assays used to analyze metabolites and/or substrates in enzyme kinetic and oral clearance studies.

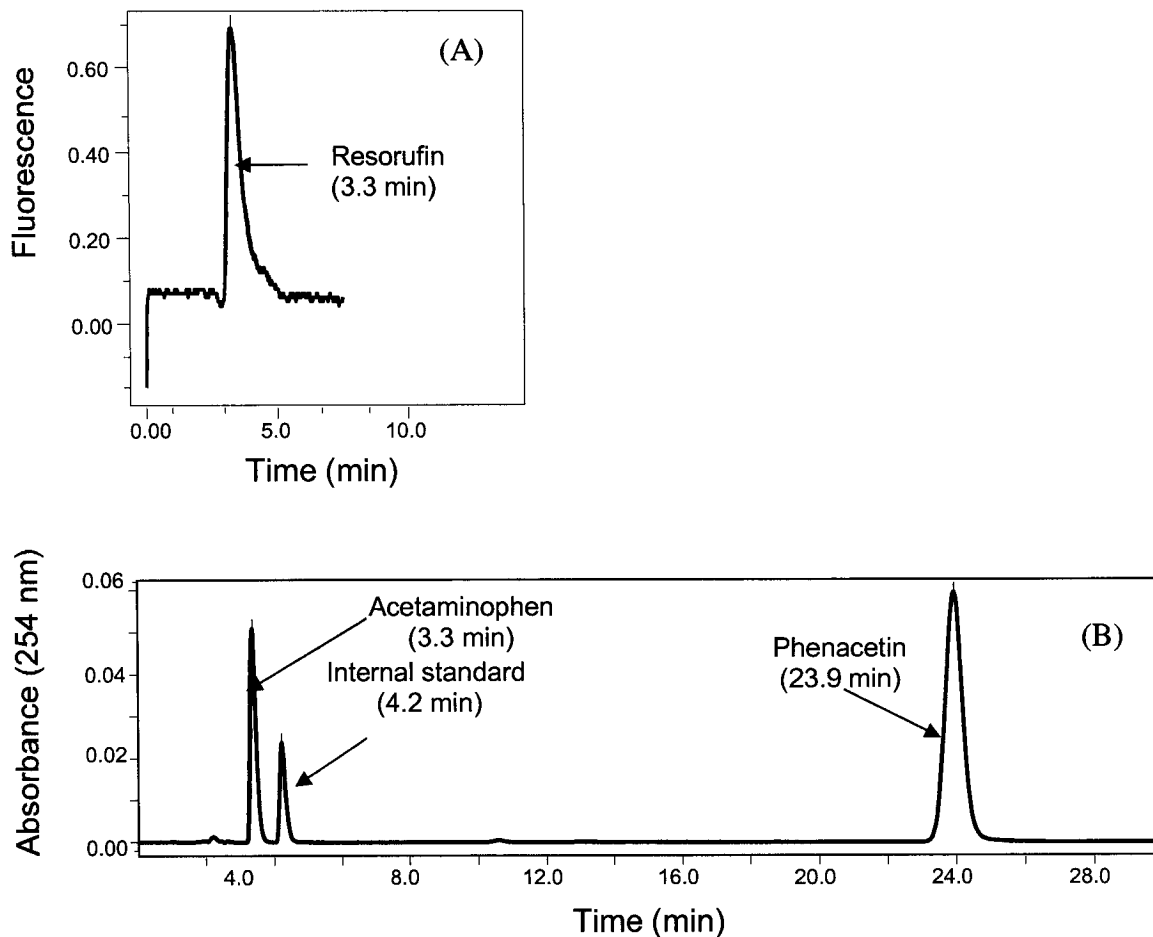
Validation parameters	Metabolites analyzed				
	6-OH CZX	4 NC	APAP <sup>e</sup>	Resorufin	CZX <sup>a</sup>
1. Linearity range ( $\mu\text{M}$ ) <sup>b</sup>	0.3-40	0.1-40	0.2-20	0.04-2	1-250
2. LOD <sup>b,c</sup>	0.15	0.02	0.05	0.01	0.25
3. LOQ <sup>b,d</sup>	0.30	0.10	0.20	0.04	1.0
4. Intra-day					
Accuracy	101-113%	95-104%	96-114%	96-113%	100-114%
Precision	<14%	<11%	<9%	<6%	<7%
5. Inter-day					
Accuracy	101-106%	91-103%	104-111%	88-110%	91-108%
Precision	<9%	<8%	<14%	<8%	<8%

<sup>a</sup>This method was used to analyze CZX in oral clearance study (section 4.4), <sup>b</sup>Resorufin units are nM, <sup>c</sup>Limit of detection, <sup>d</sup>Limit of quantitation, <sup>e</sup>acetaminophen.

Figures 4.5 and 4.6 show representative HPLC chromatograms of rat hepatic microsomal samples after being incubated with CYP-specific substrates under optimum incubation conditions (Table 4.2).



**Figure 4.5** – Representative HPLC chromatograms of rat hepatic microsomes after incubation with CYP2E1 probe substrates, chlorzoxazone (A) and p-nitrophenol (B), under incubation conditions mentioned in Table 4.2 and explained in section 3.2.3. HPLC chromatographic conditions are explained in section 3.2.4.

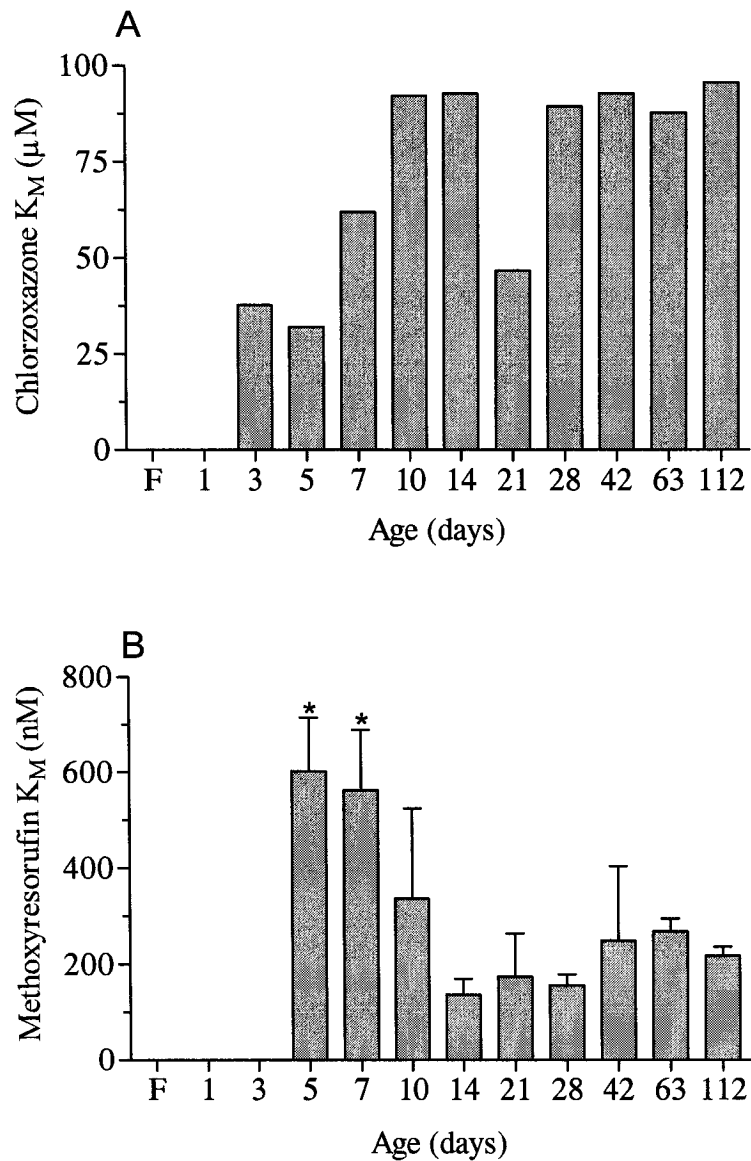


**Figure 4.6** – Representative HPLC chromatograms of rat hepatic microsomes after incubation with CYP1A2 probe substrates, methoxyresorufin (A) and phenacetin (B) under incubation conditions mentioned in Table 4.2 and explained in section 3.2.3. HPLC chromatographic conditions are explained in section 3.2.4.

#### 4.1.2.3 Developmental Changes in $K_M$ Value

To investigate whether  $K_M$  value remains constant with developmental age (the second major assumption of our ontogeny model of CYP enzyme activity),  $K_M$  values for CYP2E1-mediated CZX hydroxylation and CYP1A2-mediated MROD activities were determined in age-dependent hepatic microsomes. For CYP2E1, CZX 6-hydroxylase activity was not detected in gestation day 20 and PD1 hepatic microsomes. Chlorzoxazone  $K_M$  values (Figure 4.7 A) were only 35-40% of adult values at PD3 and PD5. By PD10,  $K_M$  values reached adult level and stayed relatively constant thereafter

except at PD21 where the  $K_M$  value was 70% that of adult value. The data may simply represent normal biological variation given the rather small sample size and pooled data.



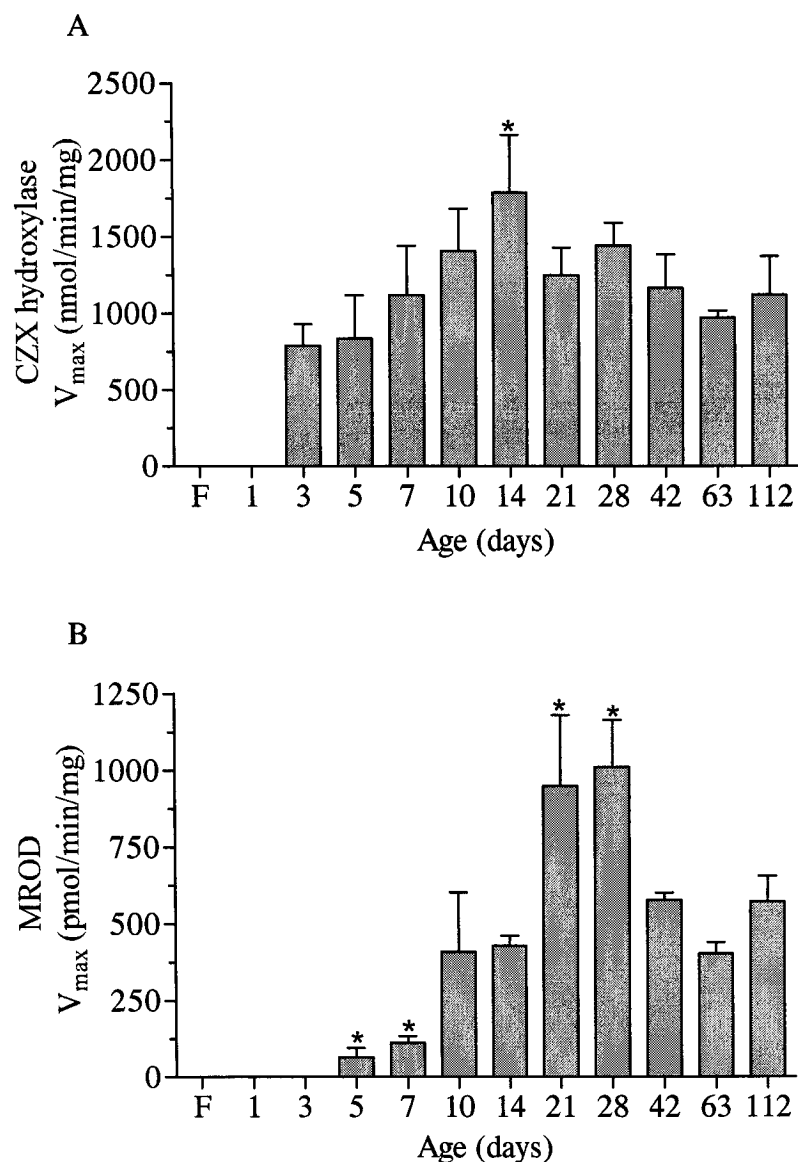
**Figure 4.7** – Michaelis-Menten constant ( $K_M$ ) estimates for CYP2E1-mediated chlorzoxazone hydroxylase activity (A) and CYP1A2-mediated methoxyresorufin-O-dealkylation (MROD) activity (B) as a function of developmental age in male Sprague-Dawley rat hepatic microsomes.  $K_M$  values for chlorzoxazone are obtained from pooled microsomes of four rats at each age group. Wilcoxon signed-rank test indicated significance.  $K_M$  values for methoxyresorufin are expressed as mean  $\pm$ SD of 4 rats except fetal livers, which were pooled from 3 different dams. The asterisk refers to those age groups that show significant difference from adult. MROD activity was detected at PD3, but the data did not allow for  $K_M$  estimation.

For CYP1A2, the methoxyresorufin  $K_M$  values (Figure 4.7 B) in PD5 and PD7 were significantly higher than adult values (~250-275% of adult values). No significant differences in  $K_M$  values were observed for PD age groups  $\geq 10$  days. The data did not allow estimation of  $K_M$  values in PD3 age group, although activity was detected.

#### 4.1.2.4 Developmental Changes in $V_{max}$ Value

To evaluate the age-dependent changes in maximum enzyme activity ( $V_{max}$ ), both CYP2E1-mediated CZX hydroxylase and CYP1A2-mediated MROD activities were measured at saturating probe substrate concentrations in rat hepatic microsomes at different developmental stages. CYP2E1 activity was not detected in fetal and PD1 livers (Figure 4.8A). A higher level of activity was detected in PD3 livers (70% of the adult value,  $P > 0.05$ ), which increased progressively to reach a maximum level at PD14 (170% of the adult value,  $P < 0.05$ ). The activity declined to the adult level at weaning (PD21) and remained similar to the adult values during puberty (Figure 4.8A). No significant difference in activity levels relative to the adult value was observed at any age group, except at PD14. The marked postnatal increase in  $V_{max}$  values for chlorzoxazone preceded hepatic scaling factor values, where the predominant age-dependent increase occurred between PD14 and PD21 and continued to increase until PD42 (Figure 4.3).

Although rat hepatic CYP1A2-mediated MROD activity was first detected at PD3, the values were below the limit of quantification of the HPLC assay. At PD5 and PD7 hepatic microsomes demonstrated significantly lower MROD activity at 11% and 20% adult activity, respectively (Figure 4.8 B). MROD activity increased significantly to reach 165% and 175% adult values by PD21 and PD28, respectively. However, after weaning (PD28) and during puberty (PD42-63)  $V_{max}$  values fell to adult values. Although, the marked increase in MROD activity between PD14 and PD21 corresponded to a marked increase in age-dependent hepatic scaling factors (Figure 4.3), activity declined after PD28 to the adult value by PD42, unlike the hepatic scaling factor.

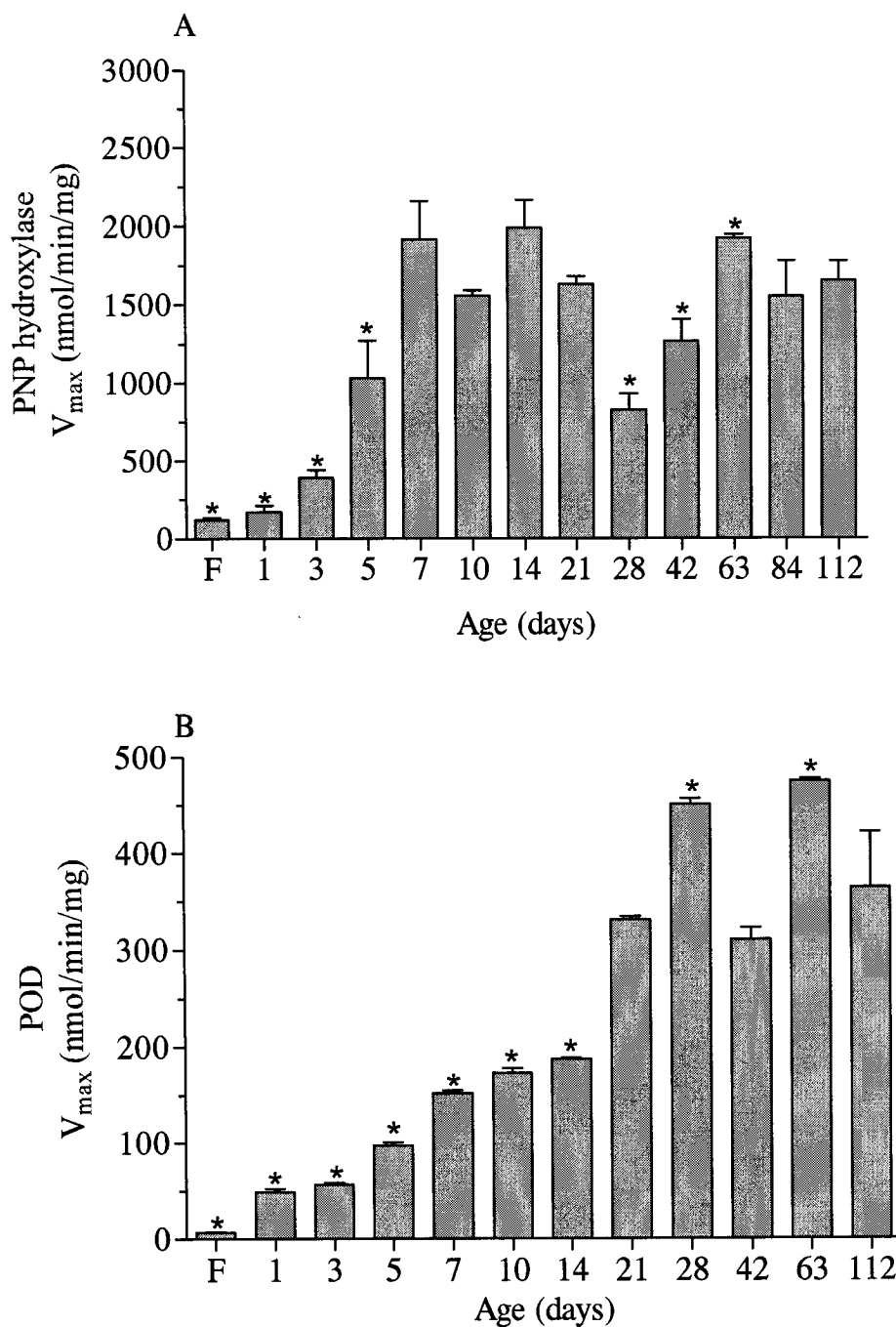


**Figure 4.8** -  $V_{max}$  estimates for CYP2E1-mediated chlorzoxazone hydroxylase activity (A) and CYP1A2-mediated methoxyresorufin-O-dealkylase activity (B) as a function of developmental age in male Sprague-Dawley rat hepatic microsomes. The data are expressed as mean  $\pm$ SD of 4 rats except fetal livers, which were pooled from 3 different dams. The asterisks refer to those age groups that show significant difference from adult.

#### 4.1.2.5 Developmental Changes in $V_{max}$ Value Using a Second Probe Substrate

To determine whether enzyme activity ( $V_{max}$ ) from one substrate predicts the level of activity for all substrates of the enzyme, developmental changes in  $V_{max}$  were estimated using a second probe substrate, *p*-nitrophenol for CYP2E1 and phenacetin for CYP1A2.

Figure 4.9 shows age-dependent changes in CYP2E1-mediated  $\rho$ -nitrophenol (PNP) hydroxylase activity and CYP1A2-mediated phenacetin-O-dealkylase activity (POD).



**Figure 4.9-**  $V_{max}$  estimates for CYP2E1-mediated PNP hydroxylase activity (A) and CYP1A2-mediated POD activity (B) as a function of developmental age in male Sprague-Dawley rat hepatic microsomes. The data are expressed as mean  $\pm$ SD of 4 rats except fetal livers, which were pooled from 3 different dams. The asterisks refer to those age groups that show significant difference from adult.



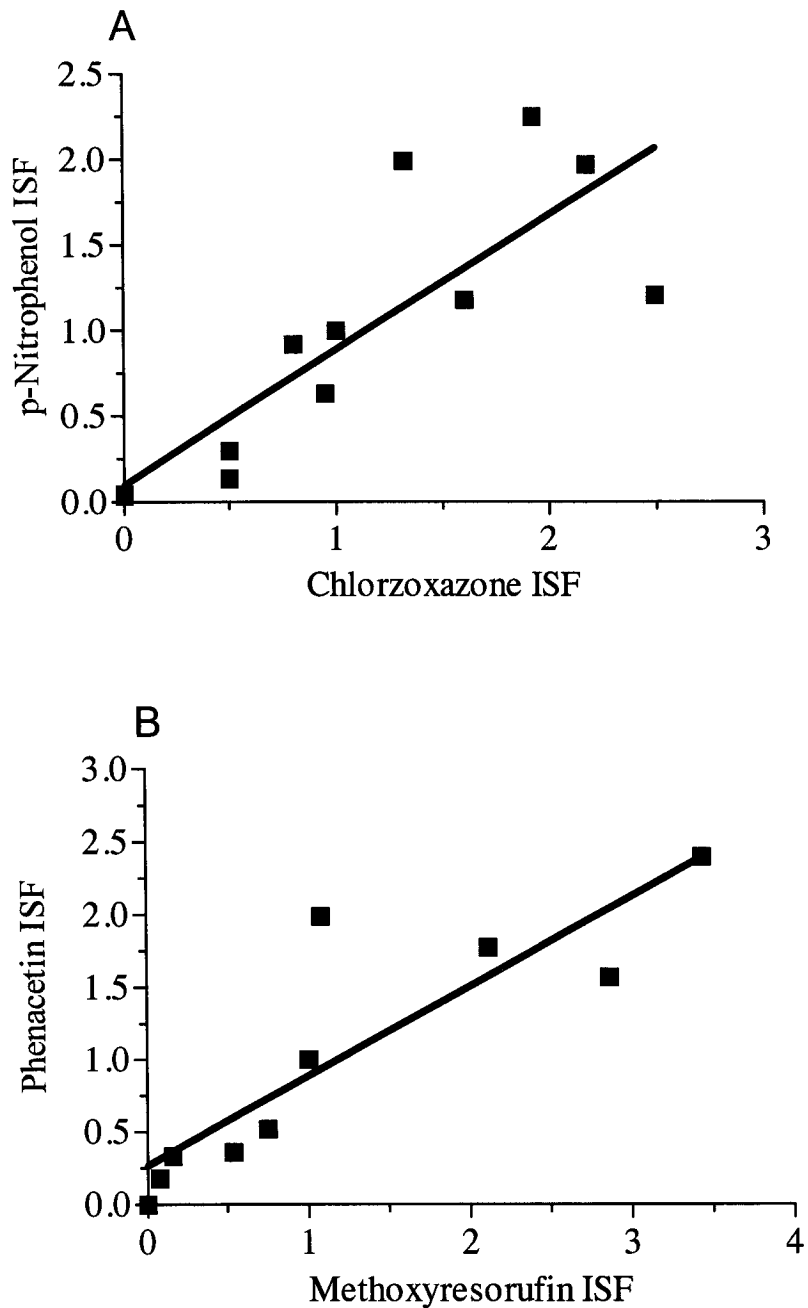
To confirm whether changes in enzyme activity of one CYP substrate covaries with changes in the activity of a second CYP substrate (the fourth major assumption of our ontogeny model of CYP enzyme activity), infant scaling factors (product of HSF and  $V_{max}$  normalized to their respective adult values) were determined at each age group (Table 4.4) using the two probe substrates for each CYP enzyme followed by correlation of the ISF values.

