Evaluation of the Assumptions of an Ontogeny Model of Rat Hepatic Cytochrome P450 Activity

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ABSTRACT:

We previously reported an ontogeny model of hepatic cytochrome P450 (P450) activity that predicts in vivo P450 elimination from in vitro intrinsic clearance. The purpose of this study was to conduct investigations into key assumptions of the P450 ontogeny model using the developing rat model system. We used two developmentally dissimilar enzymes, CYP2E1 and CYP1A2, and male rats (n = 4) at age groups representing critical developmental stages. Total body and liver weights and hepatic microsomal protein contents were measured. Following high-performance liquid chromatography analysis, apparent \( K_M \) and \( V_{\text{max}} \) estimates were calculated using nonlinear regression analysis for CYP2E1- and CYP1A2-mediated chlorzoxazone 6-hydroxylation and methoxyresorufin O-dealkylation, and \( V_{\text{max}} \) estimates for \( p \)-nitrophenol and phenacetin hydroxylations, respectively. Hepatic scaling factors and \( V_{\text{max}} \) values provided estimates for infant scaling factors (ISF). The data show microsomal protein contents increased with postnatal age and reached adult values after postnatal day (PD) 7. Apparent \( K_M \) values were similar at all developmental stages except at \(<PD7\). Developmental increases in probe substrate \( V_{\text{max}} \) values did not correlate with the biphasic increase in immunoquantifiable P450. The activity of two different probe substrates for each P450 covaried as a function of age. A plot of observed ISF values as a function of age reflected the developmental pattern of rat hepatic P450. In summation, these observations diverge from several of the model's assumptions. Further investigations are required to explain these inconsistencies and to investigate whether the developing rat may provide a predictive paradigm for pediatric risk assessment for P450-mediated elimination processes.

Intentional and inadvertent exposures (i.e., environmental contamination, breast milk) of the developing neonate to xenobiotics raises significant concerns over the potential risks posed to the exposed neonate. However, ethical impediments and limited toxicity data in human pediatric populations hinder the assessment of such risk. Furthermore, evidence for age-dependent differences in susceptibility to toxicity precludes a simple adult-to-neonate extrapolation of toxicity risk based upon toxicity studies in adult populations (Clark et al., 2004; Ginsberg et al., 2004b). A need exists, then, for predictive paradigms for risk assessment in human pediatric populations.

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A small amount of data in this article was recently published in Elbarbry FA, McNamara PJ, and Alcorn J (2007) Ontogeny of hepatic CYP1A2 and CYP2E1 expression in rat, J Biochem Mol Toxicol 21:41–50, Fig. 3. Rights to use this data have been granted by John Wiley & Sons, Inc.

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ABBREVIATIONS: P450, cytochrome P450; CZX, chlorzoxazone; 6-OH CZX, 6-hydroxychlorzoxazone; PNP, \( p \)-nitrophenol; 4NC, \( p \)-nitrocatechol; HRP, horseradish peroxidase; MROD, methoxyresorufin O-dealkylation; HPLC, high-performance liquid chromatography; PBS, phosphate-buffered saline; PBS/t, phosphate-buffered saline/Tween 20; HSF, hepatic scaling factor; ISF, infant scaling factor(s); PD, postnatal day; MP, microsomal protein.
ment purposes. Predictions of xenobiotic elimination capacity alone, though, offer little understanding on mechanisms of toxicity and mitigating genetic and environmental factors contributing to individual variation in toxicity. To facilitate our understanding of age-dependent differences in susceptibility to toxicity an appropriate developmental animal model system remains mandatory (Gaertner and Mortensen, 1996; Koren et al., 2003; Brent, 2004).