**Table 4.4** - Age-specific infant scaling factor estimates for CYP2E1 and CYP1A2 based upon age-specific relative hepatic scaling factors (RHSF) and  $V_{max}$  values normalized to adult  $V_{max}$  values (OSF) in male Sprague-Dawley rats.

Age (day)	CYP2E1					CYP1A2			
	RHSF	Chlorzoxazone		$\rho$ -nitrophenol		Methoxyresorufin		Phenacetin	
		OSF	ISF	OSF	ISF	OSF	ISF	OSF	ISF
F	0.57			0.074	0.042			0.019	0.011
1	0.47			0.10	0.049			0.13	0.063
3	0.71	0.70	0.50	0.24	0.17			0.16	0.11
5	0.67	0.74	0.50	0.62	0.42	0.11	0.076	0.27	0.18
7	0.80	0.99	0.80	1.16	0.93	0.20	0.16	0.42	0.33
10	0.76	1.25	0.95	0.94	0.71	0.71	0.54	0.47	0.36
14	1.00	1.59	1.60	1.20	1.21	0.75	0.75	0.51	0.52
21	1.72	1.11	1.92	0.99	1.70	1.66	2.86	0.91	1.57
28	1.94	1.28	2.49	0.50	0.97	1.77	3.43	1.24	2.40
42	2.09	1.04	2.17	0.77	1.60	1.01	2.11	0.85	1.78
63	1.52	0.86	1.32	1.16	1.78	0.71	1.08	1.30	1.99
112	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

RHSF =Relative Hepatic Scaling Factor calculated from age-specific HSF values normalized to adult value (Equations 3.3 and 3.4); OSF =Ontogeny Scaling Factor calculated from age-specific  $V_{max}$  values normalized to adult  $V_{max}$  value (Equation 3.5); ISF =Infant Scaling Factor calculated from the product of RHSF and OSF (Equation 3.6).

Figure 4.10 suggests a strong positive correlation between the activity of one specific substrate relative to a second specific substrate for both CYP2E1 and CYP1A2 ( $r = 0.82$  and  $r = 0.89$ , respectively).

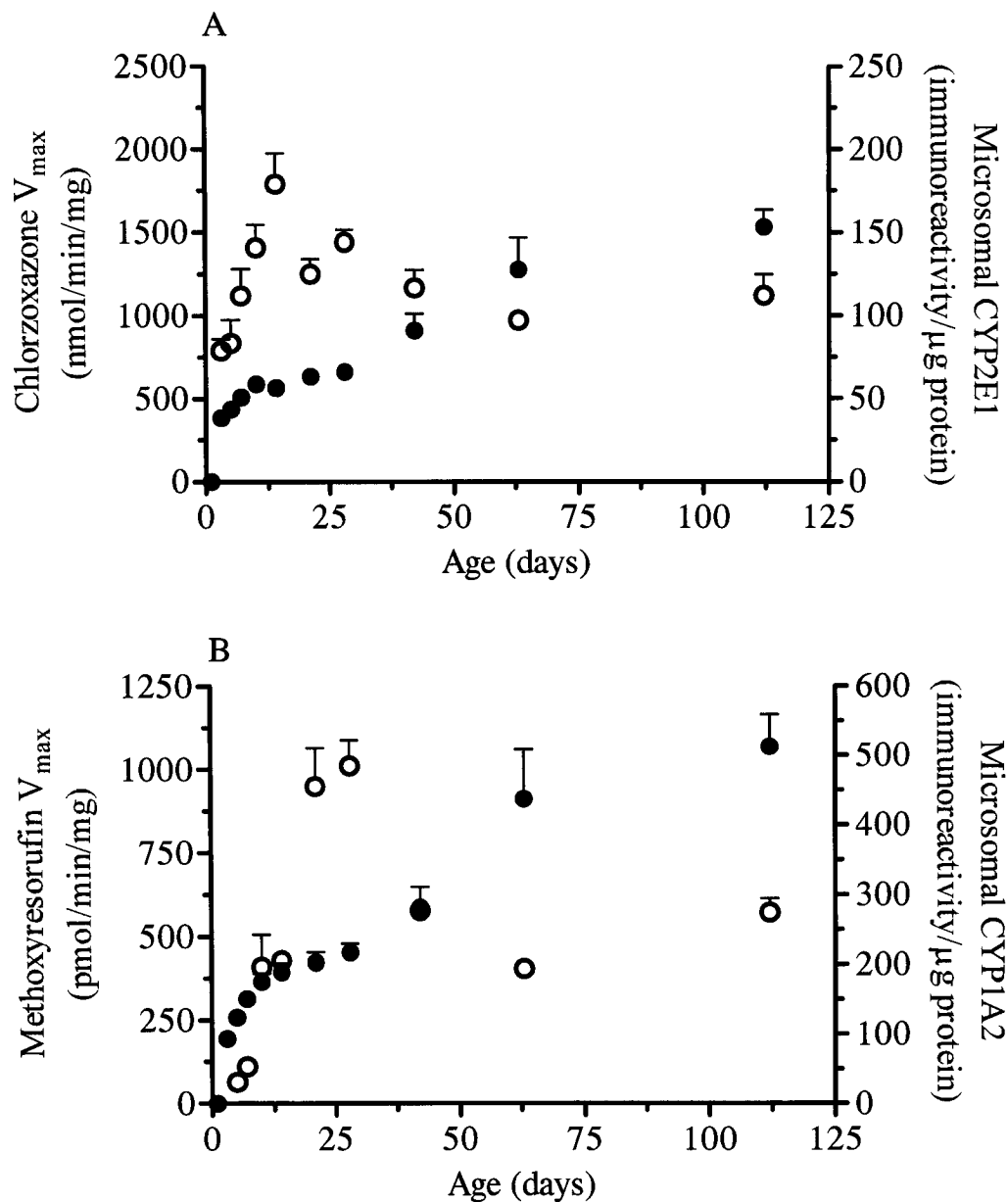


**Figure 4.10-** Correlation between infant scaling factor estimates for (A)  $\rho$ -nitrophenol as a function of chlorzoxazone ( $r = 0.82$ ); (B) Phenacetin as a function of methoxyresorufin ( $r = 0.89$ ).

#### *4.1.2.6 Correlation between Age-Dependent Changes in Catalytic CYP Activity and Immunoreactive CYP Protein level*

To investigate a proportional relationship between enzyme activity ( $V_{\max}$ ) and protein level during postnatal development (the fifth major assumption of our ontogeny model of CYP enzyme activity), CYP2E1 and CYP1A2 enzyme activity was measured at saturating probe substrate concentrations in rat hepatic microsomes at different developmental stages. Also CYP2E1 and CYP1A2 protein levels were determined in the hepatic microsomes at the same developmental stages by immunoblotting procedures (Section 4.6). When the age-dependent changes in  $V_{\max}$  were compared with changes in enzyme protein levels, CYP2E1-mediated chlorzoxazone hydroxylase  $V_{\max}$  values did not correlate with changes in CYP2E1 protein levels observed at the same age group (Figure 4.11A). CYP2E1 protein and activity were not detectable at <PD3. Although CYP2E1 protein increased following PD3, protein levels plateaued at PD10 and began increasing again at PD28. This biphasic pattern was not mirrored by CYP2E1 activity where a dramatic increase in activity was observed between PD3 and PD14 (Figure 4.8). At PD28 immunoquantifiable CYP2E1 protein levels were only ~50% of adult levels despite ~30% greater enzyme activity levels relative to adult. CYP2E1 protein continued to increase thereafter to adult values, despite the decrease in chlorzoxazone  $V_{\max}$  to adult values.

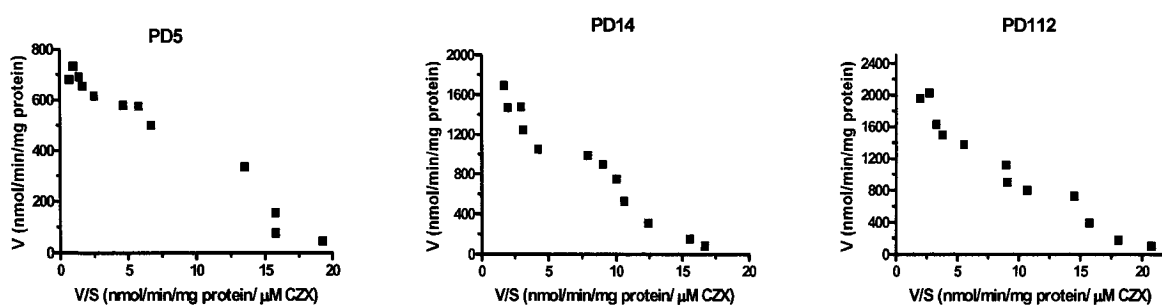
Similarly, CYP1A2-mediated methoxyresorufin  $V_{\max}$  values did not reasonably correlate with changes in immunoquantifiable CYP1A2 protein levels observed at the same age group (Figure 4.11B). A similar biphasic pattern demonstrated by CYP2E1 protein was observed with CYP1A2 protein ontogeny. However, CYP1A2 activity increased markedly between PD5 and PD28 such that MROD  $V_{\max}$  values were ~175% adult values (Figure 4.8). Thereafter, CYP1A2 protein continued to increase while MROD activity declined to adult values.



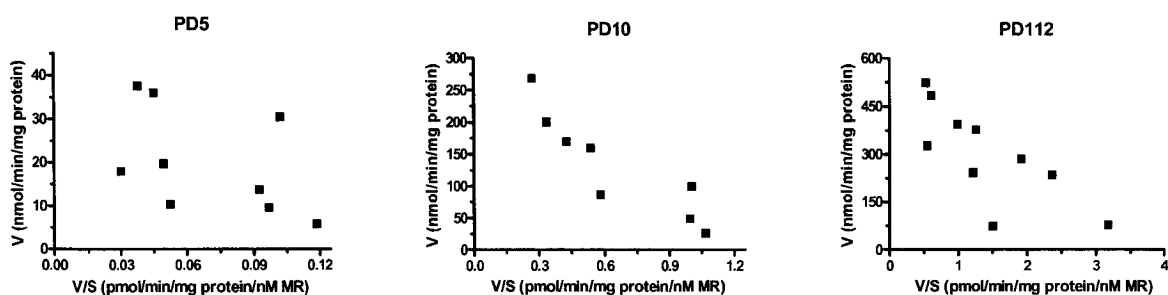
**Figure 4.11** – Age-dependent changes in CYP enzyme activity (open circle) as a function of its immunoreactive protein levels (closed circle) in rat hepatic microsomes. (A) Average CYP2E1-mediated chlorzoxazone hydroxylase  $V_{max}$  estimates as a function of average CYP2E1 protein levels. (B) Average CYP1A2-mediated methoxyresorufin-O-dealkylase  $V_{max}$  estimates as a function of average CYP1A2 protein levels. Immunoreactivity is expressed in arbitrary units per microgram of microsomal protein.

#### 4.1.2.7 Eadie-Hofstee Plots

To investigate possible involvement of more than one CYP isoform in CZX hydroxylation and MROD activities, Eadie-Hofstee plots were used. Figures 4.12 and 4.13 show representative Eadie-Hofstee plots at selected postnatal ages. The results may indicate that both CZX hydroxylation and MROD activities demonstrate at least two components, rather than a simple one component Michaelis-Menten kinetics. This possible contribution of more than one CYP pathway will have a significant impact on the estimated  $V_{max}$  and  $K_M$  parameters for these reactions.



**Figure 4.12** – Eadie-Hofstee plots for CZX hydroxylation catalyzed by rat hepatic microsomes from different ages. V =rate of metabolite formation; S =substrate concentration; PD =postnatal day; CZX =chlorzoxazone.



**Figure 4.13** – Eadie-Hofstee plots for MROD activity catalyzed by rat hepatic microsomes from different ages. V =rate of metabolite formation; S =substrate concentration; PD =postnatal day; MR =methoxyresorufin.

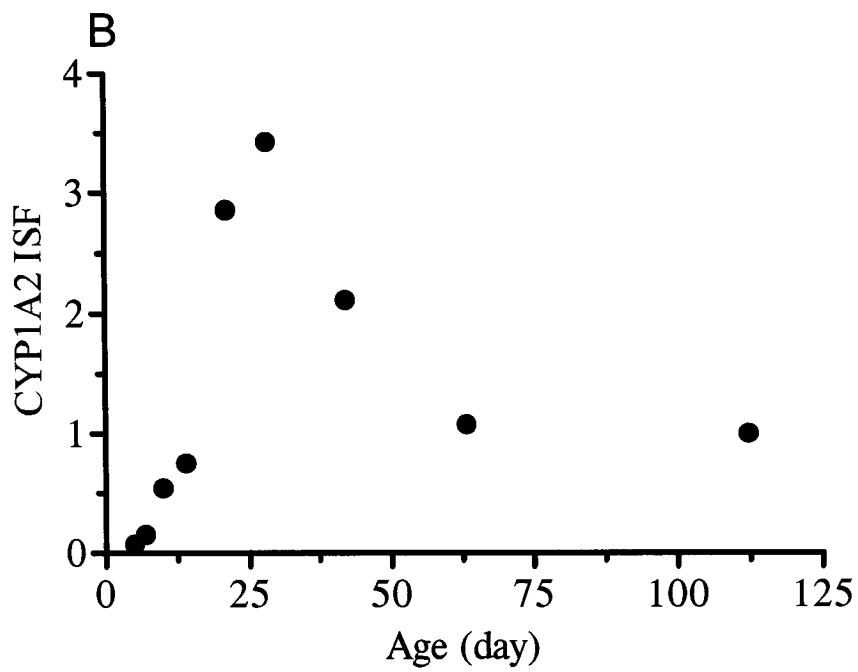
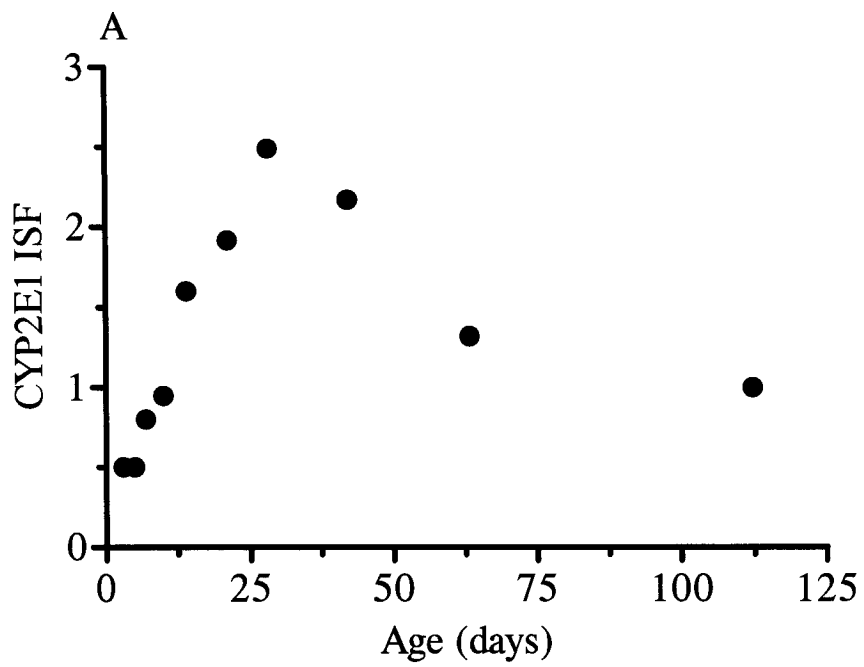
## 4.2 Development of an Ontogeny Model of CYP2E1 and CYP1A2 enzymes in Rat

The age-dependent changes in CYP2E1 and CYP1A2 enzyme activity using specific probe substrates were used to develop pharmacokinetic models to describe the developmental maturation of these enzymes in the rat.

### 4.2.1 Developmental Maturation of CYP Activity

#### 4.2.1.1 Age-Specific Infant Scaling Factor

Infant scaling factors at a particular age,  $t$ , ( $ISF_{(t)}$ ) were estimated from the product of  $HSF_{(t)}$  and  $V_{max}$  values at the same age,  $t$  ( $V_{max,(t)}$ ), normalized to the product of the respective adult values according to Equation 3.6 and as illustrated in Table 4.4. As shown in Figure 4.14, CYP2E1 ISF value is very low at birth, increases asymptotically during the first 2 weeks, and undergoes a burst during the third week before falling back to adult level after 50 days of age. Similar observations were obtained with CYP1A2 but its developmental changes were more delayed compared to CYP2E1. The asymptotic increase in activity was observed during the third week with a burst in activity during the fourth week and a sharp decline in activity during the pubertal stage (PD42-63). Given the fact that CYP2E1 and CYP1A2 are the dominant clearance pathways for CZX and MR<sup>280</sup>, respectively, pattern of age-dependent changes in ISF calculated using these substrates should reflect the pattern of CYP2E1 and CYP1A2 ontogeny.



**Figure 4.14-** Infant scaling factor (ISF) as a function of age for CYP2E1 (A) and CYP1A2 (B) enzymes.

#### 4.2.1.2 Model Development

The observed  $ISF_{(t)}$  values were fit to various models describing the developmental maturation of each CYP enzyme using pharmacokinetic modeling software (GraphPad Prism 4). Modifications to the general ontogeny model (Equation 1.16) were required to describe the age-dependent changes in CYP function from birth to adulthood as shown in Equation 4.1.

The primary modification included a parameter ( $ISF_{Burst} (e^{-k_2(t-lag)} - e^{-k_3(t-lag)})$ ) that accounted for an apparent burst of activity (greater than the adult value) in the period between weaning and puberty that overlay the general exponential increase in activity to some asymptotic level reached at adulthood ( $ISF_D (1 - e^{-k_1 t})$ ).

$$ISF_{(j,t)} = ISF_{(j,0)} + ISF_D (1 - e^{-k_1 t}) \quad (1.16)$$

$$ISF_{(j,t)} = ISF_{(j,0)} + ISF_D (1 - e^{-k_1 t}) + ISF_{Burst} (e^{-k_2(t-lag)} - e^{-k_3(t-lag)}) \quad (4.1)$$

where:  $ISF_{(j)}$  is the predicted hepatic CYP enzyme-specific infant scaling activity *in vivo* at age,  $t$  (d);  $ISF_{(j,0)}$  is the scaled *in vivo* CYP enzyme activity present at birth;  $ISF_D$  and  $ISF_{Burst}$  are a fixed and fitted parameters associated with the slow rise and burst, respectively;  $k_1$  is the first order rate constant describing the slow rate of increase in CYP enzyme pathway efficiency ( $d^{-1}$ ) to the adult value;  $k_2$  and  $k_3$  are the first order rate constants describing the onset and decline in CYP enzyme pathway efficiency ( $d^{-1}$ ) during the burst phase;  $t$  is the age in days (d); and  $lag$  is the time delay associated with the appearance of the burst phase.

#### 4.2.1.3 Model Selection

This model (Equation 4.1) was written into Prism 4 (GraphPad Software Inc.) and the number of exponential terms and inclusion of a  $t_{lag}$  parameter were determined by nonlinear regression analyses of observed ISF values to models of increasing complexity. The  $ISF_{Burst}$ ,  $lag$ , and  $k$  terms are fitted parameters and  $ISF_{(j,0)}$  is a constant based upon the scaled CYP enzyme activity determined in fetal microsomes. Upper and lower bounds were established for each estimated parameter and remained consistent between different models. Initial parameter estimates remained consistent for each analysis. Several sets of initial parameter estimates were used to ensure that the program



was not stopping at local minima. Equal weighting factors or  $1/\text{observation}^2$  weighting factors were given to all ISF data. Model selection was based on several criteria which included the Akaike Information Criterion (AIC) values, confidence intervals (CI) of parameter estimates, correlation coefficient, weighted residual plots, correspondence of observed versus predicted parameters, and visual inspection of the fitted nonlinear regression line. In general, the model equation with minimum AIC and narrowest CI values was considered the best representation of the time course of scaled CYP enzyme activity data. Table 4.5 represents the model parameter estimates (Equation 4.1) for each CYP enzyme.

**Table 4.5-** Parameter estimates for the predictive models of CYP2E1 and CYP1A2-mediated hepatic clearance in rats

Model parameter	Clearance mechanism	
	CYP2E1	CYP1A2
$ISF_{(j,0)}$ <sup>a</sup>	0	0
$ISF_D$ <sup>a</sup>	1	1
$k_1$	0.00275	0.0058
$ISF_{Burst}$	32.75	34.55
$k_2$	0.02679	0.041
lag	0.3875	6.46
$k_3$	0.03427	0.0498

<sup>a</sup> Fixed parameters

$ISF_{(j,0)}$  is the scaled in vivo CYP enzyme activity present at birth.

$ISF_D$  and  $ISF_{Burst}$  are fixed and fitted parameters associated with the slow rise and burst, respectively.

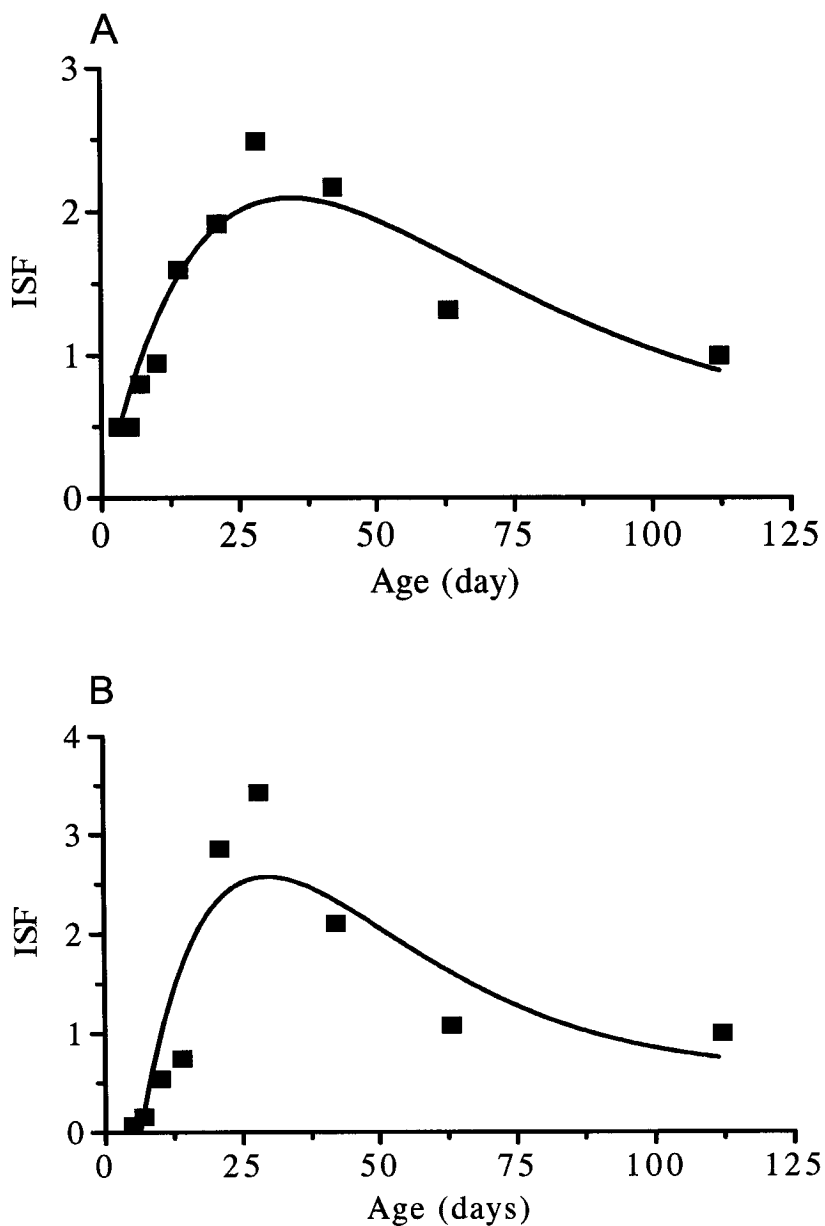
$k_1$  is the first order rate constant describing the slow rate of increase in CYP enzyme pathway efficiency ( $d^{-1}$ ) to the adult value.

$k_2$  and  $k_3$  are the first order rate constants describing the onset and decline in CYP enzyme pathway efficiency ( $d^{-1}$ ) during the burst phase.

$t$  is the age in days (d).

lag is the time delay associated with the appearance of the burst phase.

The nonlinear regression fit shown in Figures 4.15A demonstrate a reasonable correspondence between the fitted line and the observed ISF values.



**Figure 4.15-** Plot output of calculated (line) and observed (squares) ISF data versus age using the model given in equation 4.1 for CYP2E1 (A) and CYP1A2 (B). Best fits and model selection were obtained using unweighted regression analysis (GraphPad Prism). Model parameter estimates for each CYP enzyme are given in Table 4.5.

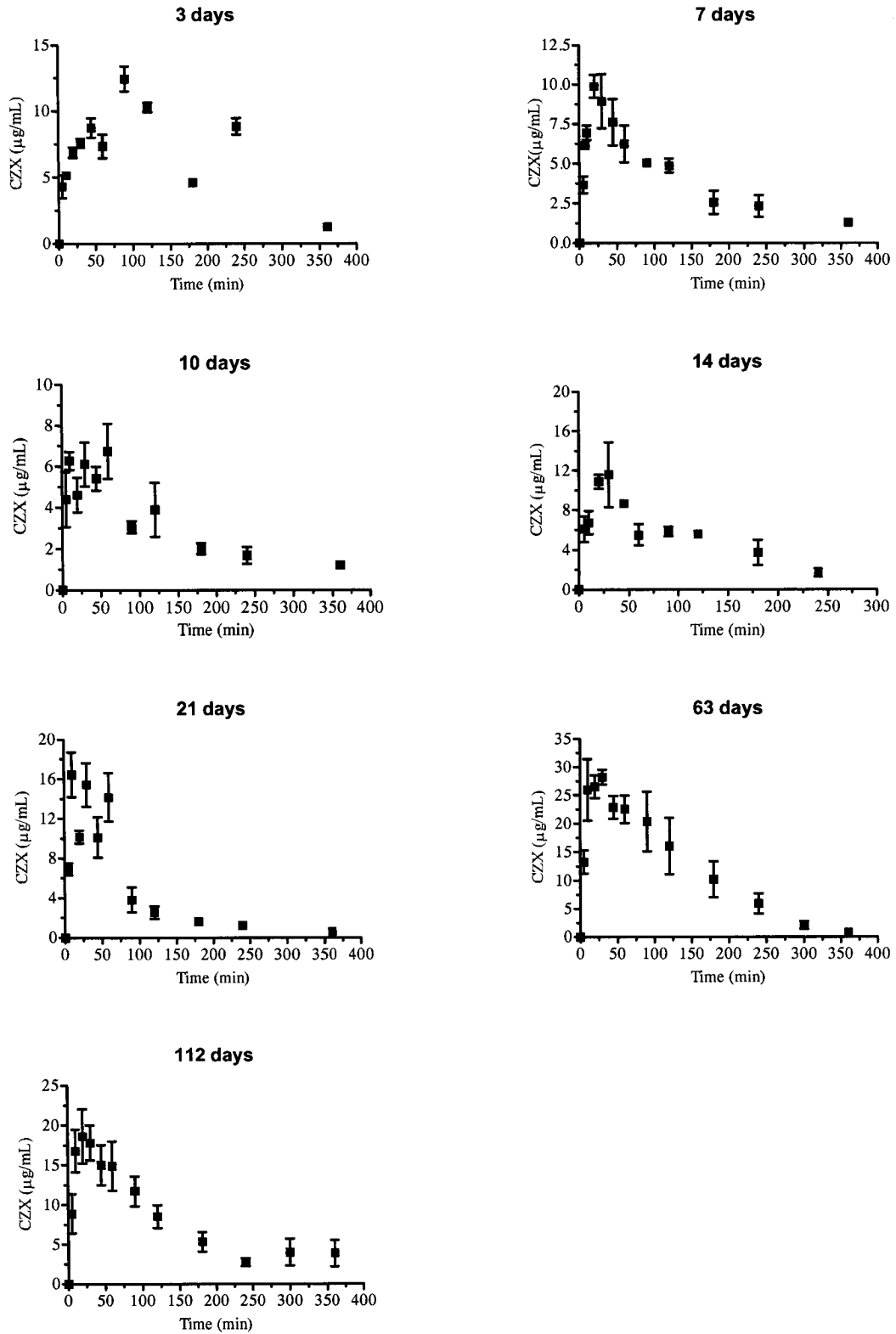
#### 4.2.2 Age-Dependent Changes in Chlorzoxazone Oral Clearance in Rats

To assess the ability of the general ontogeny PK model to make a *priori* predictions of the elimination capacity at different developmental ages as proof of concept, the oral clearance of chlorzoxazone (CZX) whose elimination is mediated by a single elimination mechanism (CYP2E1) was evaluated. Plots of average CZX serum concentration following oral administration to male Sprague Dawley rats at early neonatal (3 days), middle neonatal (7 days), late neonatal (10 and 14 days), weaning (21 days), juvenile (63 days) and adult (112 days) are shown in Figure 4.16. Pharmacokinetic parameter estimates for CZX calculated by non-compartmental analysis are shown in Table 4.6. The oral clearance (Cl/F) value of CZX at 3 days was about 130% the adult value followed by a sudden increase to 200% the adult value at 7 days and more than 230% the adult value at 10 days. This value gradually declined with age to less than 80% the adult value at puberty age (63 days). Although significant changes in oral clearance values and  $C_{max}$  values were observed, the elimination rate constant was almost similar at all examined postnatal ages. As well, other PK parameters e.g.  $AUC_{\infty}$  volume of distribution and elimination half life underwent age-dependent changes.

To test the ability of our rat CYP2E1 ontogeny model (Equation 4.1) to provide a *priori* prediction of oral clearance of chlorzoxazone (CYP2E1 substrate), age-specific ISF values were predicted from the model equation (Equation 4.1). Estimation of age-specific ISF values allowed prediction of age-specific CZX oral clearance values using a published adult oral clearance values (Cl/F) of 10.5 ml/min/kg<sup>278</sup> and using Equation 1.15.

$$Cl_{H(j,t)}^{infant} = ISF_{H(j,t)} Cl_{systemic}^{adult} \quad (1.15)$$

Visual demonstration of the correspondence between observed and calculated Cl/F values versus age (Figure 4.17A) indicates that the model underestimates the oral clearance values at the all examined ages except at the weaning age (21 days). While, calculated versus observed Cl/F data plot (Figure 4.17B) shows a reasonable correspondence ( $r = 0.75$ ), a distinct deviation from a straight line is also shown. Such deviation may indicate the need for a more complex model or more data points.

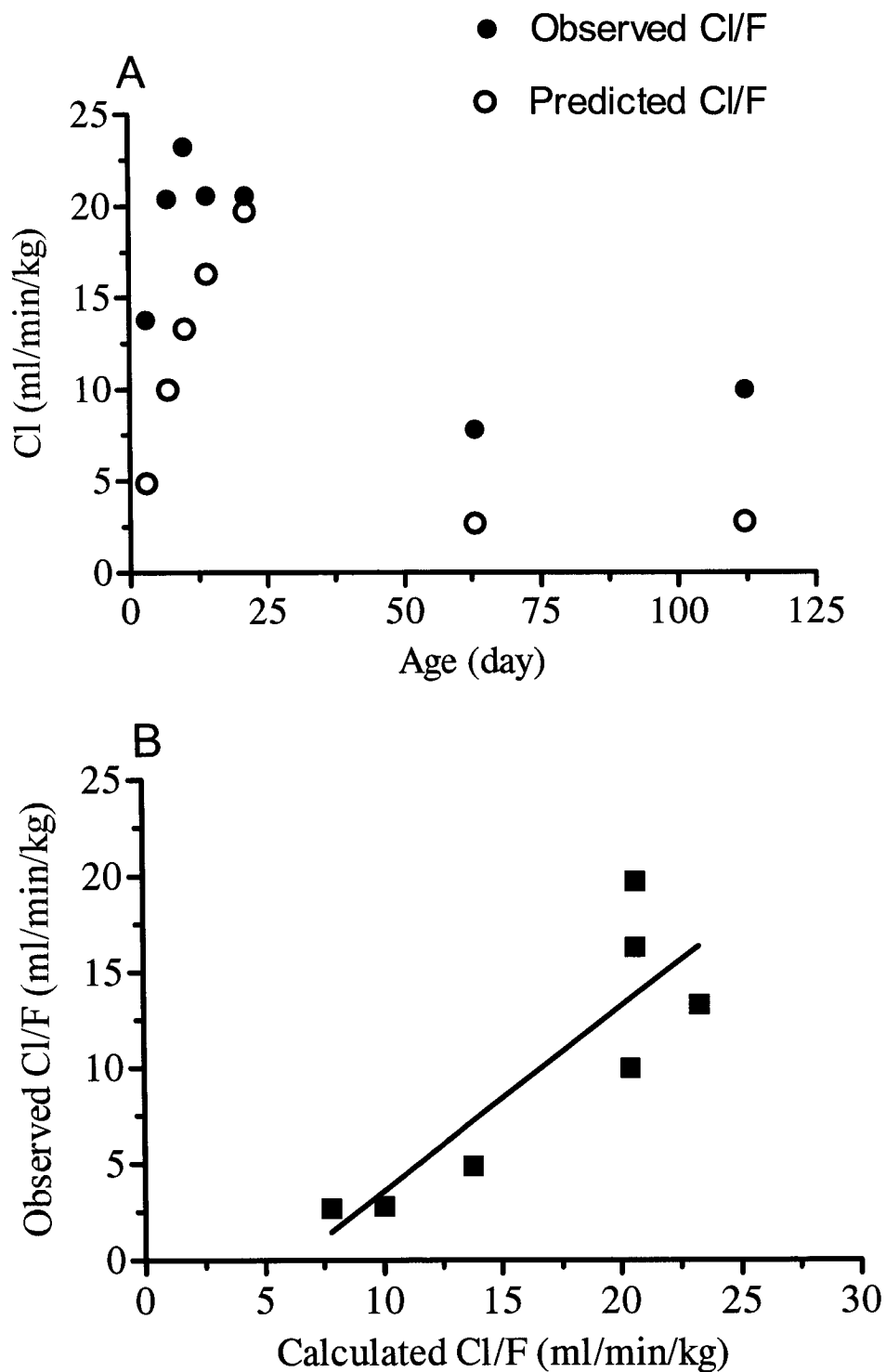


**Figure 4.16** – Mean  $\pm$ SD serum concentration versus time curves of chlorzoxazone after 30 mg/kg oral dose in male Sprague Dawley rats (n =4) at different developmental ages.

**Table 4.6** – Pharmacokinetic parameter estimates following a 30 mg/kg oral dose of CZX to male Sprague-Dawley rats (n = 4) at different developmental ages.

Age (days)	$\lambda_z$ ( $\text{min}^{-1}$ )	$C_{\text{max}}$ ( $\mu\text{g/mL}$ )	$t_{\text{max}}$ (min)	$t_{1/2}$ (min)	$AUC_{\infty}$ ( $\mu\text{g}\cdot\text{min/mL}$ )	$V_{\text{dss/F}}$ (mL/kg)	Cl/F (mL/min/kg)
3	0.0086	12.5	90	80	2169	1611	13.8
7	0.0083	11.4	29	83	1561	2578	20.4
10	0.0075	7.6	22.5	92	1286	5045	23.3
14	0.00868	14.1	25	80	1463	2483	20.6
21	0.00658	19.9	40	105	1500	3434	20.6
63	0.012 (0.004)	32.4 (4)	37.5 (35)	59 (16)	4207 (1382)	688 (338)	7.8 (2.7)
112	0.009 (0.001)	20 (5.3)	22.5 (9.5)	78 (9.7)	3166 (1048)	1153 (410)	10 (2.8)

<sup>a</sup>Due to limited blood volume of neonatal pups, a destructive sampling schedule (four neonates/age/sacrifice time) was employed and the results are expressed as average at each age. <sup>b</sup>PK parameter estimates were determined in four animals and the results are expressed as average ( $\pm$ SD).



**Figure 4.17** – Age-related correspondence between calculated and observed CZX oral clearance (Cl/F) values (A) and plot output of calculated versus observed CZX Cl/F data using CYP2E1 ontogeny model (Equation 4.1) (B).

### 4.3 Characterization of the Ontogeny of CYP2E1 and CYP1A2

#### 4.3.1 Ontogeny of Rat Hepatic CYP2E1 and CYP1A2 mRNA

To assess developmental changes in mRNA expression levels of rat hepatic CYP2E1 and CYP1A2, real-time RT-PCR was performed using 20-residue oligonucleotide primers (Table 3.2) specific to rat CYP2E1 and CYP1A2, respectively.

##### 4.3.1.1 Optimization of Real-Time RT-PCR Conditions

Table 4.7 lists the optimal annealing temperatures and corresponding primer efficiencies and standard curve ranges for *CYP2E1* and *CYP1A2* genes. Primer sequences and amplicon sizes are detailed in Section 3.2.8.2. Optimal annealing temperatures were selected based on primer efficiency (E) (a theoretical value of 2 is optimal) and lack of primer dimer formation. For primer and assay optimization, the slope was measured from a three-point calibration curve using serial dilutions of control RNA (adult female Wistar rat).

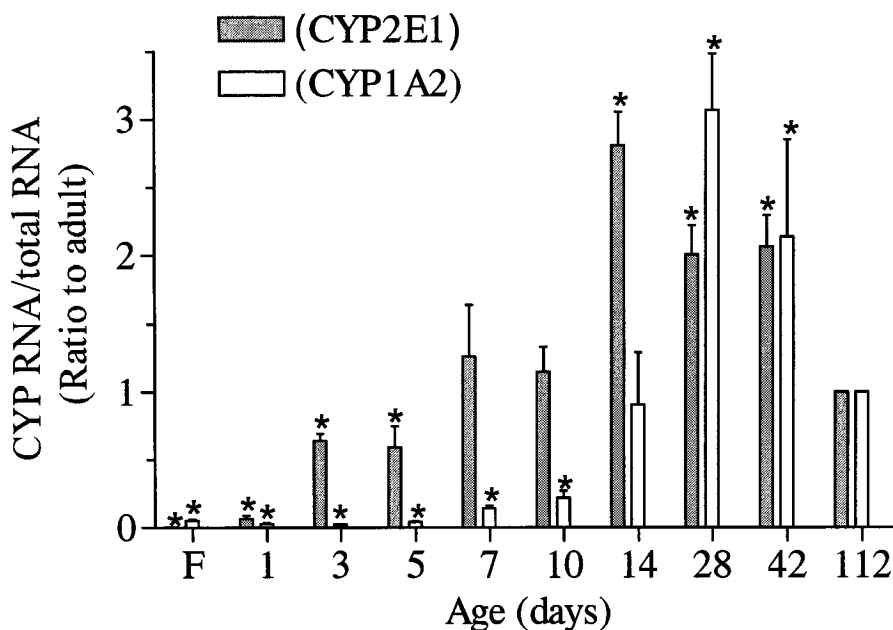
**Table 4.7** – Optimal annealing temperatures and standard curve ranges for *CYP2E1*, *CYP1A2* and *β-actin* genes for real-time RT-PCR analysis.

Gene	Annealing Temperature (°C)	Primer E	Standard Curve Range (ng)
<i>CYP2E1</i>	55	1.9	0.05-500
<i>CYP1A2</i>	58	2.04	0.05-500
<i>β-actin</i>	55	1.93	0.05-500

##### 4.3.1.2 Age-Dependent Changes in mRNA Expression of Rat Hepatic CYP2E1 and CYP1A2

Results of age-dependent changes in CYP mRNA expression levels are expressed as mean  $\pm$ SD of the ratio (CYP mRNA/total RNA) as compared to adult (Figure 4.18). CYP2E1 mRNA expression was low in fetal livers (approximately 0.005 fold the adult value) and increased after birth to reach more than 2.5- fold the adult value at PD14. The expression at puberty (PD42) was 2-fold the adult value with P value  $<0.05$ . As well, CYP1A2 mRNA expression was very low in fetal livers (approximately 0.05 fold

the adult value), and increased gradually after birth until PD10. Dramatic increases in CYP1A2 mRNA expression occurred after PD10 to reach more than three fold the adult value at 28 days. The expression at puberty (PD42) was more than two fold the adult value with  $P < 0.05$ .



**Figure 4.18** – Age-related variations in rat hepatic CYP2E1 and CYP1A2 mRNA expression levels. Total RNA was extracted from rat livers ( $n = 4$ ) and 40 ng was reverse transcribed and amplified as described in materials and methods. Data are expressed as mean  $\pm$ SD of the ratio CYP RNA/total RNA and as ratio to adult. Fetal livers were pooled from 3 different dams. The asterisks refer to those age groups that show significant difference from adult.

#### 4.3.2 Ontogeny of Rat Hepatic CYP2E1 and CYP1A2 Protein Expression

To assess changes in protein expression levels of hepatic CYP2E1 and CYP1A2 with maturation and investigate the correlation between CYP enzyme activity and CYP protein level, immunoblot analyses were performed using specific polyclonal antibodies against rat hepatic CYP2E1 and CYP1A2. The polyclonal antibodies used in this study recognized unique bands at 56 and 58 kilo Daltons (KD) which were the same sizes



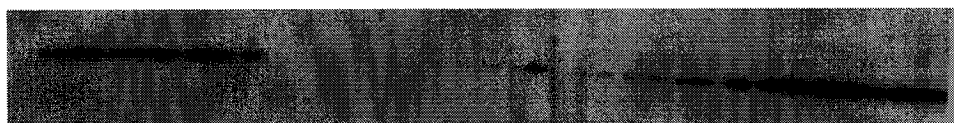
recognized by the purified rat CYP2E1 and CYP1A2 proteins loaded with the hepatic microsomes, respectively (Figures 4.19 and 4.20).

Lanes 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16



**Figure 4.19** –Immunoblots of rat hepatic microsomes and rat CYP2E1 purified protein. 10.5  $\mu$ g microsomal protein and different CYP2E1 protein amounts (20, 50 and 100 pmol) were electrophoresed on a 10% SDS-PAGE, transferred onto a PVDF membrane, and probed with a polyclonal antibody to rat CYP2E1. Lanes (1-3) are purified rat CYP2E1 protein (100, 50 and 20 pmol, respectively). Lanes (4-16) are rat hepatic microsomes from fetal (lane 4) and postnatal days 1, 3, 5, 7, 10, 14, 21, 28, 42, 63, 84 and 112, respectively.

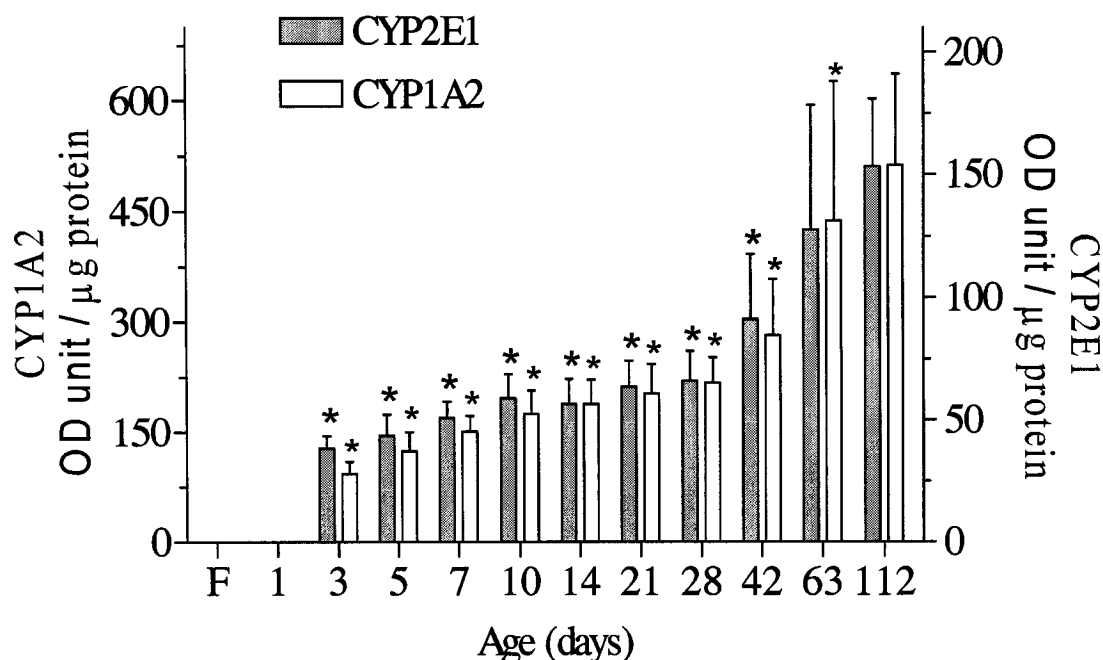
Lanes 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16



**Figure 4.20** – Immunoblots of rat hepatic microsomes and rat CYP1A2 purified protein. 0.4  $\mu$ g microsomal protein and different CYP1A2 protein amounts (20, 80, 200 and 500 pmol) were electrophoresed on a 10% SDS-PAGE, transferred onto a PVDF membrane, and probed with a polyclonal antibody to rat CYP1A2. Lanes (1-4) are purified rat CYP1A2 protein (500, 200, 80 and 20 pmol, respectively). Lanes (5-16) are rat hepatic microsomes from fetal (lane 5) and postnatal days 1, 3, 5, 7, 10, 14, 21, 28, 42, 63 and 112, respectively.

The amount of protein present in hepatic microsomes and cross-reacting with the polyclonal antibody is shown in Figure 4.21. The results are expressed as absorbance unit/amount of microsomal protein. When examined as a function of age, expression of CYP2E1 and CYP1A2 proteins showed a biphasic pattern. Proteins were not visible in fetal and postnatal day 1 hepatic microsomal samples. Expression for both CYP2E1 and CYP1A2 proteins was detected at 3 days of age where it accounted for no more than 25

% of the adult content,  $P < 0.05$ . Expression increased progressively and plateaued at PD 10 followed by gradual increase at PD28 (30% of the adult value,  $P < 0.05$ ). Protein levels reached 75% of the adult value at the puberty age (63 days).



**Figure 4.21** – Age-related changes in rat hepatic CYP2E1 and CYP1A2 protein expression. Microsomal proteins were separated on a 10% SDS-PAGE and transferred to a PVDF membrane. CYP proteins were immunochemically detected with polyclonal antibodies raised against rat CYPs. The antigen-antibody complex was visualized after addition of a peroxidase-conjugated IgG, and quantified by scanning with an image analysis system. Data are expressed as mean  $\pm$ SD of 4 rats except fetal livers, which were pooled from 3 different dams. The asterisks refer to those age groups that show significant difference from adult.

### 4.3.3 Ontogeny of Rat Hepatic CYP2E1 and CYP1A2 Activity

Developmental changes in CYP2E1 and CYP1A2 maximal enzyme activities ( $V_{max}$ ) were assessed by measuring the enzyme activity at saturating chlorzoxazone and methoxyresorufin concentrations in age-dependent rat hepatic microsomes as shown in Figure 4.8. Both activities were undetected in fetal and PD1 hepatic microsomes.

Activities were low at early neonatal ages followed by a marked increase between PD3 and PD21 (CYP2E1) or between PD5 and PD28 (CYP1A2). Subsequently, activities declined to adult values by puberty with both enzymes (see section 4.1.2.4 for more detailed description for the ontogeny of CYP2E1 and CYP1A2 activity).

#### **4.3.4 Correlation between Different Ontogeny Patterns of CYP2E1 and CYP1A2 Enzymes in Rats**

##### *4.3.4.1 CYP Immunoreactive Protein and its mRNA Level*

A poor correlation was observed for CYP2E1. Although CYP2E1 protein was not detected in fetal and PD1 livers, its mRNA was detected. Postnatally, mRNA levels rose rapidly to achieve approximately 1-fold, 3-fold, and 2-fold the adult level at PD10, PD14 and PD28, respectively. On the other hand, CYP2E1 protein levels increased slowly from PD3 (25% the adult value) to PD28 (30% the adult value). After PD28, CYP2E1 protein increased progressively to achieve the adult level at PD42 (Figure 4.22).

As well, developmental changes in CYP1A2 mRNA expression did not correlate with the immunoquantifiable CYP protein, except for the early postnatal period where both CYP1A2 protein and its transcript were low and increased gradually (Figure 4.22).

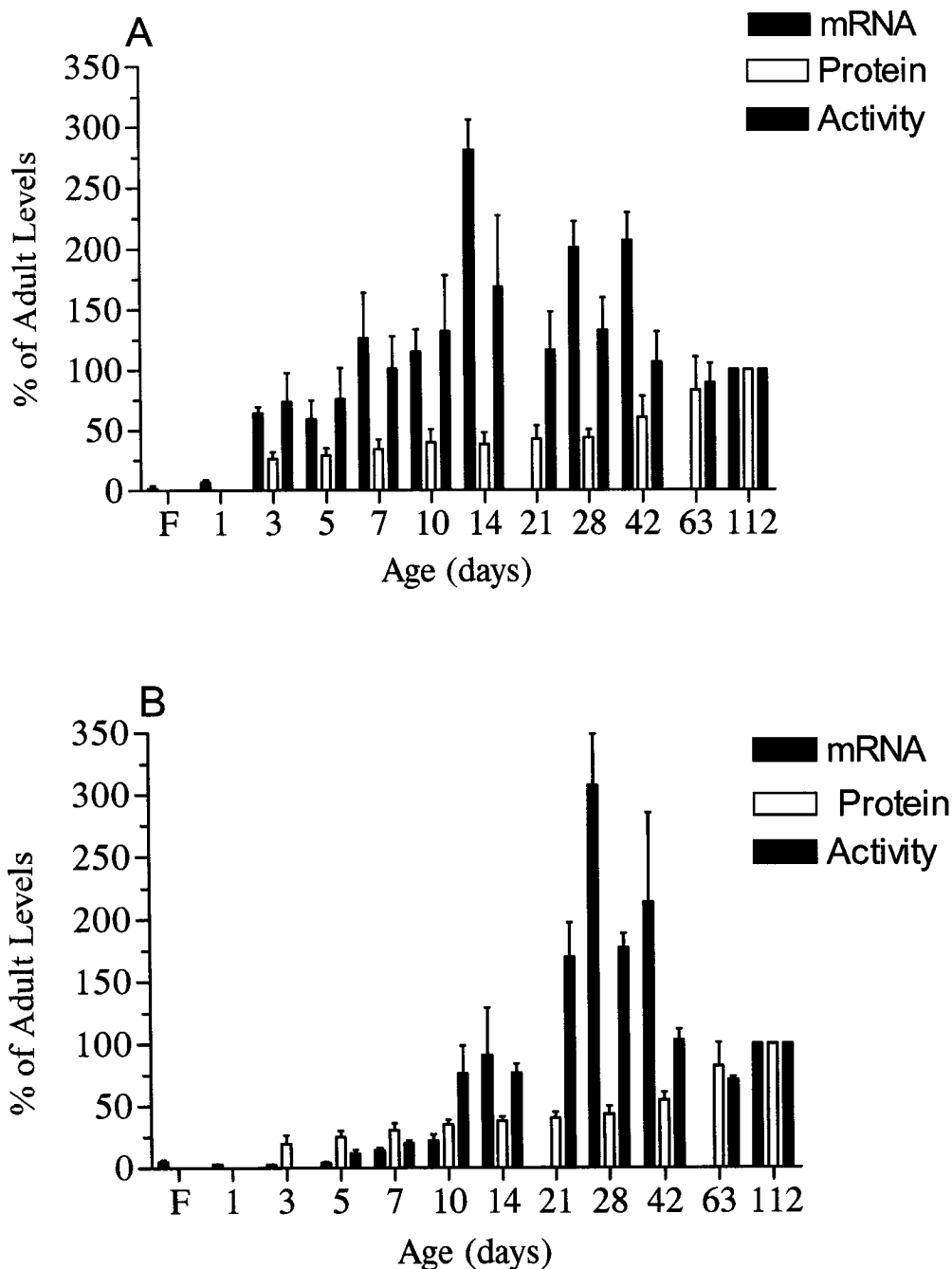
##### *4.3.4.2 CYP Immunoreactive Protein and its Catalytic activity*

For both CYP enzymes, their protein and activity were not detected in fetal and PD1 livers. However, dramatic increase in catalytic activity was observed between PD3 and PD21 (CYP2E1) or between PD5 and PD28 (CYP1A2) followed by a decline to the adult level during puberty. These dramatic changes in activity were not mirrored by changes in protein levels for both enzymes, as mentioned in section 4.2.6 and shown in Figure 4.11.

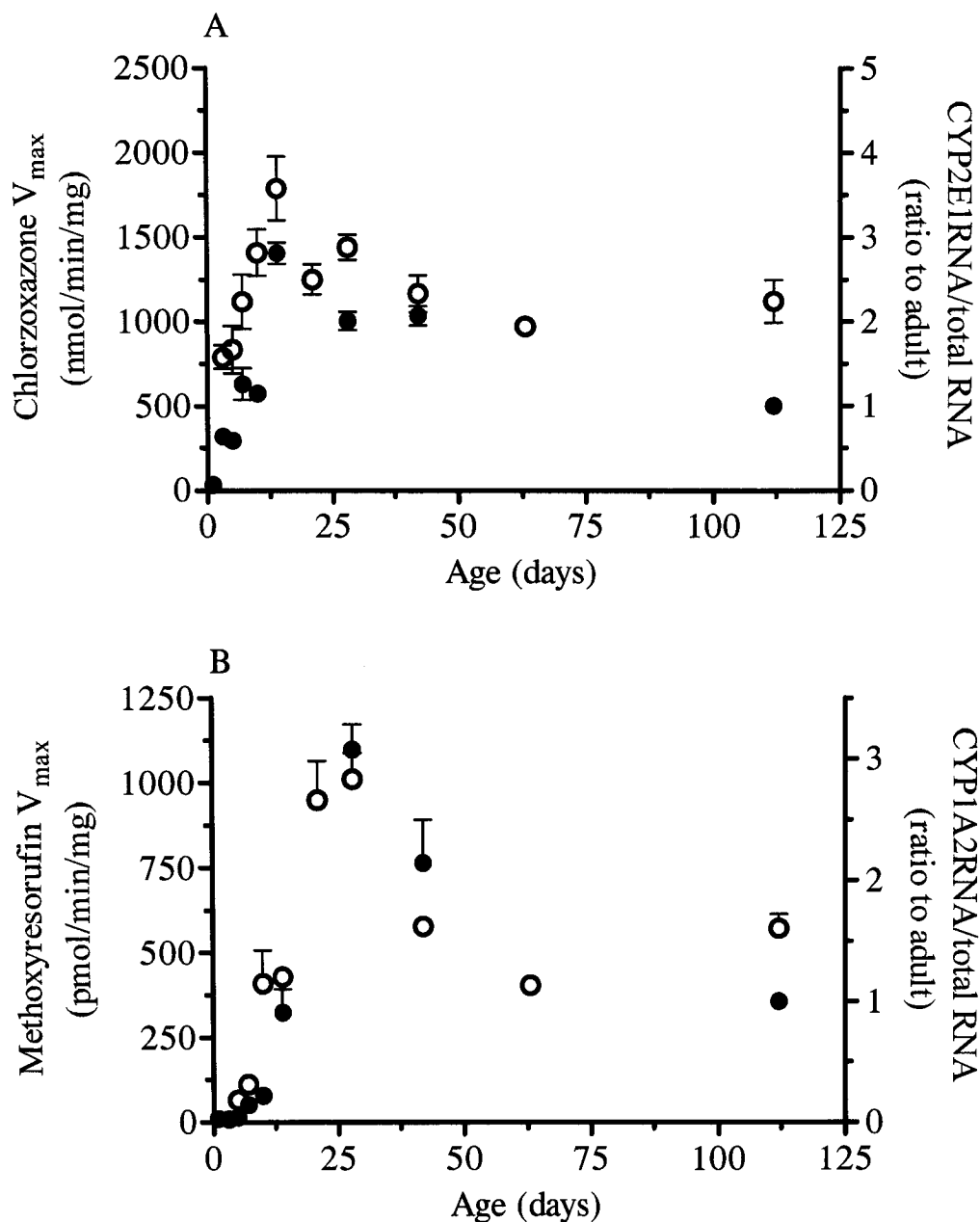
##### *4.3.4.3 CYP mRNA and its Catalytic Activity*

On the other hand when age-dependent changes in CYP enzymes activities were compared with changes in mRNA levels obtained from the real-time RT-PCR data,

CYP2E1-mediated CZX hydroxylase activity and CYP1A2-mediated MROD activity correlated reasonably well with changes in CYP2E1 and CYP1A2 mRNA levels, respectively (Figure 4.23).



**Figure 4.22-** Developmental changes in expression of (A) CYP2E1 and (B) CYP1A2 mRNA, protein and activity levels in rat livers. All data are expressed as a percentage of adult levels.

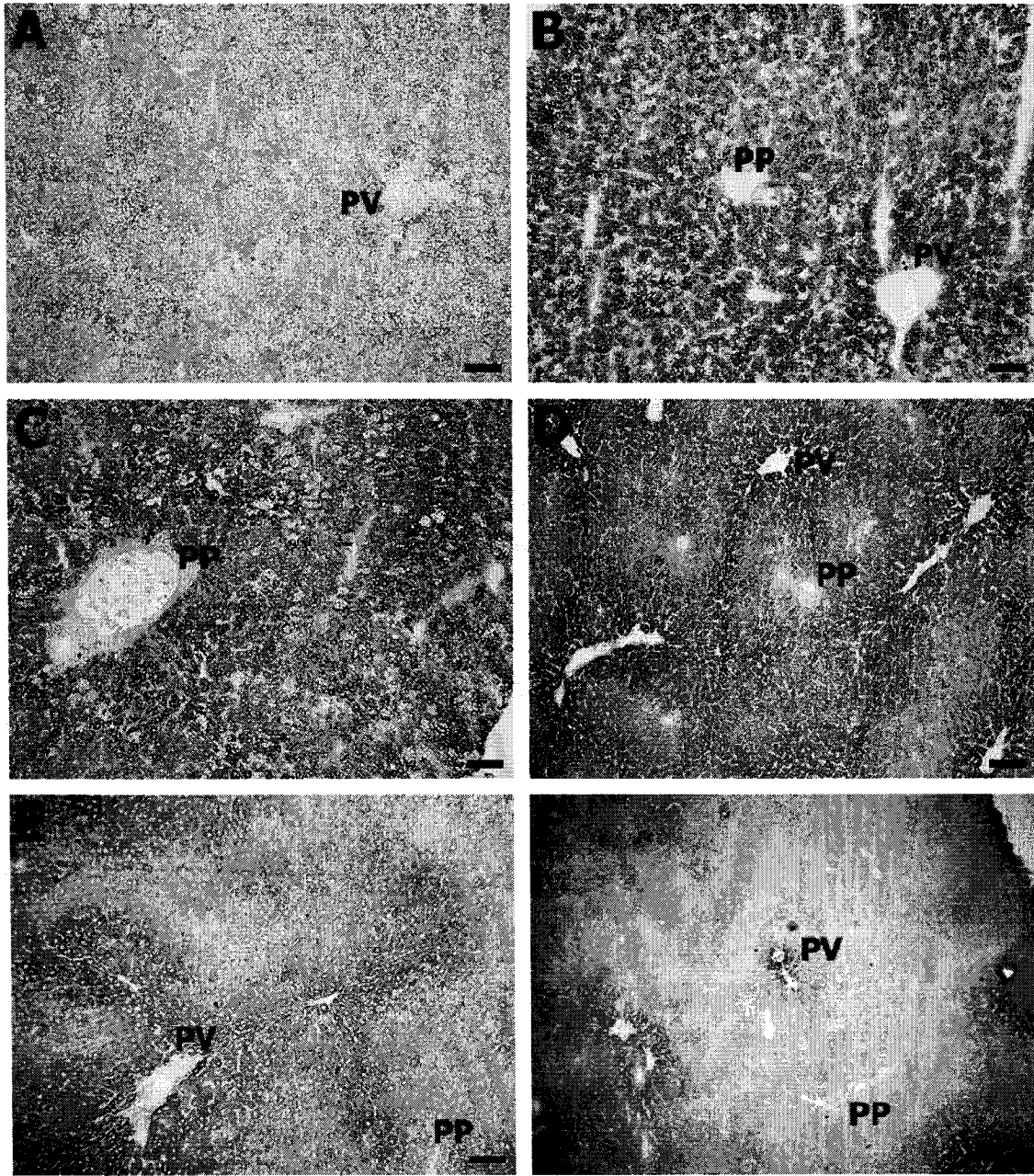


**Figure 4.23**— Age-dependent changes in CYP enzyme activity (open circle) as a function of mRNA levels (closed circle) in rat hepatic microsomes. (A) Average CYP2E1-mediated chlorzoxazone hydroxylase  $V_{max}$  estimates as a function of average CYP2E1 mRNA levels. (B) Average CYP1A2-mediated methoxyresorufin-O-dealkylase  $V_{max}$  estimates as a function of average CYP1A2 protein levels. mRNA data were expressed as (CYP RNA/total RNA) as a ratio to adult values.

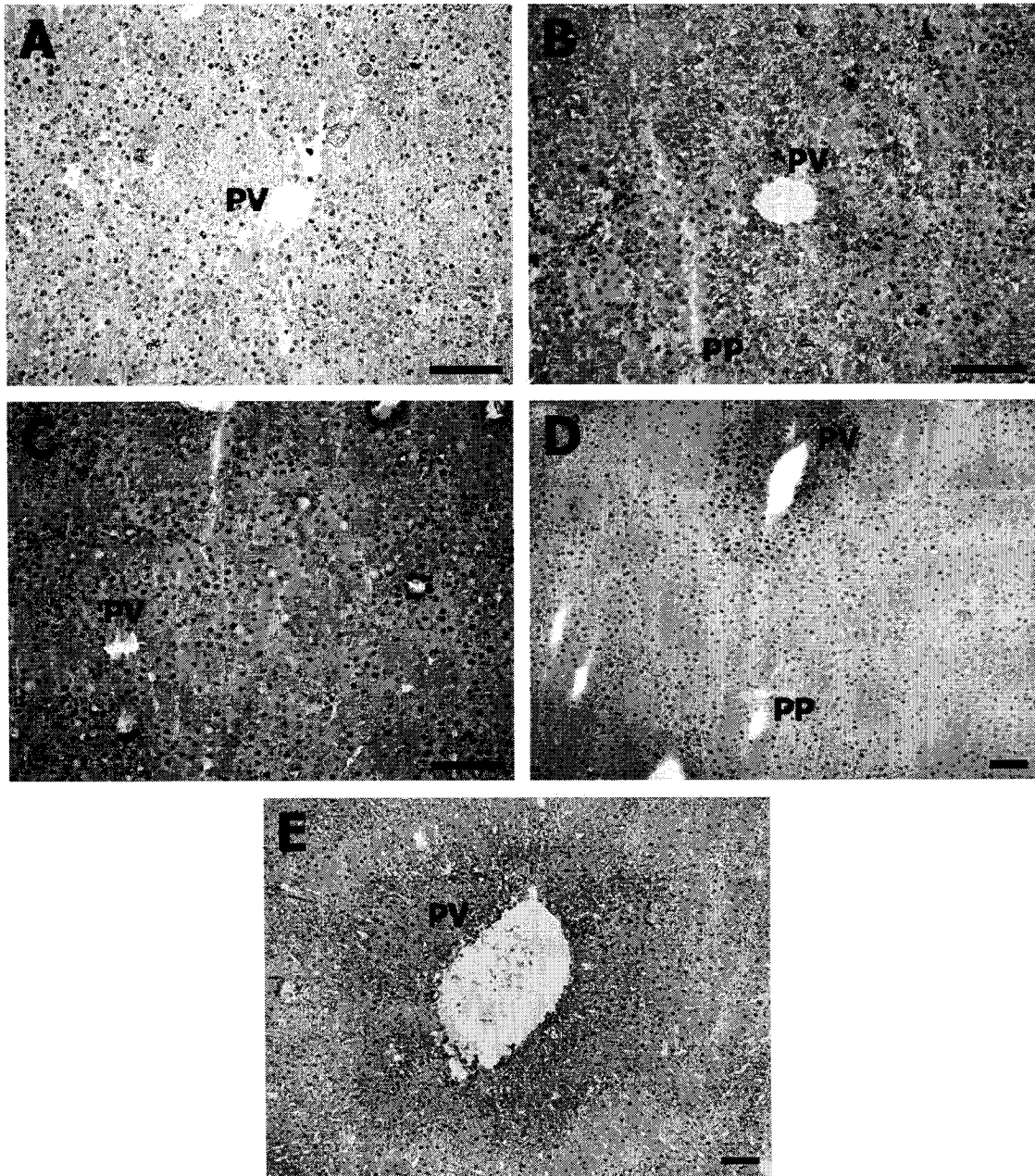
Although activities were not detected at <PD3, mRNA expression was detected, yet very low levels. Both enzyme activity and mRNA levels increased markedly following PD3 to reach maximum levels at PD14 (CYP2E1) or PD28 (CYP1A2) before declining to adult values. While activity levels at early puberty (PD42) were similar to adult values, mRNA levels were about 2-fold the adult values for both CYP enzymes (Figure 4.23).

#### **4.3.5 Ontogeny of Zonal Expression of Rat Hepatic CYP2E1 and CYP1A2**

To characterize the age-dependent changes in intrahepatic distribution of CYP2E1 and CYP1A2 protein I performed an immunohistochemical analysis with polyclonal antibody to CYP2E1 and CYP1A2 in gestation age 20, postnatal ages 1, 5, 10, 42 and adult livers. Immunoreactivity in fetal and PD1 livers was homogeneously distributed throughout the liver and the intensity of staining appeared similar regardless of the intralobular location of the hepatocyte (Figures 4.24 A-B and 4.25 A-B). At postnatal days 5 (only for CYP2E1) and 10 immunostaining showed slightly greater intensity in the perivenous hepatocytes (Figures 4.24 C-D and 4.25 C). A more distinct loss of immunoreactivity was observed in periportal hepatocytes at postnatal day 42 (Figures 4.24 E and 4.25 D). In adult liver, immunoreactivity is focally distributed in the perivenous hepatocytes with almost no staining in the periportal region (Figures 4.24 F and 4.25 E).



**Figure 4.24** – Immunohistochemical localization of CYP2E1 in representative rat livers from (A) fetus; (B) postnatal day 1; (C) postnatal day 5; (D) postnatal day 10 (late neonatal); (E) postnatal day 42 (early puberty); and (F) adult. In fetal and neonatal livers, hepatocytes throughout the entire hepatic acini exhibit staining. Increasingly more intense staining is observed in the perivenous hepatocytes in the livers from postnatal day 42 rats with very intense staining in perivenous hepatocytes and almost negative staining in the periportal hepatocytes in the adult. (PV =perivenous; PP =periportal.). Scale Bar, 100  $\mu$ m.



**Figure 4.25** – Immunohistochemical localization of CYP1A2 in representative rat livers from (A) fetus; (B) postnatal day 1; (C) postnatal day 10; (D) postnatal day 42 (early puberty); and (E) adult. In fetal and neonatal livers, hepatocytes throughout the entire hepatic acini exhibit staining. Increasingly more intense staining is observed in the perivenous hepatocytes in the livers from postnatal day 42 rats with very intense staining in perivenous hepatocytes and almost negative staining in the periportal hepatocytes in the adult. (PV =perivenous; PP =periportal.). Scale Bar, 100  $\mu$ m.

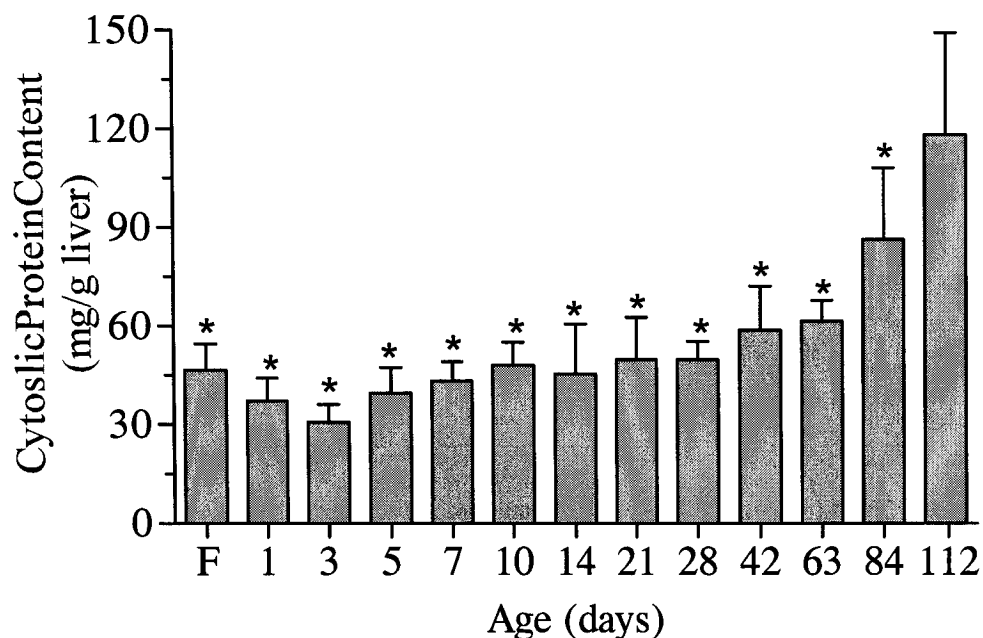


#### 4.3.6 Ontogeny of Rat Hepatic Glutathione-S-Transferase (GST) Activity

To determine the age-dependent changes in GST activity in rat livers, cytosolic protein content (mg/gram liver) was determined and the cytosolic GST activity was assessed spectrophotometrically using a GST general probe substrate.

##### 4.3.6.1 Age-Dependent Changes in Cytosolic Protein Content

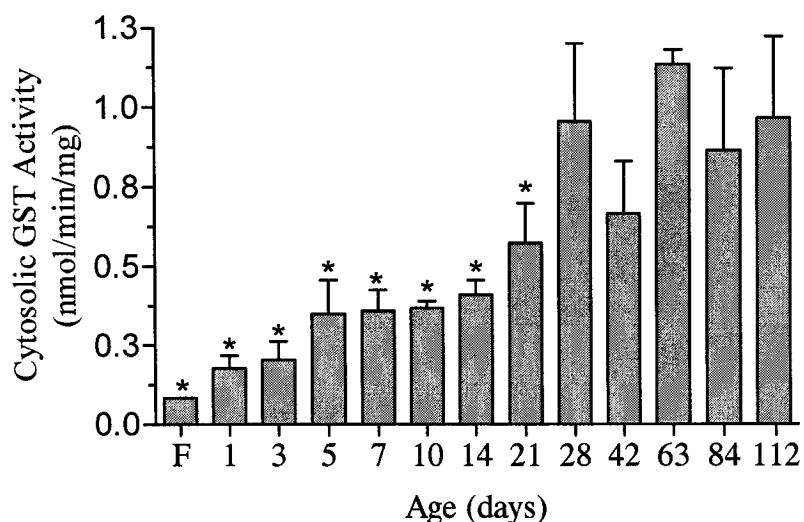
Hepatic cytosolic protein content for each age group (n =4) was measured by the method of Lowry *et al*<sup>270</sup>. The results shown in Figure 4.26 indicate that average cytosolic protein content (mg/gram liver weight) was low at birth (30 % of the adult value, P<0.05) and increased gradually to puberty (63 days) where it was 50 % of the adult value, P<0.05.



**Figure 4.26** – Mean ( $\pm$  SD) hepatic cytosolic protein content in male Sprague-Dawley rats (n=4). Asterisks indicate statistical difference from adult (112 days), P < 0.05. F= gestation day 20.

#### 4.3.6.2 Age-Dependent Changes in Total Cytosolic GST Activity

Liver cytosolic GST activity was determined using the general substrate 1-chloro-2,4-dinitrobenzene (CDNB). As shown in Figure 4.22, general GST activity was detected at gestational age 20, (10% of the adult value;  $P < 0.05$ ), and increased gradually until PD21 where activity was about ten fold the fetal value. By PD28 general GST activity reached adult values.



**Figure 4.27** –Age-related changes in hepatic cytosolic GST activity. The activity was monitored by spectrophotometric analysis of glutathione conjugation of 1-chloro-2,4-dinitrobenzene, a general GST probe substrate. Data are expressed as mean  $\pm$ SD of the hepatic GST activity of 4 animals. Fetal livers were pooled from 3 different dams. The asterisks refer to those age groups that show significant difference from adult.

## 5. DISCUSSION

### 5.1. Evaluation of the Assumptions of an Ontogeny Model of CYP Enzyme Activity

#### *5.1.1 Development and Relevance of the Model*

Breastfeeding is the most significant route of exposing the developing infant to a wide variety of drugs and environmental toxicants<sup>16,30,47</sup>. Most of the commonly used exposure risk assessment approaches usually underestimate the toxicity risk to the exposed infant because these approaches consider mainly the amount of exposure dose in breast milk and ignore the contribution of infant pharmacokinetics in determining the exposure outcome on the child<sup>16,43,47-49</sup>. Early life stages exhibit significant and dynamic developmental changes in the physiological and biochemical processes that govern the pharmacokinetics and pharmacodynamics of xenobiotics and such changes can significantly affect the toxicological outcome of an exposure<sup>44,221</sup>. However, only recently have efforts intensified to enhance our understanding of age-related changes in pharmacokinetic processes. Databases containing paediatric clinical pharmacokinetic data are in development to provide relevant information for more successful adult-to-infant extrapolation of therapeutic compounds and applicable toxicants<sup>224,281</sup>. Furthermore, physiologically based pharmacokinetic models are improving the extrapolation of animal developmental toxicology data for human paediatric risk assessments<sup>223,282</sup>, which is particularly important for those toxicants for which no human data are available.

Although the general lack of information regarding paediatric pharmacokinetics compels the use of toxicological information available from adult populations<sup>221,224</sup> or animal developmental toxicology studies<sup>282,283</sup>, both adult-to-infant and animal-to-human extrapolations can lead to under- or overestimations of exposure risk in paediatric populations<sup>222,284</sup>. An alternative approach is to use pharmacokinetic models that are based upon relevant biochemical and physiological data from paediatric

populations. These models offer the promise to provide more quantitative predictions of internal dosemetrics (i.e. steady state plasma concentration, area-under-the-plasma concentration vs. time-curve) and the potential for toxicity. Such models, then, may provide critical information for use in risk/benefit assessments of the safety of breastfeeding during maternal therapy or following exposure of the nursing mother to environmental toxicants.

Several modeling approaches have been proposed using either human clinical or animal data<sup>221,222,224,235</sup>. In our laboratory, a general mathematical model describing the ontogeny of hepatic CYP enzyme-mediated elimination in the first 6 months of age was developed and applied to predict infant metabolic clearance<sup>77</sup>. The model involves an *in vitro-in vivo* extrapolation of CYP enzyme activity data from age-specific human hepatic microsomes using CYP-specific probe substrates (see Appendix I for the theoretical framework of the model). The model enables prediction of age and CYP-specific infant scaling factors (ISF) that correlate adult intrinsic clearance with the ability of the infant to eliminate drugs by CYP pathways (Equations 1.15 and 1.16). Accordingly, this model could be a promising approach to provide appropriate assessments of the toxicological risk to infants following exposure to toxicants that undergo CYP-mediated bioactivation or detoxification.

Application of this model requires an evaluation of its underlying assumptions and its ability to make robust predictions of infant metabolic clearance. Given the logistic/ethical boundaries of obtaining *in vitro/in vivo* metabolism data in human infants<sup>60,285</sup>, the rat was used as an animal model system to evaluate the model's assumptions. I used the rat because this species shows sufficient similarities to humans regarding developmental physiology (i.e. liver development), enzymology and molecular mechanisms of drug metabolism<sup>1,96,259</sup>. As well, the rat is the most widely studied model in preclinical and toxicology studies when initial information regarding drug metabolism in humans is required<sup>1,116,261,262</sup>. Accordingly, its shortcomings are highly understood and characterized.

### 5.1.2 Developmental Changes in Hepatic Scaling Factors (HSF)

Extrapolation of the *in vitro* metabolism data to the *in vivo* situation is based on the use of the parameter, intrinsic clearance ( $Cl_{int}$ ). This parameter is a pure measurement of the enzyme activity towards a substrate without influence of the other physiological determinants of hepatic clearance, hepatic blood flow and drug binding within the blood<sup>225</sup>. *In vitro/in vivo* extrapolation of intrinsic clearance ( $Cl_{int}$ ) is achieved when appropriate hepatic scaling factors (HSF) are applied (Appendix I). HSF (mg microsomal protein per g body weight) accounts for the contribution of hepatic microsomal protein content (MP) and liver weight (LW) normalized to body weight (BW) to the metabolic function of the whole liver (Equation 3.3).

In the human CYP ontogeny model (see Appendix I), literature data of age-dependent changes in human LW and BW were obtained and used to develop an empirical equation describing the developmental changes in LW and BW from birth to the first year of life (Equation 11, Appendix I). However, due to the limited information about age-dependent changes in MP, it was assumed that microsomal protein content (mg/g liver) remains constant throughout postnatal maturation<sup>77</sup>. Accordingly, an adult human value of 52.2 mg/g liver<sup>258</sup> was used as MP value for all infant age groups. By assuming constant MP value in building the general model of CYP ontogeny, any change in  $HSF_{(t)}$  will be solely due to the developmental changes in LW normalized to BW (Equation 3.3).

#### 5.1.2.1 Developmental Changes in Microsomal Protein Content (MP) in Rat Livers

In my attempt to evaluate this assumption, hepatic MP was determined in age-specific rat hepatic microsomes. Results shown in Figure 4.2 reveal that rat hepatic microsomal protein content was significantly lower in fetal (20% the adult value) and early neonatal (25-60% the adult value) livers and continued to increase until reaching the adult value at postnatal day 7 (PD7) with the maximum value obtained at the onset of puberty (PD42) (125 % the adult value). Several studies have reported developmental changes in rat hepatic MP content, but these studies were conducted using a limited number of liver samples or age groups that do not cover all developmental stages in

rats<sup>95,115,286,287</sup>. The observed MP data in my study are in agreement with the MP results reported in similar age groups used in these studies.

To my knowledge, there are few data reporting developmental changes of MP content in human. Most of the reported studies were conducted in adult human (35-75 years) hepatic microsomes. While these reports show that hepatic concentration of microsomal protein remained virtually unchanged, they also show significant interindividual variability<sup>288-290</sup> as well as gender differences<sup>289</sup>. Other studies reported adult values of 45 mg/g<sup>252</sup>, 53 mg/g<sup>258</sup>, 77 mg/g<sup>291</sup>, or 27-108 mg/g<sup>290</sup> for MP content in human. These studies determined MP content from a limited number of liver samples (3-20 livers) with some studies using samples from patients with liver cancer. Generally, this significant interindividual variability could have implications for the accurate extrapolation of *in vitro* data on drug metabolism to predict *in vivo* metabolic clearance. However, a recent article using hepatic microsomes from 38 liver donors proved that microsomal scaling factor from human livers to be a robust method and recommended the use of a microsomal scaling value of 40 mg of microsomal protein per gram of liver as a generic value for predicting *in vivo* drug metabolic clearance from *in vitro* human hepatic microsomal data<sup>292</sup>.

Whether such changes in rat hepatic MP content can be extrapolated to human development is unknown, but compared to human, rats exhibit important differences in biogenesis of the endoplasmic reticulum (ER) and hepatocellular differentiation during development<sup>293-295</sup>. Liver parenchymal cells during late gestation and early neonatal stages in rats contain predominantly rough ER<sup>294</sup>. The total amount of ER per cell increases between 3 days before birth and birth where rough ER remains predominant<sup>294</sup>. Smooth ER becomes a quantitatively significant component only a few days after birth<sup>293,294</sup>. Such developmental changes in hepatocellular content of smooth ER may explain the age-related changes in MP in rats.

#### 5.1.2.2 Developmental Changes in Rat Liver Weight (LW) and Body Weight (BW)

An examination of the developmental changes in the ratio of liver weight to body weight reveals that this ratio remains relatively constant throughout rat postnatal

development (Figure 4.1). This suggests the age-dependent changes in HSF (Figure 4.3) are primarily a function of developmental changes in MP content. The similarity observed between the developmental profiles of MP and HSF confirms this assertion (Figures 4.2 and 4.3, respectively).

#### 5.1.2.3 Consequences on the Model

While the above observations demonstrate a relatively constant (LW/BW) ratio throughout development, they show developmental changes in MP with significantly low values at early postnatal stages compared to adult. Although these findings contradict one of the assumptions of the ontogeny model of CYP enzyme activity, this contradiction does not necessarily weaken the potential value of the model. These results merely suggest the need to measure hepatic microsomal protein content in paediatric livers to incorporate age-dependent changes in hepatic microsomal protein content in the modeling process to provide predictive estimates of the ISF values.

#### 5.1.3 Developmental Changes in Metabolic Intrinsic Clearance ( $Cl_{int}$ )

Application of the ontogeny model is limited only to low hepatic clearance drugs where systemic clearance is principally a function of hepatic enzyme activity and the unbound fraction, and not hepatic blood flow (Equation 1.10). As well, the model assumes that efflux and uptake transporters do not contribute significantly to the intrinsic clearance ( $Cl_{int}$ ) of the compound. Transport processes can markedly influence  $Cl_{int}$  and act as the rate limiting process of metabolic clearance<sup>296-298</sup>. At present, the model may provide predictions for a wide variety of low clearance compounds when their systemic clearance is not affected by transport processes. However, the model can accommodate the contribution of transport processes other than passive diffusion and future investigations in this laboratory are planned to address this issue.

CYP2E1 and CYP1A2 were selected to evaluate the model assumptions due to the following reasons:

1. Each enzyme demonstrates markedly different ontogeny patterns<sup>44</sup>. This will enable the recognition of critical model elements that are necessary to improve the general ontogeny model of CYP-mediated hepatic clearance.

2. Reasonable predictions made for these two CYP enzymes, with different developmental patterns, may indicate the potential ability of the model to describe the developmental maturation of a wide variety of CYP enzymes.
3. Both CYP2E1 and CYP1A2 have significant toxicological relevance as they mediate bioactivation and/or detoxification of many commonly used therapeutic drugs and environmental toxicants and procarcinogens<sup>100</sup>. Consequently, developmental changes in the activity of these enzymes could have profound and age-dependent effects on the ultimate toxicological outcome of xenobiotic exposures.

Considering the genetically similar complement of hepatic CYP enzymes between infant and adults, a basic underlying model assumption is that affinity for a substrate for a given CYP enzyme should not be different between infant and adults for the specific enzyme-substrate pair<sup>77</sup>. Affinity, in this context, refers to the specific affinity between an enzyme and substrate pair in the absence of factors that may influence the apparent affinity of a compound, i.e. non-specific binding and contribution of other enzymes to the reaction pathway<sup>252</sup>. Accordingly, the developmental differences in maturation of the specific CYP enzyme activity exclusively determines the age-dependent differences in  $Cl_{int} \left( \frac{V_{max}}{K_M} \right)$ , i.e. substrate affinity ( $K_M$ ) for the enzyme remains constant during postnatal development and the developmental changes in  $Cl_{int}$  is only due to changes in functional enzyme activity ( $V_{max}$ ).

#### *5.1.3.1 Developmental Changes in Substrate Affinity ( $K_M$ )*

To evaluate the critical assumption of constant  $K_M$  values during development, probe substrates for CYP2E1 and CYP1A2 were selected with low hepatic extraction ratios and hepatic intrinsic clearances<sup>180,272,280</sup>. Additionally, no reports are available to indicate that uptake or efflux transporters can affect hepatic intrinsic clearance of these compounds. Upon examination of the developmental changes in  $K_M$  values of CYP2E1-mediated CZX hydroxylation and CYP1A2-mediated MROD activities, lower values for CYP2E1 and higher values for CYP1A2 were observed in postnatal ages  $\leq 7$  days compared to adult (Figure 4.7).



Due to the limited human and rat data, it is difficult to confirm whether such developmental changes in  $K_M$  values occur during human neonatal development. One study<sup>133</sup> reported a 50%  $K_M$  value for CYP2E1-mediated CZX hydroxylation in adult human hepatic microsomes compared to newborns. Also some studies reported different  $K_M$  values in microsomes prepared from livers in neonates relative to adult liver microsomes for other substrates<sup>156,272,299</sup>. Although I did not conduct further investigations to explain the age-dependent differences in the apparent  $K_M$  values, three possible explanations could be suggested. These include inefficient probe substrate specificity, non-specific protein binding in hepatic microsomes, and age-related changes in microsomal membrane composition.

#### 5.1.3.1.1 Inefficient probe substrate specificity

Enzyme kinetic parameters were estimated using accepted probe substrates and age-dependent rat hepatic microsomes. This *in vitro* system consists of heterogeneous population of CYP enzymes. No single probe substrate is 100% specific for a particular CYP of interest, and the contribution of other CYP enzymes to a reaction pathway is very possible<sup>280,300</sup>. Consequently, the measured  $K_M$  value is in fact the apparent  $K_M$ , a hybrid value that takes into account the contribution of all enzymes in the particular reaction pathway, and not the  $K_M$  value for a specific CYP enzyme-substrate pair. Depending upon their relative contributions and affinities for the substrate and stage of development, other CYP enzymes could markedly impact apparent  $K_M$  values<sup>301</sup>.

Specificity for all CYP probe substrates has been established using adult hepatic microsomes. Studies have documented that most probe substrates can be selective for the enzyme of interest but are not 100% specific<sup>280,300</sup>. Although CYP2E1 contributes extensively to the metabolism of both chlorzoxazone (CZX) and  $\rho$ -nitrophenol (PNP), CYP1A2, CYP2C11, CYP3A1 and CYP3A2 also contribute to some extent<sup>280</sup>. Furthermore, the CYP1A2 probe substrates, ethoxyresorufin and phenacetin, undergo extensive metabolism by CYP1A2, but with minor contributions from CYP2C6, CYP2C11, CYP2E1, CYP3A1 and CYP3A2<sup>280</sup>. While these studies show a major contribution from one particular pathway (e.g. CYP2E1 or CYP1A2) and minor contributions from others, these results were observed in adult hepatic microsomes, a

situation that may be significantly different from neonatal microsomes. Since most CYP enzymes undergo different rates and patterns of maturation towards adulthood<sup>114,268</sup>, the major elimination pathway observed in adult livers can be functionally immature in the developing infant. Consequently, the minor contributing pathways seen in adult may contribute significantly to substrate elimination in the developing infant<sup>77</sup>. Some of these CYP pathways, especially CYP3A1 and CYP3A2, are expressed during gestational stages in rats and increase dramatically after birth, whereas activities of CYP2E1 and CYP1A2 are either undetected or very low<sup>1</sup>. Eadie-Hofstee plots (Figures 4.12 and 4.13) gave clear evidence of a deviation from a single site Michaelis-Menten model for chlorzoxazone hydroxylation and MROD activity, which may indicate the possible contribution of other CYP enzyme(s), in addition to CYP2E1 and CYP1A2, respectively.

#### *5.1.3.1.2 Non-specific protein binding in hepatic microsomes*

The apparent  $K_M$  value can be significantly affected by developmental changes in non-specific protein binding of drug substrate in the hepatic microsomal system, which affects the unbound, free fraction ( $f_{u,mic}$ ) that is available to interact with the enzyme of interest<sup>252,302</sup>. Michaelis-Menten (MM) kinetics assumes that all substrate available in the system is in the unbound form, and parameter estimates are generated assuming that all substrate is freely available to the enzyme active site<sup>303</sup>. Many substrates violate this assumption, and for some substrates, non-specific binding is extensive<sup>251,252</sup>. Furthermore, the extent of non-specific binding and its influence on the apparent  $K_M$  value has never been assessed in paediatric hepatic microsomes.

The degree to which non-specific protein binding influences the M-M parameter estimates depends upon the amount of protein in the system<sup>304</sup>. At equilibrium and with non-saturable binding, Equation 5.1 describes the relationship between the microsomal free fraction ( $f_{u,mic}$ ), the dissociation constant ( $K_d$ ), and the concentration of microsomal protein binding component  $[B]$ <sup>301,304</sup>

$$f_{u,mic} = \frac{K_d}{[B] + K_d} \quad (5.1)$$

Assuming age-dependent changes in hepatic microsomal protein components responsible for non-specific binding and using Equation 5.1 to define the ratio of free fractions expected at two different developmental stages with different concentrations of non-specific binding components, the fraction unbound is expected to be constant only when the concentrations of the binding components are similar between ages or much less than  $K_d^{301}$ . The apparent  $K_M$  ( $K_{M, app}$ ) will be influenced by the degree of non-specific binding in a manner shown in Equation 5.2.

$$K_{M, app} = \frac{K_M}{f_{u, mic}} \quad (5.2)$$

Assuming different concentrations of non-specific binding components with development, the apparent  $K_M$  value is expected to be similar between different age groups only when the concentration of microsomal protein binding components are similar or much less than  $K_d^{301}$ . On the other hand, if the concentrations of these components are much greater than  $K_d$ , a proportional relationship is expected between the apparent  $K_M$  and concurrent changes in protein concentration<sup>301</sup>.

In light of my own data, an age-dependent difference in the extent of non-specific binding is possible. Total hepatic microsomal protein (MP), which constitutes a heterogeneous population of proteins to which CYP substrates can bind specifically and/or non-specifically, shows age-dependent changes (Figure 4.2). Accordingly, age-dependent differences in the extent of non-specific binding is possible. Total CYP protein content constitutes a major component of the microsomal fraction and provides both specific and non-specific binding sites for CYP substrates<sup>100</sup>. My results, shown in Figure 4.4, indicate that total CYP protein undergoes developmental changes with age. This observation is supported by many studies that report age-dependent changes in total hepatic microsomal CYP content in rats<sup>95,114,115,286</sup> and humans<sup>93,166,108</sup>. Such changes can lead to variations in the extent of non-specific binding with age and could significantly affect the apparent  $K_M$  value during development.

#### 5.1.3.1.3 Microsomal membrane composition

One last possible reason for the observed age-dependent changes in the experimentally determined  $K_M$  values may relate to age-related changes in the phospholipid composition of the microsomal membrane<sup>293,294</sup>. The apparent viscosity of the microsomal membrane is much higher in fetal and newborn livers than in adult livers due to changes in phospholipid/cholesterol molar ratios<sup>305,306</sup>. Endogenous glucocorticoids were found to play a major role in this phenomenon through their effect on the unstauration of microsomal fatty acids leading to increasing microsomal membrane fluidity in the perinatal period<sup>305,306</sup>. This can modify the catalytic efficiency of CYP enzymes either directly, through changing the conformation of the CYP protein, or indirectly, by improving substrate accessibility to the enzyme active site or by increasing the rate of electron transfer from NADPH-cytochrome P450 reductase to the CYP enzyme active site<sup>242</sup>.

Such uncertainty regarding the developmental changes in  $K_M$  values needs further investigation. The use of cDNA-expressed CYP enzymes both singly or mixed in proportions similar to those of CYP enzymes in hepatic microsomes at different age groups may facilitate the assessment of the contribution of different CYP isoforms to the estimated  $K_M$  value at a given age<sup>280,307</sup>. Furthermore, the influence of non-specific protein binding on the experimentally estimated  $K_M$  values can be assessed by determining the apparent  $K_M$  value at different microsomal protein concentrations. A proportional change in the  $K_M$  value with a change in microsomal concentration indicates that the substrate has high protein binding capacity and non-specific binding may have a significant effect on the estimated  $K_M$  value.

#### 5.1.3.2 Developmental Changes in Functional Enzyme Activity ( $V_{max}$ ) and Correlation to Functional CYP Protein.

To monitor the ontogeny of the catalytically active CYP2E1 and CYP1A2 enzymes in rats, *in vitro* CZX hydroxylation and methoxyresorufin-O-delkylation (MROD) activities were monitored, respectively (Figure 4.8). For both CYP enzymes, activity was not detected in the perinatal period. Postnatally, enzyme activity was significantly lower than adult levels and steadily increased after postnatal day 5 (PD5) to reach

maximum levels prior to (CYP2E1) or during (CYP1A2) the weaning period (PD21-28), where the activity was significantly higher than the adult level. While the obtained  $V_{\max}$  values are markedly higher than few reported studies<sup>180,182,274</sup>, the ontogeny profiles for CZX hydroxylation and MROD activities demonstrate similar patterns with these studies, even though they utilized few developmental ages<sup>1,308</sup>. The pattern of changes in  $V_{\max}$  values is most critical since all  $V_{\max}$  values are normalized to adult values for the purpose of the model.

The observed surge in CYP2E1 activity before the weaning period and the peak in CYP1A2 activity around the time of weaning is probably regulated by endogenous hormones (e.g. growth hormone and thyroxin) that are involved in the weaning process<sup>125,183</sup>. Also this weaning-related surge in activity may be an adaptation to ingestion of solid food and exposure to different xenobiotics that could affect the endogenous hormones levels and consequently alter CYP expression<sup>125,245</sup>. However, these observed developmental changes in functional enzyme activity could also be due to one or more of the aforementioned reasons in section 5.1.3.1. For example, inefficient probe substrate specificity means that the observed activity could be a hybrid function of the CYP enzyme of interest (e.g. CYP2E1 or CYP1A2) in addition to one or more CYP enzymes that contribute to some extent in the metabolic reaction under investigation. This is more prominent in hepatic microsomes from younger ages where the major CYP pathway is immature or underdeveloped leading to considerable contributions from other CYP pathways that play only a minor role in adult hepatic microsomes. Since these CYP enzymes have different onsets and patterns of development<sup>44</sup>, their influence on the observed changes in functional enzyme activity could be significant.

The profiles of developmental changes in catalytic activity of each CYP enzyme were correlated to its specific immunoreactive protein level at each age to assess the proportional relationship between enzyme activity ( $V_{\max}$ ) and its functional protein level (one of the major assumptions of the general ontogeny model and a basic assumption of the MM equation)<sup>303</sup>. However, my results show that developmental changes in  $V_{\max}$  did not completely parallel the biphasic patterns observed with its respective CYP protein level (Figure 4.11). Although some human CYP enzymes (e.g. CYP2E1,

CYP1A2 and CYP2D6) show a reasonable correlation between the maturation of CYP activity and CYP protein level<sup>133,165,242</sup>, other CYP enzymes (e.g. CYP3A and CYP2C) demonstrate a discordance<sup>93,241</sup>.

Before I go further and discuss these unexpected findings, recall that the model assumes a proportional relationship between enzyme activity and functional protein level. Accordingly, evaluation of this assumption requires:

1. Measurement of the developmental changes in CYP activity using a specific probe substrate for this particular CYP enzyme.
2. Monitoring the developmental changes in the functional protein level of that CYP enzyme.

As discussed previously, one may question the substrate specificity of the probe substrates (i.e. not 100% specific)<sup>280,300</sup>. Furthermore, quantitative immunoblotting techniques using CYP-specific antibodies do not differentiate functional from non-functional CYP protein. Therefore, the observed discordance between CYP enzyme activity and its protein level could be attributed to two main reasons:

1. During the early postnatal period, discordance may be due to the contribution of other CYP enzymes whose development precedes CYP2E1 or CYP1A2 pattern of development and contributes significantly to probe substrate metabolism at particular postnatal ages. For example CYP3A1 is involved in the hydroxylation of CZX<sup>280</sup>. This enzyme is well expressed in rat fetal livers, whereas CYP2E1 activity is not detected<sup>1</sup>. CZX hydroxylase activity in the early neonatal period may reflect CYP3A1 (and others) activity rather than solely CYP2E1. Consequently, a poor correlation between CZX hydroxylase activity and CYP2E1 protein level could be expected during early stages of development.
2. During the neonatal, weaning and post weaning periods, discordance may be due to posttranslational mechanisms that markedly influence enzyme activity during development. Posttranslational modification of CYP proteins may diminish or inactivate its catalytic activity. For example, protein phosphorylation may rapidly inactivate CYP2B and CYP2E1 enzymes<sup>309,310</sup>. The decline in CYP enzyme activity relative to the increase in CYP content at or after weaning may be due to the possible accumulation of non-functional protein.

Interestingly, a recent study reported a model of CYP2E1 ontogeny in human liver microsomes based upon evaluations of CYP2E1 protein content with immunoblotting techniques<sup>311</sup>. My data questions the potential application of models that are developed to predict *in vivo* intrinsic clearance of CYP substrates based on human CYP protein levels<sup>311</sup>, rather than CYP probe substrate activity. Hence, immunodetectable protein levels may not act as a suitable surrogate to CYP enzyme activity when specific probe substrates are not available.

#### *5.1.3.3 Consequences on the Model*

As mentioned in sections 5.1.3.1 and 5.1.3.2, significant changes were observed in  $K_M$  and  $V_{max}$  values during development. Although the first observation (developmental changes in  $K_M$  values) seems inconsistent with the model's assumption, it suggests a need to incorporate developmental changes in  $K_{M(j)}$  estimates in the model building process (i.e. model the  $V_{max}/K_M$  ratio) instead of modeling only changes in  $V_{max(j)}$ . The incorporation of a new parameter, which undergoes interindividual variability during development, will increase the model's complexity and possibly result in greater uncertainty in its parameter estimates.

#### *5.1.4 Correlation between CYP Enzyme Activity Levels Determined Using Two Probe Substrates*

My findings, on the other hand, have satisfied another critical assumption of our CYP ontogeny model. The developmental changes in CYP enzyme activity using one specific substrate was found to correlate well with changes in activity using a second, somewhat less specific substrate (Figures 4.9 and 4.10). Some minor differences were observed, especially in the detection of enzyme activity in fetal and early neonatal stages using the second probe substrate. This may suggest the contribution of other CYP enzymes that express relatively significant activity in the early neonatal period relative to CYP2E1 and CYP1A2 enzymes<sup>280</sup>. Such similarity between different substrates for the same CYP enzyme indicates that the model can be applied generally to all substrates of the CYP enzyme of interest.

### 5.1.5 Conclusion

The general ontogeny model of human CYP enzyme activity is a promising risk assessment tool for pediatric populations, particularly following exposures to xenobiotics in breast milk. In my attempt to evaluate its underlying assumptions, I recognized potentially important discrepancies with the stated assumptions. Microsomal protein (MP) content may not remain constant with development, which will require incorporation of age-related changes in MP content into the model building process. Changes in  $K_M$  values during early postnatal development may reflect lack of probe substrate specificity or changes in non-specific binding to microsomal components during CYP enzyme ontogeny, rather than age-dependent changes in the inherent properties of substrate-CYP enzyme interactions. Further assessments of probe substrate specificity and  $K_M$  evaluations are required for further evaluation of the model assumptions. Postnatal increases in  $V_{max}$  occur for both CYP2E1 and CYP1A2, with maximal activity prior to or at weaning stage, respectively. The activity of one substrate seems to correlate with the level of activity of a second substrate of the CYP enzyme. However, a poor correlation was observed between catalytic activity and protein level of the tested CYP enzymes during development, and further investigations are needed to explain this discordant development.

Until further investigations provide appropriate explanations for these discrepancies that dispute the validity of the model, one can not dismiss the potential value of the CYP ontogeny model as a potential risk assessment tool. As a proof of concept, Section 5.2 discusses the ability of the model to predict *in vivo* oral clearance of a specific CYP2E1 substrate, CZX, in rats.

## 5.2. Development of a Pharmacokinetic Model of Rat CYP2E1 and CYP1A2

### Ontogeny

Despite the documented similarities between rats and humans concerning the biochemistry and enzymology of CYP2E1 and CYP1A2<sup>2,312</sup>, comparison of the developmental changes in ISF estimates between the rat (Figure 4.14) and human<sup>77</sup> reveals important differences in the onset, rate and pattern of maturation of CYP



activity. These differences in developmental ISF profiles indicate species differences in CYP regulation with development and this information should be carefully considered when extrapolating rat toxicokinetic information to human paediatric populations. Due to their significant role in mediating bioactivation or elimination of xenobiotics, developmental maturation of CYP enzymes may determine the toxicological outcome following exposure to xenobiotics. Accordingly, different ontogeny patterns of CYP activity between rats and human could result in different toxicological responses to the same xenobiotic during development. Such differences in developmental patterns necessitate the development of an unique model that describes the ontogeny of CYP enzymes in rats.

To develop a model describing the ontogeny of CYP enzymes in rats, the following scaling factors were estimated (see Appendix I and section 3.2.6 for more details):

1. Hepatic scaling factors ( $HSF_{(t)}$ ) that account for the contribution of hepatic microsomal protein content (MP) and liver weight (LW) normalized to body weight (BW) to the metabolic function of the whole liver, Equation 3.3.
2. Normalization of HSF of a particular developmental age to HSF in adult to produce age-specific relative hepatic scaling factor ( $RHSF_{(t)}$ ) that relates the abundance of microsomal protein content per body weight in infant to the adult, Equation 3.4.
3. Ontogeny scaling factors ( $OSF_{(t)}$ ) were determined by normalizing functional enzyme activity ( $V_{max(t)}$ ) observed at a particular age (t) to that observed in the adult, Equation 3.5. Accordingly, OSF represents the elimination capacity of a specific enzyme in the infant relative to the adult.
4. The product of the scaling factors,  $RHSF_{(t)}$  and  $OSF_{(t)}$ , is the age-specific infant scaling factor ( $ISF_{(t)}$ ) that represents the ontogeny of an individual functional enzyme relative to the whole body, Equation 3.6, and correlates adult *in vivo* intrinsic clearance due to an individual CYP enzyme to the capacity of the infant at age (t) to eliminate drugs by the same metabolic pathway, Equation 1.15.

The observed  $ISF_{(t)}$  values were fit to various models describing the developmental maturation of each CYP enzyme using the modeling software GraphPad Prism 4.

Modifications to the general human ontogeny model (Equation 1.16) were required to describe the age-dependent changes in CYP function from birth to adulthood in rats as shown in Equation 4.1. The primary modification included a parameter

$(\text{ISF}_{\text{Burst}} (e^{-k_2(t-\text{lag})} - e^{-k_3(t-\text{lag})}))$  that accounted for an apparent burst of activity (greater than the adult value) in the period between weaning and puberty that overlay the general exponential increase in activity to some asymptotic level reached at adulthood  $[(\text{ISF}_D (1 - e^{-kt}))]$ .

While the visual inspection of the fitted non-linear regression lines shows adequate fits (Figure 4.15), the parameter estimates (Table 4.5) are associated with wide confidence intervals around the estimates. This may be due to the limited number of data points (average of 4 at 12 developmental ages) and the need for a more complex equation to describe the data.

The primary importance of establishing a PK model of the ontogenesis of rat CYP activity is to 1) provide a proof-of-concept that a model can be established based on appropriate *in vitro* data to make reasonable predictions of CYP-mediated intrinsic clearance and 2) allow predictions of internal dosimetry metrics (i.e. steady-state plasma concentrations, AUC) at different developmental rat stages for developmental toxicology studies and extrapolation to humans. The model was tested for its ability to provide *a priori* predictions of chlorzoxazone (CZX) oral clearance in male Sprague-Dawley rats. Chlorzoxazone is a probe substrate commonly used to monitor CYP2E1 activity *in vivo*<sup>3,123</sup>. Although the original model predicts a systemic clearance, chlorzoxazone was administered orally for the following reasons:

1. Oral ingestion is the most common route of exposure in infants (e.g. breastfeeding).
2. In toxicology studies, test compounds are usually administered to animals orally in solutions or suspensions.
3. Oral bioavailability (F) of CZX is high<sup>278,313</sup>.
4. Difficulty of CZX administration by IV route to rat pups. Attempts to administer CZX by intracardiac administration resulted in a mortality rate >50% following injection.

Table 4.6 shows the age-dependent changes in pharmacokinetic parameters of CZX in rats. The table demonstrates similar elimination rate constant ( $\lambda_z$ ) values between the different ages, while different values were observed for maximum serum concentration ( $C_{max}$ ) and area under serum concentration time curve (AUC). One would expect age-dependent changes in the  $\lambda_z$  values, since changes in  $V_{max}$  (or ISF) values were observed with development. The age-related changes in  $C_{max}$  and AUC in conjunction with the similar  $\lambda_z$  values may suggest age-dependent changes in the oral bioavailability of CZX. Also, while the table shows similar elimination rate constants across all tested ages, dramatic developmental changes in ISF and oral clearance estimates were observed. Since the elimination rate constant is a hybrid function of systemic clearance and volume of distribution, these observations may be the result of developmental changes in body composition (e.g. water content, lipid content) and extent of tissue and protein binding, which can induce age-dependent changes in CZX volume of distribution. Comparison of the predicted and observed oral clearance (Figure 4.17) indicates that the model reasonably predicted CZX oral clearance values at all tested postnatal ages with the poorest prediction observed at juvenile and adult stages where the predicted values were 3- and 3.5-fold the observed values, respectively. Predicted oral clearance values at other ages were within 2-fold the observed values.

The two most commonly used approaches to predict clearance in neonates are physiologically based pharmacokinetic (PBPK) modeling and allometric scaling<sup>78,235,250</sup>. Compared to our pharmacokinetic model of CYP ontogeny, PBPK models require estimation of age-dependent changes in drug absorption, elimination capacity, plasma protein binding, physiological characteristics of the subject and physicochemical parameters of the drug under investigation<sup>78</sup>. Accordingly, generation and validation of a PBPK model is resource and time intensive. However, when these data are available this approach can provide reasonable predictions of systemic clearance<sup>78,257</sup>. Although our general ontogeny model does not account for age-related changes in body composition and extent of protein and tissue binding, it can be incorporated into these PBPK models as it provides essential information regarding the developmental changes in CYP activity.

The second approach of predicting systemic clearance is allometric scaling<sup>235,250</sup>. However, this is an empirical approach that does not provide physiological relevance and usually fails to make adequate predictions for low and intermediate hepatic extraction ratio drugs<sup>235</sup>. Since CYP enzymes undergo a developmental maturation, many substrates are likely to exhibit low hepatic extractions during early postnatal development despite their possible intermediate to high extraction ratios in the adult<sup>250</sup>. Accordingly, allometric scaling may not be a suitable approach to predict drug clearance in neonatal populations.

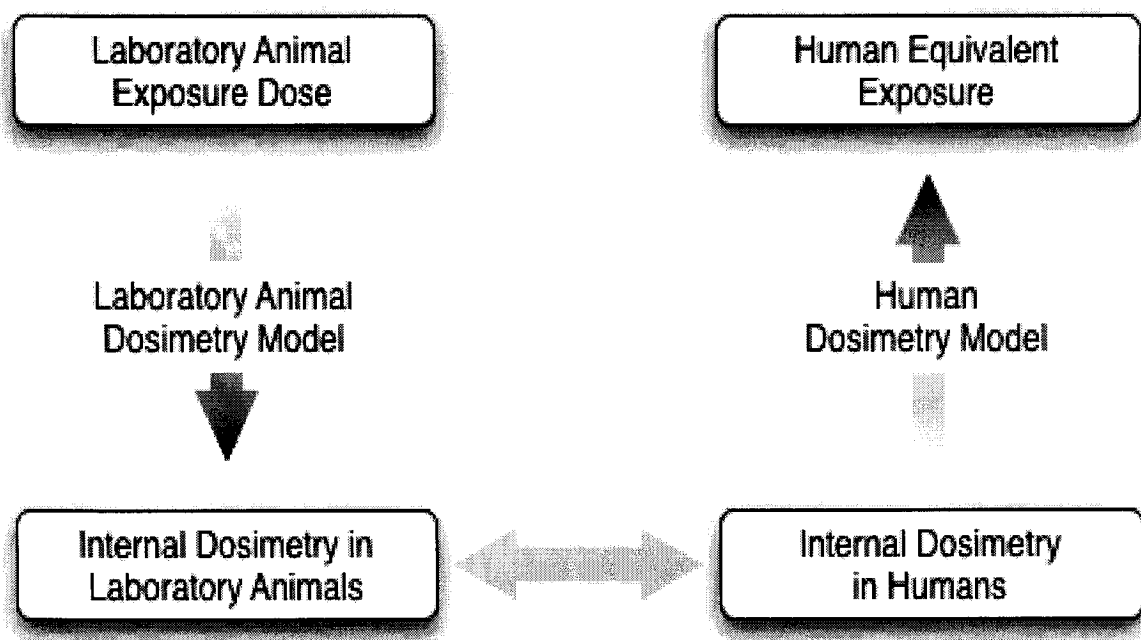
A principal goal of our ontogeny model of CYP enzyme activity is to provide risk-relevant information regarding the potential for toxicity in paediatric populations following exposure to toxicants, especially from breast milk. Evaluation of exposure risks requires knowledge of the developmental window of susceptibility (the onset of toxicodynamic processes that lead to toxicity), the internal biologically effective dose (tissue dosimetry) that occurs during that window and dose-response behavior within that window<sup>229</sup>. Due to the difference in timing of birth across species in relation to the development of organs and physiological systems, often differences exist in the pharmacokinetics that occur during the relevant developmental period in each species<sup>229</sup>.

Such differences may create difficulties in relating rodent critical developmental stages to human stages. However, our ontogeny model may still make predictions of internal dosimetry following a given toxic dose during the exposure period (e.g. *in utero* exposure, postlactational exposure), which may help to further explain dose-response behavior and age-related changes in susceptibility to toxicity.

Due to age-dependent changes in pharmacokinetic processes<sup>314</sup>, the same dose/kg body weight may result in different values for internal dosimetry metrics at different developmental stages and, therefore, different risks of toxicity. This illustrates the uncertainties associated with extrapolating adult dosimetry estimates to paediatric populations, especially in the early neonatal ages where the physiological and biochemical changes are rapid and significant<sup>224</sup>. As toxicant elimination is a major determinant of tissue concentration (tissue dosimetry), a PK model that provides an *a priori* prediction of elimination capacity at different developmental ages is of great

significance in defining age-dependent internal doses and correlating that dose to the response observed during the exposure period<sup>221,224</sup>.

Calculation of the age-dependent changes in internal dosimetry in laboratory animals used in pre-clinical and toxicology studies can potentially improve the accuracy of estimating the exposure risk in humans<sup>315</sup>. Such inter-species extrapolation requires estimation of exposure levels that result in a comparable effective internal dosimetric value in humans. Assuming similar toxicodynamic processes between rats and humans, a human PBPK model based upon human parameters can be used to simulate an exposure level that achieves the same internal dosimetric value as observed in the rat (Figure 5.1).



**Figure 5.1-** Calculation of a human equivalent exposure dose from laboratory animal toxicity data using dosimetry/physiologically based pharmacokinetic (PBPK) models. Adapted from reference<sup>315</sup>.

This approach has been recommended by Health Canada (HC) and US Environment Protection Agency (EPA) to determine internal dose-response relationships in calculating target dose levels<sup>223</sup>.

This general PK model of rat CYP enzyme activity ontogeny is an initial proof-of-concept investigation to demonstrate that an ontogeny model based on a physiologically-based (PB) ISF can be developed and applied in predicting age-dependent systemic clearance due to CYP-mediated metabolism if appropriate *in vitro* data is available. Future investigations with more age groups can increase the reliability of the model.

A serious limitation of the model is its inability to accommodate those compounds whose concentration at the CYP enzyme is controlled by uptake or efflux membrane transporters. Future investigations using primary hepatocytes are required to assess the potential role of membrane transport processes during development and incorporate them into the model building process to extend the spectrum of model application.

### 5.3. Characterization of Rat CYP2E1 and CYP1A2 Ontogeny

The lack of pharmacokinetic/toxicokinetic data in paediatric populations represents a critical obstacle to accurately assess the pharmacological/toxicological outcome of exposure to chemicals, e.g. from breast milk<sup>316</sup>. In many cases this risk assessment is based upon extrapolation of toxicity data from adult populations to the infant<sup>227,234,250</sup>. However, due to the immature physiological and biochemical processes that determine the pharmacokinetics (or toxicokinetics) and pharmacodynamics (or toxicodynamics) of toxicants, the developing infant may respond to the toxicant exposure in a different way than the adult<sup>229,233</sup>. This age-dependent difference in vulnerability to toxicants casts serious uncertainties to the simple adult-to-infant extrapolation approach and necessitates having toxicokinetic data from paediatric populations<sup>221,233</sup>. However, obtaining such data from infants is usually associated with numerous ethical and logistic limitations<sup>60,285</sup>. Extrapolation of developmental toxicity data from animal models to human may be an useful approach to elucidate the influence of developmental changes in toxicokinetic processes on the susceptibility to toxicity<sup>229,233</sup>.

The CYP enzyme system, is considered one of the most critical toxicokinetic mechanisms that determine the pharmacological/toxicological outcome following exposure to xenobiotics<sup>96</sup>. The ontogeny of these enzymes, then, could have a significant impact on the age-related differences in susceptibility to toxicity<sup>268</sup>. Due to

the significant species differences in the rate and pattern of CYP enzymes ontogenesis, toxicity outcomes from animal models can not necessarily be extrapolated directly to humans<sup>110</sup>. However, the limited available information regarding the ontogeny of CYP enzymes in human necessitates the use of animal model system for risk extrapolation and to identify the toxicokinetic mechanisms of age-related differences in susceptibility to toxicity<sup>222,283</sup>.

Due to the above mentioned significance of studying the ontogeny of CYP enzymes in a relevant animal model, I have extensively characterized the ontogeny of two toxicologically significant CYP enzymes (CYP2E1 and CYP1A2)<sup>100</sup> in a rat model system at the levels of mRNA, protein, catalytic activity and hepatic tissue expression. Collectively, this extensive characterization may identify an animal model system of human CYP ontogeny that will ultimately have the following benefits:

1. Identifying a toxicokinetic basis responsible for age-dependent changes in susceptibility. For example, an immature CYP pathway in the developing neonate could lead to accumulation and toxicity risk following exposure to a toxicant that undergoes elimination/detoxification by that CYP pathway, even when the exposure level is very minimal (e.g. with breastfeeding). No adverse effects will be observed in adult after exposure to the same toxicant if that CYP pathway is well developed and mature.
2. This characterization will enhance our understanding of CYP regulation and the impact of various endogenous and exogenous factors on CYP enzyme expression during maturation<sup>317</sup>. Exposure of the fetus or the young neonate to certain chemicals can induce permanent alterations in CYP activity (metabolic imprinting)<sup>223,233</sup>.
3. Comparison of the observed ontogeny patterns in rat to the available ontogeny data in humans will assess the appropriateness of the rat as an animal model for developmental toxicology studies.
4. Characterization of the maturation profile of CYP enzymes, that determine bioactivation potential and the detoxification capacity of numerous xenobiotics, can facilitate prediction of the pharmacological/toxicological outcome of xenobiotic exposure during development.

The rat was selected because it is often used in developmental toxicology and pre-clinical studies and demonstrates sufficient similarities to human CYP2E1 and CYP1A2 enzymes regarding their enzymology and biochemistry<sup>2,312,318,319</sup>. In addition to the significant similarities in nucleotide sequence between rats and human *CYP2E1* and *CYP1A2* genes<sup>2,263</sup>, these enzymes catalyze similar reactions and are induced or inhibited by similar mechanisms<sup>174</sup> in both species. Consequently, characterization of rat CYP2E1 and CYP1A2 ontogeny can facilitate the extrapolation of rat developmental toxicology studies using substrates for these enzymes and provide invaluable risk-relevant information for human paediatric risk assessment<sup>316</sup>.

Although both CYP2E1 and CYP1A2 are minimally affected by the sexually dimorphic effects of endogenous hormones (e.g. growth hormone)<sup>117,118</sup>, I chose to limit my studies to male rats to accommodate future investigations with CYP enzymes that may be affected by such factors.

### *5.3.1 Ontogeny of mRNA Expression*

#### *5.3.1.1 CYP2E1*

Sparse information is available in the literature on the ontogeny of CYP2E1 mRNA in the rat and my results using real-time RT-PCR (Figure 4.22) are consistent with the few published reports<sup>1,98,115,261</sup>. CYP2E1 mRNA was detected at very low levels in fetal and early neonatal livers and elevated levels were detected few days after birth with peak levels at PD14 (approximately 3-fold the adult value), then declined to 2-fold the adult level at early puberty (PD42). The mechanism governing this regulation is unknown, but changes in the methylation status of certain cytosine residues at the 5' end of the *CYP2E1* gene correlate with transcriptional activation of this gene<sup>98,264,320</sup>. Demethylation of these cytosine residues was detected within 24 hours after birth, and at one week and ten weeks after birth<sup>264</sup>. Whether this demethylation is a result of transcriptional activation of the gene or whether demethylation itself causes gene expression is not presently known.

While several studies failed to detect CYP2E1 mRNA in human fetal livers<sup>157,321</sup>, Vieira *et al*<sup>133</sup> reported very low levels in fetal liver samples from late gestation. Similar



to the observed significant rise of mRNA in the preweaning period in rats, human CYP2E1 mRNA levels significantly rise during the first three months of age and reach 50% of the adult value in by 3-12 months<sup>133</sup>. Unfortunately, mRNA levels were not measured in children aged 1-10 years and during puberty and, therefore, it is difficult to know exactly when mRNA levels peak before declining to the adult value. As in rats, the methylation status of the *CYP2E1* gene has been correlated with transcriptional activation<sup>133,134</sup>.

#### 5.3.1.2 *CYP1A2*

The very few literature reports regarding the ontogeny of CYP1A2 mRNA in rats could not detect any mRNA levels in fetal and perinatal livers<sup>115,157</sup>. Other studies, consistent with my findings (Figure 4.22), detected CYP1A2 mRNA from as early as 2 weeks of gestation or few days prior to parturition followed by a postnatal increase to adult levels around weaning age (PD 21-28)<sup>1,322,323</sup>. In addition to the role of aryl hydrocarbon receptors (*AhR*) in the inducible expression of *CYP1A2*, members of the nuclear factor 1 (NF-1) family of transcription factors may play an important role in the hepatic constitutive expression of the *CYP1A2* gene<sup>169,170</sup>.

Few studies have reported the ontogeny of human CYP1A2 mRNA expression. In contrast to rats, the available reports suggest the absence of detectable CYP1A2 mRNA expression in gestational age from 11 to 24 weeks and low levels were detected at early neonatal ages<sup>110,111,157,165</sup>. Adult mRNA levels were attained in children aged 1-15 years<sup>110</sup>.

Comparison of the results of CYP2E1 and CYP1A2 mRNA ontogeny data in rats with those available in humans reveals some similarities between the two species, where the transcript is not detected or expressed at a very low level during gestation or early neonatal period and undergoes a significant rise during the preweaning (CYP2E1) or late weaning (CYP1A2). The sparse information regarding mRNA ontogeny in human during childhood and puberty limits the comparison to rats during these developmental stages.

### 5.3.2 Ontogeny of Immunochemical CYP Protein Expression

#### 5.3.2.1 CYP2E1

When the immunoreactive protein levels were examined as a function of age, the results shown in Figure 4.21 indicate that no CYP2E1 protein was detected in microsomes from fetal and PD1 livers. The immunoreactive protein level in PD3 hepatic microsomes was very low (less than 25 % the adult level), but gradually rose with postnatal development. The protein level did not change significantly between PD10 and PD28, but increased progressively thereafter. My results are in agreement with the few studies that examined CYP2E1 protein expression in rats at selected developmental ages<sup>116,183,261</sup>. However, and inconsistent with my findings, one study reported expression of CYP2E1 in rat fetal livers<sup>161</sup> and another study reported peak CYP2E1 protein levels prior to weaning with adult level achieved after weaning<sup>127</sup>.

A similar pattern of rat CYP2E1 protein development was reported in humans<sup>133,324,311</sup>. As with the rat, human CYP2E1 protein was not detected in fetal liver microsomes, but unlike the rat, it rose immediately in the first 24 hours after birth<sup>133</sup>. Postnatally, human CYP2E1 protein levels rose gradually to reach about 30 % the adult value during the first month, after which it remained almost steady until 12 months<sup>133,324</sup>. Thereafter, CYP2E1 increased progressively<sup>133,166,324</sup>.

In contrast to human, rat CYP2E1 mRNA and protein expression increase dramatically during the early neonatal period. Thereafter, developmental changes in mRNA did not correlate with the immunoquantifiable protein levels. In humans, changes in CYP2E1 mRNA levels correlate reasonably with protein levels, especially after one month of age. Such data suggests species-differences in CYP2E1 regulation during development. Transcriptional mechanisms may regulate the initial rise in CYP2E1 level during the early neonatal period in rats. However, posttranscriptional mechanisms may play a larger role in governing CYP2E1 levels in rats throughout postnatal development. On the other hand, transcriptional mechanisms probably play a larger role in governing CYP2E1 level in humans after the first month of life. Such species-differences in CYP2E1 regulation can lead to species-differences in susceptibility to toxicants and need further investigations.

### 5.3.2.2 CYP1A2

When the immunoreactive protein levels were examined as a function of age, the results shown in Figure 4.21 indicates that no CYP1A2 protein was detected in microsomes from fetal and PD1 livers. These findings are consistent with some studies<sup>115,183,322</sup> but inconsistent with one study that reported very low levels of CYP1A2 protein in PD1 liver, but not in fetal livers<sup>1</sup>. The immunoreactive protein level in PD 3 hepatic microsomes was very low (less than 20% the adult level) but gradually rose with postnatal development to reach the adult value at late pubertal age (PD63). Inconsistent with my observations, Johnson *et al.*<sup>183</sup> reported a surge in CYP1A2 protein expression at PD21 (5 fold the adult value) followed by a decline to reach the adult value at early pubertal age (PD40). On the other hand, and consistent with my findings, Couroucli *et al.* reported a gradual increase in hepatic CYP1A2 protein levels in rats at postnatal days 8, 15, 22, and 38<sup>323</sup>.

This pattern of steady state increase in CYP1A2 immunoreactive protein is similar to my observation with CYP2E1 and to other CYP enzymes in rats<sup>1,325</sup>. Interestingly, the observed pattern of rat hepatic CYP1A2 protein ontogeny is similar to the reported expression pattern in human, where CYP1A2 protein was not detected in fetal and early neonatal hepatic microsomes<sup>165,243</sup>. During the first month after birth, the protein concentration was less than 5% the adult level, but rose slowly during the third month to reach only 10% the adult value<sup>165,158,243</sup>. A 50% of the adult level was reported in infants aged more than one year<sup>165</sup>.

Similar to the observed ontogeny patterns for rat CYP2E1 mRNA and protein, developmental changes in CYP1A2 mRNA did not correlate with changes in the immunoquantifiable CYP1A2 protein levels, except during the early postnatal period. This may suggest that CYP1A2 expression is principally determined by transcriptional mechanisms, which may be predetermined by the ontogeny pattern of nuclear hormone receptors<sup>326</sup>. However, posttranscriptional mechanisms likely play a larger role in governing CYP1A2 expression in rats throughout postnatal development. In human, the very little information regarding the ontogeny of CYP1A2 mRNA makes it difficult to postulate the regulatory mechanisms governing the ontogeny of human CYP1A2.

In light of my results with rats and the available human data, a reasonable similarity was observed as the immunoreactive CYP protein levels showed a similar biphasic pattern in both species. Such findings can support the suitability of rats as a model to describe the ontogeny of human CYP2E1 and CYP1A2 proteins. The absence of immunoreactive CYP2E1 protein in fetal microsomes and its immediate surge after birth in both rats and human may suggest the implication of a physiological event that is directly related to parturition in the regulatory process rather than temporal maturation by itself. CYP2E1 is thought to be involved in two gluconeogenesis pathways from acetone<sup>126,132</sup>. This putative role in glucose metabolism may be related to the early onset of CYP2E1 expression when the newborn becomes independent of the mother's glucose and has a high demand of glucose<sup>133,160</sup>. While not yet clearly investigated, the delayed evolution of CYP1A2 in both human and rats may be regulated by a HNF-1 binding site in the 5' flanking region of the gene<sup>167,169</sup>. In addition to the contribution of this site to the tissue-specific expression of CYP1A2, it might be implicated in the age-specific expression as well<sup>169</sup>.

### *5.3.3 Ontogeny of Catalytic CYP Enzyme Activity*

#### *5.3.3.1 CYP2E1*

As shown in Figure 4.8, CZX, a CYP2E1 probe substrate, hydroxylase activity was not detected in fetal and PD1 hepatic microsomes. High activity (70% the adult value) was detected at early postnatal ages with progressive increase to reach the maximum level (more than 150% the adult value) at PD14. The activity declined to the adult value at the weaning age (PD21-28) and stayed constant thereafter. Similar results were reported for the developmental changes in CYP2E1-mediated demethylation of dimethylnitrosamine activity in rats where activity peaked at PD14 and declined at PD21<sup>127</sup>. Consistent with this pattern of CYP2E1 activity changes with age, greater hepatotoxicity due to carbon tetrachloride bioactivation was observed in PD15 compared to PD60 male rats<sup>327</sup>. Developmental changes in rat hepatic CYP2E1 activity reasonably paralleled its mRNA expression levels, but not the immunoquantifiable protein.

In humans, very low hepatic CYP2E1 activity was detected from the fetal liver samples at late gestation stage (less than 5% the adult value) and immediately after birth

(approximately 20% the adult value)<sup>133</sup>. In the neonatal period, the activity gradually elevated to reach the adult value at ages 1-10 years<sup>133</sup>. Unlike rats, the ontogeny pattern of CYP2E1 activity in humans parallels the increase in its protein levels during development. Such data may suggest different regulatory mechanisms that control CYP2E1 levels between rats and human.

Regardless of species, greater risk of hepatotoxicity is expected at weaning, puberty and adult periods for xenobiotics that undergo CYP2E1-mediated bioactivation. However, substrates which need CYP2E1 for their elimination may accumulate in early postnatal ages leading to toxicity<sup>3</sup>.

#### 5.3.3.2 CYP1A2

The results shown in Figure 4.8 demonstrate undetectable methoxyresorufin-O-dealkylase (MROD) activity, a CYP1A2 activity marker, in fetal and early postnatal ages. At PD5, MROD activity was very low (less than 20% the adult value) and gradually increased to attain a maximum level at PD28 (more than 150% the adult value). Similar to these findings, Couroucli *et al* reported 8-, 15- and 26-fold increase in rat hepatic MROD activity at PD15, PD22 and PD38, respectively, compared to PD8<sup>323</sup>. This compares reasonably well with the 4.5-, 9.5- and 10.5-fold increase in MROD activity at PD10, PD21 and PD28, respectively, compared to PD7 in my study. Another recent study using deltamethrin as a CYP1A2 substrate<sup>328</sup> reported a 4-, 9.5- and 9-fold change in intrinsic clearance values at PD21, PD40 and PD90, respectively, compared to PD10 in male rats<sup>329</sup>.

Very limited data are available to describe the developmental maturation of CYP1A2 in human. The existing literature reports indicate similar human CYP1A2 ontogeny pattern to the above characterized pattern for rats. Human CYP1A2 activity was not detected in fetal and early neonatal hepatic misrosomes<sup>165,91</sup>. Postnatally, CYP1A2 shows delayed ontogenesis with the first significant rise in activity observed at ages 1-3 months<sup>165</sup>. Thereafter, the activity continued to rise following the ontogenic profile of CYP1A2 protein until reaching 33% the adult value at ages 1-10 years<sup>165</sup>.

Such observed pattern of delayed CYP1A2 ontogenesis in both rats and human may have a great toxicological impact on both species. Higher risk of CYP1A2-mediated

xenobiotic bioactivation is expected in rats at weaning and in human at adult stages. However, in both species risk of drug accumulation is expected at early postnatal periods for substrates whose elimination is principally mediated by CYP1A2.

#### *5.3.4 Correlation between the CYP Ontogeny Profiles in Rats*

Comparison of the ontogeny profiles of mRNA and protein of both CYP enzymes in rats (Figure 4.22) reveals a discordant relationship during postnatal development. While mRNA levels were detected in fetal livers, proteins were not detected. Postnatally, a rapid increase in mRNA levels was observed with levels exceeding 250% the adult levels by PD14 (CYP2E1) or PD28 (CYP1A2). This was accompanied by slow rise in protein levels that did not exceed 50% of the adult levels until PD42. Collectively, this could suggest that regulation of both CYP2E1 and CYP1A2 in rats occurs principally at the post transcriptional level. This discordance between mRNA and protein levels is similar to that observed with human CYP enzymes<sup>166,241,330</sup>.

A reasonable correlation was obtained when the developmental changes in CYP activity were compared to changes in corresponding mRNA levels for both CYP2E1 and CYP1A2 enzymes (Figure 4.23). These findings are inconsistent with those reported in human where the maturation of CYP activity does not follow that of mRNA levels, especially during the early developmental stages for both CYP2E1 and CYP1A2<sup>133,165</sup>. On the other hand, and also inconsistent with human studies, the activity of rat CYP2E1 and CYP1A2 enzymes did not follow the immunodetectable CYP protein (Figure 4.11). Possible reasons of this discordance are possible contribution of other CYP enzymes, especially at pre-weaning ages, and posttranslational mechanisms that can influence CYP activity, especially at post-weaning ages (section 5.1.3.2).

#### *5.3.5 Ontogeny of Zonal Expression of CYP2E1 and CYP1A2*

A number of studies have demonstrated the zonal expression of CYP enzymes in the livers of adult human and laboratory animals<sup>244-246,249</sup>. However, few studies have investigated intrahepatic expression of CYP enzymes in the developing human or

animal. The results of my study on the hepatic zonal expression of CYP2E1 and CYP1A2 in rat livers show remarkable development-related differences in intrahepatic expression. A homogenous distribution of the enzymes in all parenchymal cells was observed during fetal and early postnatal stages (Figures 4.24 and 4.25 A and B). A more heterogeneous intralobular expression was observed at juvenile and adult stages with the enzymes preferentially distributed in the perivenous region (PV) (Figure 4.24 E and F and Figure 4.25 D and E).

My results are quite comparable to that observed in human for CYP1A2, CYP3A and CYP2C<sup>243</sup>. This may indicate a similar regulatory mechanism is controlling this age-dependent zonal expression in both species. Many studies have reported the involvement of hormonal regulation in the zonal-specific expression of CYP enzymes<sup>7,201,249</sup>. Hypophysectomy of rats resulted in higher expression of CYP2B and CYP3A in the periportal (PP) zone with complete disappearance of zonation, while treatment with growth hormones re-established the zonation pattern<sup>245,249</sup>. This may suggest the potential role of growth hormone in down regulating the periportal transcription of these genes in the liver. Oinonen and Lindros<sup>245</sup> suggested the existence of regulatory factors located in the upstream region of the *CYP* gene. These factors could be either negative response elements, activated periportally, or transcriptional activators acting in the perivenous region<sup>245,326</sup>.

Age-dependent changes in the zonal expression of CYP enzymes could affect zone-specific damage caused by many hepatotoxicants. Focal expression of CYP2E1 and CYP1A2, which generally mediate generation of reactive intermediates following exposure to many chemicals, in the PV region could increase the vulnerability of this region to hepatotoxicity<sup>245,249,331</sup>. Another factor that increases such vulnerability is the insufficient expression of glutathione peroxidase, an enzyme that detoxifies reactive peroxides, in this region<sup>247</sup>. Although the two major groups of detoxification enzymes, glutathione-S-transferases and UDP-glucuronyl transferases, exhibit higher expression in the PV than the PP region, the gradient appears to be less steep compared to CYP enzymes<sup>247</sup>. Accordingly, the regional imbalance between bioactivation and detoxification mechanisms can be the basis for PV-specific damage following exposure to chemicals that undergo CYP-mediated bioactivation to highly reactive and toxic

metabolites. For example; acetaminophen-induced damage to the PV zone after a high dose is inhibited by isoniazide, a specific inhibitor to CYP2E1 that catalyzes acetaminophen bioactivation to reactive intermediate<sup>331</sup>. In another study animals deficient in the expression of both CYP2E1 and CYP1A2 (double-null) were found highly resistant to acetaminophen-induced cellular damage compared to the wild-type animals<sup>154</sup>.

In paediatric livers, where CYP expression level is usually low and zonation is absent, hepatocellular damage induced by CYP-mediated generation of reactive metabolites may be unlikely to happen. However, several reports have documented the possibility of transplacental and translactational induction of CYP enzymes in the fetus or nursing neonates following chronic exposure of the pregnant or lactating mother to CYP inducers, e.g. ethanol and tobacco smoke<sup>116,127,159,161,262</sup>. Whether the induction of CYP enzymes in fetal and neonatal livers is zone-specific is unclear.

Several studies in rats and human have described zoned induction of CYP enzymes (CYP1A, CYP2B, CYP2E1 and CYP3A) with larger induction in the PV region and higher relative (fold) induction in the PP zone<sup>246,248,249</sup>. This regional selectivity of CYP induction is independent of the kind of inducer. The induction of glutathione-S-transferases and UDP-glucuronyl transferases was found to be at least as strong in PP cells as in the PV cells<sup>248</sup>. Accordingly, perivenous zone is generally characterized by dominance of CYP-mediated bioactivation and lower capacity for detoxification. Zonation of CYP enzymes in the downstream PV zone seems somewhat unexpected. Considering the endogenous role of these enzymes as protectors against toxicants and their need for oxygen to achieve this role, a higher expression in the upstream periportal region is expected. The mechanisms and toxicological impact of developmental changes in intrahepatic zonation of CYP enzymes need further investigation.

### *5.3.6 Toxicological Relevance of Characterization of CYP Ontogeny*

This extensive characterization of CYP2E1 and CYP1A2 ontogeny at the levels of mRNA, protein and activity shed some light on possible regulation mechanisms of these toxicologically-significant enzymes. Together with the pharmacokinetic models that



describe their ontogeny (see section 5.2), this extensive characterization will aid toxicological assessments and provide greater insights into the role of toxicokinetics in determining exposure risk.

While these studies recognized some differences in the postnatal development of CYP2E1 mRNA, protein and activity between rats and human, a reasonable degree of similarity was observed between rat and human hepatic CYP1A2 ontogeny. Recognition of such species-differences could help explain species differences in susceptibility to toxicants and mechanisms of toxicity. Identification of these species differences in toxicokinetics will facilitate extrapolation of developmental toxicity data from animals to human. Furthermore, similar ontogeny pattern between rats and humans could suggest the suitability of rats as an animal model to investigate the influence of postnatal exposure to xenobiotics and endogenous compounds on CYP ontogeny and for identification of biomarkers of exposure.

Finally, having the ontogeny of CYP enzymes fully characterized in this important developmental model will help to identify the underlying toxicokinetic basis for the observed age-dependent differences in susceptibility to toxicity.

I have to mention that the observed conflicts between my results and some data reported in the literature regarding the ontogeny of CYP enzymes could be due to the influence of different animal strain, diet and bedding composition. For example, the rat chow used in my study contains soy protein and amino acids that were found to alter endogenous hormones levels (e.g. growth hormone and thyroxin<sup>332,333</sup>), which in turn could affect the CYP expression<sup>125,245</sup>. Also strain associated alteration in enzyme expression has been reported<sup>178,200</sup>. The interlaboratory variation for measurement of enzymatic activity are highly sensitive to factors that frequently vary from one laboratory to another (e.g. hepatic microsomal and cytosolic preparation, storage of hepatic microsomes and cytosolic fractions, analytical methods, probe substrate selection and substrate concentrations and incubation conditions for enzyme kinetics experiments).

Statistical analysis of activity, protein and mRNA expression data at all developmental stages were compared to that at PD112 (young adult). Comparison of my data at PD112 to published data at ages beyond PD112 shows close similarities

which may indicate values observed at this age may be considered as a stable reference for mature rats.

#### 5.4. Characterization of the Ontogeny of Cytosolic GST Enzymes

In spite of the dual bioactivation / detoxification nature of both phase I and phase II enzymes, phase I enzymes (mainly CYP enzymes) are generally seen as bioactivating<sup>100</sup> while phase II enzymes are generally considered to detoxify xenobiotics<sup>106</sup>. Glutathione-S-Transferases (GST) are one of the elaborate phase II mechanisms evolved by the mammalian cells to protect against toxic insults due to electrophilic metabolites or reactive oxygen species that usually arise during CYP-mediated drug metabolism<sup>46</sup>.

The balance between CYP enzymes and GST enzymes can dramatically affect the toxicological outcome of exposure to chemicals that undergo CYP-dependent bioactivation. Given the significant and dynamic changes in activities of these enzymes during development<sup>45,46</sup>, toxicological risk estimates developed for adults may not apply for paediatric populations following exposure to the same chemical, especially from breast milk. Therefore, a thorough understanding of the ontogeny of both CYP enzymes and GST enzymes is necessary to accurately assess the age-dependent exposure risk.

In addition to characterizing the ontogeny of CYP2E1 and CYP1A2, two toxicologically significant CYP enzymes, I examined the ontogeny of cytosolic GST activity in a rat model using a general probe substrate. Figure 4.27 shows detectable GST activity during late gestation (about 20% the adult level). Postnatally, the activity increases steadily with age to achieve the adult level at weaning age (PD28). To my knowledge, this is the first study to analyze the ontogeny of cytosolic GST activity in rats using large number of liver samples that cover a broad range of ages from late gestation to adulthood. Consistent with these findings, Borlakoglu *et al* reported detectable, yet low, levels of GST activity at day 10 of gestation that increased more than 5-fold at day 20 of gestation<sup>115</sup>. Following birth, and consistent with my observations, the activity progressively increased to attain the adult level at PD60<sup>115</sup>. Another study reported 1.75-, 2-, and 2-fold increase in GST activity at PD75, PD300

and PD690, respectively compared to PD14<sup>200</sup>. Other studies have reported similar GST activity values at similar developmental stages used in my study<sup>200,210,276,334,214</sup>.

Comparison of the ontogeny profile of GST enzymes, as detoxification enzymes, to the profiles of CYP2E1 and CYP1A2, as bioactivation enzymes, can provide some insight into the exposure outcome to xenobiotics. Since CYP2E1 and CYP1A2 exhibited undetectable activity in the perinatal period yet moderate activity of GST enzymes suggests that paediatric populations would have a lower risk of CYP-mediated cellular damage than compared with older ages, where CYP enzymes are well developed. However, direct exposure (e.g. drug therapy) or inadvertent exposure (e.g. breastfeeding) can potentially enhance (i.e. through induction) the activity of low levels of CYP enzymes<sup>127,161</sup>. These changes could significantly affect the response of the developing child to xenobiotic exposures, which could be further amplified if GST enzymes are not induced concomitantly. Additional studies are needed to fully characterize the ontogeny of other phase II enzymes (e.g. UGT and sulfotransferases) and understand the molecular mechanisms that modulate their expression and activity.

## **5.5. CONCLUSION AND GENERAL REMARKS**

The ethical and technical difficulties associated with conducting toxicity studies in children resulted in inadequate understanding of the impact of age and development on pharmacokinetics (or toxicokinetics) and pharmacodynamics (or toxicodynamics) of compounds. Therefore, assessment of exposure risk to toxicity in neonates is largely dependent on toxicity information from adults. Due to the significant and dynamic maturation of physiological determinants of drug pharmacokinetics (or toxicokinetics), especially in the early neonatal stages, infants can be more susceptible to the same toxicant exposure (on a body weight basis) than adults. Accordingly, simple adult-to-infant extrapolation can lead to inaccurate assessments of the exposure risk in the developing infant.

In my attempt to improve assessment of exposure risk in paediatric populations, I evaluated the underlying assumptions of a general ontogeny model that was developed

to describe the postnatal maturation of CYP enzyme activity that mediated the elimination or formation of potential toxicants. These assessments were made in the rat and the data recognized some discrepancies with the stated model assumptions.

Microsomal protein (MP) content did not remain constant with development. Rather, MP content was significantly low at ages younger than postnatal day (PD) 7, compared to adult. Liver weight normalized to body weight was relatively constant throughout postnatal development and age-related changes in hepatic scaling factors (HSF) become primarily a function of changes in MP. This suggests the need to incorporate developmental changes in MP content into the model building process.

The Michaelis-Menten constant ( $K_M$ ) did not remain constant with development. Rather, apparent  $K_M$  values were significantly different from adult values at developmental stages younger than PD7. The changes in apparent  $K_M$  values during early postnatal development may indicate inadequate probe substrate specificity during CYP enzyme ontogeny, influence of developmental changes in non-specific binding in hepatic microsomes or changes in microsomal membrane composition. Although these findings may suggest the need to incorporate these  $K_M$  changes in the model building process, further investigations are required to provide more definitive information regarding the potential of  $K_M$  changes with development. These investigations need to assess the influence of non-specific protein binding and the contribution of different CYP isoforms to the estimated  $K_M$  value at a given age.

Consistent with the model assumptions, age-dependent changes in  $V_{max}$  were observed with maximum enzyme activity occurring before (with CYP2E1) or after (with CYP1A2) weaning (PD21-PD28). However, these changes did not correlate with the developmental changes in immunoquantifiable protein level for both CYP2E1 and CYP1A2. Such discordance between activity and protein levels may indicate possible contribution from other CYP enzymes (especially during the early neonatal stages) to the apparent  $V_{max}$  value or posttranslational mechanisms that may affect enzyme activity (especially during the postweaning stages). Further investigations are needed to assess this discordance. Again consistent with the model assumptions, different probe substrates for the examined CYP enzymes (CYP2E1 and CYP1A2) exhibited similar

patterns in age-dependent changes in activity. This may indicate the potential applicability of the model to all substrates metabolized by the CYPs under investigation.

An *in vitro-in vivo* extrapolation of rat hepatic microsomal activity data was used to develop a model to describe rat hepatic CYP2E1 and CYP1A2 ontogeny. The model provided a reasonable prediction of the oral clearance of chlorzoxazone, a CYP2E1 probe substrate. This indicates that despite the above mentioned discrepancies with the model assumptions, the model is robust and has potential as a risk assessment tool.

Extensive characterization (mRNA, protein, activity and hepatic tissue expression) of the ontogeny of rat hepatic CYP enzymes may provide a model system to identify toxicokinetic mechanisms underlying age-dependent differences in susceptibility to toxicity and to enhance our understanding the impact of exposure to exogenous or endogenous compounds on CYP ontogeny. The results of rat CYP ontogeny were compared to the available human data to determine the appropriateness of the rat for developmental toxicology studies.

Unlike the ontogeny pattern in human, temporal changes in CYP catalytic activity correlate well with changes in transcript levels, but not with the immunoquantifiable protein levels. On the other, the ontogeny pattern of rat hepatic CYP protein was similar to those reported for human. Similar temporal and hepatic zonal expression of CYP enzymes between rats and human was observed where the expression was more homogenous in fetal and early neonatal stages then underwent heterogeneous expression towards the perivenous zone with maturation. Recognizing such similarities and differences between rats and human regarding onset, rate and pattern of CYP ontogeny is critical for improving the accuracy of rat-to-human extrapolation of developmental toxicokinetic data.

The activity of phase II enzymes, as a significant detoxification mechanism, plays an important role in determining the toxicological outcome of exposure. Accordingly, the ontogeny of glutathione-S-transferases (GST), an important group of phase II enzymes, was determined in age-specific rat hepatic cytosolic fractions using a general probe substrate. GST activity was detected at low levels in late gestation and early neonatal ages followed by gradual increase with age. Compared to the pattern of CYP activity that generally mediates xenobiotic bioactivation, infant populations are expected to be

under a low risk of CYP-mediated cellular damage relative to older age groups. However, characterization of the ontogeny of other phase II and antioxidant systems requires more extensive investigation.

My dissertation research provided a critical evaluation of two potential model systems capable of providing risk relevant information in paediatric populations, namely a pharmacokinetic model that may predict an infant's capacity to eliminate toxicants by CYP mechanisms and the developing rat as a model of human CYP2E1 and CYP1A2 ontogeny. The information obtained offers mounting and supportive evidence for the use of such model systems to identify whether toxicokinetic mechanisms may underlie age-dependent differences in susceptibility to toxicity and the impact of genetic and environmental factors on CYP enzyme ontogeny.

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## Appendix I

# Ontogeny of Hepatic and Renal Systemic Clearance Pathways in Infants

## Part II

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### Abstract

Maturation of drug systemic clearance mechanisms during the postnatal period produces dramatic and rapid changes in an infant's capacity to eliminate drugs. A tentative general mathematical model describing the ontogeny of hepatic cytochrome P450 (CYP) enzyme-mediated clearance and renal clearance due to glomerular filtration in the first 6 months of life was elaborated from age-specific

*in vitro* hepatic microsomal activity data (normalised to amount of hepatic microsomal protein) for enzyme-specific probe substrates and *in vivo* probe substrate data for glomerular filtration (normalised to bodyweight), respectively. The model predicts an age- and clearance pathway-specific Infant Scaling Factor (ISF) for the first 6 months of life. The ISF reflects functional maturation of a specific clearance pathway (normalised to bodyweight) relative to adult values. Therefore, the ISF directly correlates adult clearance values with an infant's capacity to eliminate drugs. Substitution of appropriate model parameter estimates and the age of the infant into the model provides an estimated ISF value, which may then be used to predict the contribution of a particular clearance pathway to total systemic clearance in the infant when adult systemic clearance values are known.

The model was tested for its ability to predict infant systemic clearance of drugs whose elimination is principally mediated by a single CYP enzyme or by glomerular filtration. The model performed reasonably well for CYP1A2 and CYP3A4, but poorer predictions were obtained for CYP2D6 and CYP2C because of lack of model complexity and/or inadequate hepatic microsomal activity data to fully describe the maturational process of functional enzyme. For renal clearance due to glomerular filtration, data normalised to bodyweight (kg) showed a limited maturational trend, suggesting that adult renal clearances normalised to bodyweight might reasonably predict infant renal clearances in the first 6 months of life. The model provided reasonable predictions of renal clearance due to glomerular filtration in the infant.

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As described in part I of this review,<sup>[1]</sup> dramatic and rapid improvements in the efficiency of drug elimination occur during the first months of post-natal development. The consequences of clearance pathway ontogenesis remain incompletely known because of the paucity of clinical pharmacokinetic data specific for infant populations. Recently, *in vitro* data for cytochrome P450 (CYP) enzyme activity in fetal and infant livers and *in vivo* data for probe substrates of renal function have become available.<sup>[1]</sup> Part II of this review uses data presented in part I to develop a tentative general mathematical model that describes the ontogeny of individual systemic clearance pathways in the infant up to the first 6 months of life. This model may help to estimate an infant's capacity to eliminate a drug when age-specific clinical pharmacokinetic data are lacking.

As highlighted in part I of the review, developmental patterns of most hepatic and renal elimination pathways exhibit an age-dependent increase until an asymptotic state is reached at the adult level. This maturation pattern parallels the devel-

opmental pattern observed with many other biological processes. Consequently, mathematical equations that describe the developmental pattern of biological processes in general<sup>[2]</sup> were modified to mathematically describe the maturation of renal glomerular filtration and hepatic CYP enzyme-mediated metabolism in the first 6 months of life. *In vivo* marker substrate data for glomerular filtration efficiency and *in vitro-in vivo* extrapolation of hepatic microsomal activity data in infant livers were used to develop this tentative model describing the development of these systemic clearance pathways. The theoretical basis for extrapolating *in vitro* hepatic microsomal enzyme activity data to *in vivo* CYP enzyme-specific hepatic clearance is well established in the literature,<sup>[3-13]</sup> and similar approaches were applied in the present paper.

## 1. Theoretical Framework of Clearance Concepts

Drug response often correlates well with steady state plasma drug concentrations. Following an in-

fusion regimen, the steady-state concentration ( $C_{ss}$ ) is determined by the infusion rate ( $R_0$ ) and systemic clearance (CL) [equation 1a]. Similarly, bioavailability (F), dose (D), administration interval ( $\tau$ ) and CL determine the average steady-state plasma concentration of a drug ( $\bar{C}$ ) during sustained oral administration (equation 1b). Systemic clearance reflects the efficiency of elimination pathways and plays a pivotal role in determining the steady state plasma drug concentration and corresponding response in the infant.

Equation 1a:

$$C_{ss} = \frac{R_0}{CL}$$

Equation 1b:

$$\bar{C} = \frac{FD/\tau}{CL}$$

Since most drugs are eliminated from the body by hepatic and/or renal clearance mechanisms, systemic drug clearance becomes an additive function of hepatic ( $CL_H$ ) and renal ( $CL_R$ ) elimination pathways (equation 2). The exact contribution of each pathway to systemic clearance is drug-specific.

$$CL = CL_H + CL_R$$

A comparison of clearance pathways between adults and infants must consider the large differences in body size and elimination organ mass. To minimise size-associated differences in pharmacokinetic estimates, the literature generally reports clearance estimates normalised to bodyweight. Clearance pathway comparisons between the adult and the infant must not only consider differences in body and organ size, but must additionally acknowledge the influence of elimination pathway ontogenesis. This developmental phenomenon is the main focus of the paper.

### 1.1 Hepatic Clearance

In addition to hepatic enzyme activity, nonenzymatic factors determine the *in vivo* hepatic clear-

ance of a drug. The well-stirred model of hepatic clearance offers a simple model describing the interrelationships between all physiological determinants that govern hepatic clearance (equation 3):

$$CL_H = \frac{Q_H f_u CL_{int}}{Q_H + f_u CL_{int}}$$

where  $Q_H$  represents hepatic blood flow,  $f_u$  is the fraction of drug unbound in plasma and  $CL_{int}$  is intrinsic clearance.<sup>[14,15]</sup> Each physiological determinant undergoes maturation whose rate of change may be described by some developmental rate process. This paper principally focuses on the development of enzyme activity as the primary determinant of hepatic clearance in infants.

#### 1.1.1 Intrinsic Clearance as a Function of Enzyme Activity

Most drugs are low hepatic clearance drugs. Hepatic enzyme activity, and not hepatic blood flow, is the rate-limiting step in the hepatic elimination of low clearance drugs (equation 3). Intrinsic clearance ( $CL_{int}$ ) is a measure of the total metabolic enzyme activity towards a drug and represents the sum of all individual pathways of metabolism involved in hepatic elimination of that drug ( $\Sigma CL_{int}$ ). Consequently, for low clearance drugs, hepatic clearance becomes the sum of all individual intrinsic clearance terms ( $CL_{int(j)}$ ) governing the various pathways of drug metabolism (equation 4):

$$CL_H \Rightarrow f_u CL_{int} = f_u \sum_{j=1}^n CL_{int(j)}$$

Typically, one or more metabolic processes (i.e. hydroxylation; reduction) or enzymes contribute to the hepatic metabolic elimination of most drugs.

*In vivo* intrinsic clearance may be expressed in terms of the *in vivo* Michaelis-Menten enzyme parameters shown in equation 5:

$$CL_{int(j)} = \frac{V_{max(j)}^*}{K_m(j) + f_u C}$$

where the *in vivo*  $V_{\max(j)}$  represents the maximal enzyme rate,  $K_{m(j)}$  is the plasma drug concentration at half maximal activity,  $f_u$  is the unbound fraction of drug and  $C$  is the plasma drug concentration. Under linear conditions (i.e.  $K_{m(j)} > f_u C$ ), equation 5 simplifies to equation 6:

$$CL_{int(j)} = \frac{V_{\max(j)}^*}{K_{m(j)}}$$

*In vivo*  $CL_{int(j)}$  may be predicted from *in vitro* estimates of  $CL_{int}$  using *in vitro-in vivo* extrapolation methods described in the literature.<sup>[3-13]</sup>

### 1.1.2 Hepatic Scaling Factor

Intrinsic clearance serves as the foundation for the *in vitro-in vivo* extrapolation of hepatic microsomal enzyme activity data.<sup>[3]</sup> Extrapolations are possible because, in general, a proportional relationship exists between both the *in vivo*  $V_{\max(j)}$  parameter (which has units of activity per kg bodyweight) and the *in vitro*  $V_{\max(j)}$  parameter (which has units of activity per mg of microsomal protein). The *in vitro*  $CL_{int}$  (i.e.  $V_{\max(j)}/K_{m(j)}$ ) may predict the *in vivo*  $CL_{int}$  when appropriate hepatic scaling factors (HSF) are applied (equation 7):

$$CL_{int(j)} = \frac{V_{\max(j)}^*}{K_{m(j)}} = \frac{HSF V_{\max(j)}}{K_{m(j)}}$$

The HSF accounts for the contribution of hepatic microsomal protein content and liver weight normalised to body size to the metabolic function of the whole liver and has units of mg of microsomal protein per kg bodyweight. Hepatic scaling factors must be calculated for each infant age group and for adults prior to extrapolation of *in vitro* metabolism data to account for age-dependent growth processes in infants.

Infants possess a genetically similar complement of hepatic drug-metabolising enzymes as the adult. A basic underlying assumption in the development of the model is the  $K_{m(j)}$  value for a particular CYP enzyme remains constant with adult values throughout infant development. As well, infants and adults express the same complement

of hepatic CYP enzymes; therefore, activity levels do not reflect differences in the affinity of the enzyme for a particular substrate. Rather, functional immaturity of the specific CYP enzyme and age-dependent growth processes (HSF) explain the observed differences in  $CL_{int(j)}$  between the infant and adult. If these assumptions hold true, then equation 7 yields equation 8a and equation 8b for the infant and the adult, respectively:

Equation 8a:

$$CL_{int(j)}^{infant} = \frac{HSF^{infant} V_{\max(j)}^{infant}}{K_{m(j)}}$$

Equation 8b:

$$CL_{int(j)}^{adult} = \frac{HSF^{adult} V_{\max(j)}^{adult}}{K_{m(j)}}$$

Since adult and infant  $K_{m(j)}$  values are assumed to be similar, rearrangement of equation 8b and substitution for  $K_{m(j)}$  in equation 8a yields equation 9:

$$\begin{aligned} CL_{int(j)}^{infant} &= \frac{HSF^{infant} V_{\max(j)}^{infant}}{HSF^{adult} \frac{V_{\max(j)}^{adult}}{V_{\max(j)}^{infant}}} CL_{int(j)}^{adult} \\ &= RHSF_{(t)} \frac{V_{\max(j)}^{infant}}{V_{\max(j)}^{adult}} CL_{int(j)}^{adult} \end{aligned}$$

where  $RHSF_{(t)}$ , the relative hepatic scaling factor, is a unitless parameter that relates the abundance of microsomal protein content per bodyweight in the infant to the adult. Due to organ and body growth processes,  $RHSF$  may be a function of developmental age ( $t$ ).

To calculate a  $RHSF_{(t)}$  for a particular infant age,  $HSF_{(t)}$  is estimated from the product of microsomal protein yield (MP; mg of microsomal protein per g of liver) and liver weight (LW; g) normalised to a bodyweight (BW; kg) as a function of  $t$  (equation 10):

$$HSF_{(t)} = MP \frac{LW_{(t)}}{BW_{(t)}}$$

Liver and body weight change as a function of age, and equation 11 approximates liver or body weight at any age,  $t$  (in days), from birth to the first year of life:

$$W_{(t)} = W_0 + W_1(1 - e^{-k_1 t}) + (W_a - W_0 - W_1)(1 - e^{-k_2 t})$$

where  $W_{(t)}$  is the liver (g) or body (kg) weight at any age (days),  $W_0$  is the liver or body weight at birth,  $W_1$  is a fitted parameter,  $W_a$  is the average adult liver or body weight and  $k_1$  and  $k_2$  are the first-order rate constants governing the age-dependent increase in the liver or body weight.

### 1.1.3 Ontogeny Scaling Factor

Equation 9 suggests that infant intrinsic clearance may be predicted from knowledge of the age-specific RHSF, the relative magnitude of infant and adult  $V_{\max}$  terms, and the adult intrinsic clearance value for a given hepatic enzyme pathway. Unfortunately, this type of detailed *in vitro* (or *in vivo*) assessment is rarely available for paediatric populations. However, the literature presents some *in vitro* data on the relative abundance and functional activities ( $V_{\max}$ ) of enzyme-specific model substrates for a number of hepatic drug-metabolising enzymes (see part I of this review).<sup>[1]</sup> If it is assumed that enzyme activity reflects the relative abundance of functional enzyme ( $J$ ), then the ratio of  $V_{\max}$  values (infant to adult) of one substrate ( $s1$ ) metabolised by enzyme  $J$  will be proportional to another  $J$  substrate ( $s2$ ) as shown in equation 12:

$$OSF_{(j,t)} = \frac{V_{\max}^{\text{infant}}(j,t,s1)}{V_{\max}^{\text{adult}}(j,s1)} \propto \frac{V_{\max}^{\text{infant}}(j,t,s2)}{V_{\max}^{\text{adult}}(j,s2)} \propto \frac{J^{\text{infant}}}{J^{\text{adult}}}$$

where OSF is defined as an ontogeny scaling factor and represents the elimination capacity of a specific enzyme in the infant relative to the adult.  $OSF_{(j,t)}$  becomes a unitless fraction of adult activity and is a function of both infant age and the particular enzyme or elimination pathway ( $j$ ) measured. If the assumption holds true, then differences in functional enzyme levels between infant

and adult livers account solely for the differences in infant and adult enzyme activities ( $V_{\max}$ ), and maturation of CYP enzymes relates directly to enhancements in functional enzyme content.

### 1.1.4 Infant Scaling Factor

Equation 13 proposes a model to estimate intrinsic clearance in the infant for a specific hepatic pathway:

$$CL_{\text{int}(j,t)}^{\text{infant}} = RHSF_{(t)} OSF_{(j,t)} CL_{\text{int}(j)}^{\text{adult}} = ISF_{(j,t)} CL_{\text{int}(j)}^{\text{adult}}$$

where  $ISF_{(j,t)}$ , the infant scaling factor, is the product of  $RHSF_{(t)}$  and  $OSF_{(j,t)}$ .  $ISF_{(j,t)}$  reflects the development of a specific functional enzyme normalised to a bodyweight. Therefore, a prediction of infant clearance due to a specific hepatic metabolic pathway may be made based on the relative contribution of that clearance pathway and the scaling factor for liver content ( $RHSF_{(t)}$ ) and ontogenesis of a specific enzyme ( $OSF_{(j,t)}$ ).

### 1.1.5 Developmental Model for the Infant Scaling Factor

The mathematical concepts underlying the ontogenesis of clearance pathways are similar to those described in modelling phenomena of developmental biology.<sup>[2]</sup> A general model describing the age-dependent increase in CYP enzyme-mediated systemic clearance in infants from birth to 6 months of age is shown in equation 14:

$$ISF_{(j,t)} = ISF_{(j,0)} + ISF_D(1 - e^{-k_1 t})$$

where  $ISF_{(j,t)}$  is the predicted hepatic CYP enzyme-specific infant scaling activity *in vivo* at age  $t$  (in days),  $ISF_{(j,0)}$  is the scaled *in vivo* CYP enzyme activity present at birth,  $ISF_D$  is a fitted parameter and  $k_1$  is the first-order rate constant describing the rate of increase in CYP enzyme pathway efficiency ( $\text{day}^{-1}$ ). Insufficient data prevented the development of a predictive model that could describe the ontogeny of CYP enzyme-mediated metabolism beyond the first 6 months of age, or predictive models for other non-CYP-mediated pathways.