Our laboratories previously developed an ontogeny model of hepatic P450-mediated elimination based on an in-vitro-in vivo extrapolation of published P450 activity data from age-specific human fetal and infant hepatic microsomes. Our review outlines the theoretical framework for the model (Alcorn and McNamara, 2002b). Briefly, the model involves scaling in vitro hepatic intrinsic clearance data to in vivo intrinsic clearance and the extrapolation of known adult intrinsic clearance values to any age during postnatal development. This extrapolation occurs via a scaling factor (the infant scaling factor), which incorporates age-dependent changes in hepatic scaling factors and P450 activity. Critical assumptions of the model include 1) microsomal protein content is constant and independent of developmental age; 2) $K_M$ values are constant and not a function of developmental age; 3) $V_{max}$ is proportional to functional enzyme level and activity from one substrate will accurately predict activity for all substrates of that enzyme; 4) uptake and efflux transporters do not contribute significantly to intrinsic clearance; 5) the drug is a low extraction ratio drug; and 6) the elimination pathways in the adult are known and the infant shares the same pathways.

Since interspecies extrapolation of developmental toxicity data remains a practical option to elucidate the impact of development on susceptibility to toxicity, we propose a similar model approach for rat hepatic P450 ontogeny as a means of providing a predictive paradigm for risk assessment. Comparative analyses of human and rat P450 enzyme maturation (Cresteil, 1987; Rich and Boobis, 1997) and P450 enzyme substrate specificity and regulation highlight important species differences between the rat and human. Furthermore, differences in the rates and patterns of P450 maturation (Alcorn and McNamara, 2002a) may result in species differences in the parameterization of the model. Nevertheless, P450 enzyme mechanisms are similar across species, which suggests model assumptions remain the same regardless of species. However, before any toxicokinetic model of P450 ontogeny in rat can be developed and used to provide robust predictions of hepatic P450-mediated elimination, we must first evaluate the appropriateness of the model’s underlying assumptions.

The purpose of this study is to conduct investigations into the key assumptions of the model of hepatic P450 ontogeny. We use two developmentally dissimilar P450 enzymes, CYP2E1 and CYP1A2, and male Sprague-Dawley rats at various fetal, neonatal, juvenile, and adult age groups to provide critical information on the assumptions of the model. The implications of the findings on the validity of the model are discussed.

**Materials and Methods**

**Chemicals and Reagents.** Chlorozoxone (CZX), 6-hydroxychlorozoxone (6-OH CZX), umbelliferone (7-hydroxycoumarin), p-nitrophenol (PNP), p-nitro catechol (4NC), salicylamide, phenacetin, acetaminophen, caffeine, resorufin, methoxyresorufin, 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). Rat CYP1A2 and CYP2E1 supersomes were acquired from BD Biosciences (Woburn, MA). Peroxidase-conjugated antibody (goat anti-rabbit IgG HRP conjugate), prestained SDS-polyacrylamide gel electrophoresis standards, Immun-Star HRP kit, and Trans-Blot cell and polyvinylidene difluoride membranes (0.2 μm) were obtained from Bio-Rad (Hercules, CA). Rabbit anti-human/rat CYP2E1 and sheep anti-rat CYP1A2 polyclonal antibodies and rabbit anti-sheep IgG HRP conjugate antibody were purchased from Chemicon International (Temecula, CA). All other chemicals used were analytical grade.

**Animals.** Sprague-Dawley rats were obtained from Charles River Canada (St. Constant, PQ, Canada). Eight pregnant rats were received at day 14 of pregnancy and allowed to acclimatize for 1 week. At day 21 of pregnancy, four pregnant rats were anesthetized under isoflurane, and the fetuses were collected. Given the difficulty with sex-typing fetal rats, pups from both genders were used. 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. Other age groups (2, 3, 4, 6, 9, 12, and 16 weeks) ($n = 4$) were obtained from Charles River Canada 1 week before the required age during which time rats were allowed to acclimatize. All rats received standard laboratory rat chow and water ad libitum throughout the acclimatization period. At the appropriate age, body weights were recorded. Rats were then killed by guillotine under light isoflurane anesthesia, and the livers were rapidly excised and rinsed in cold 0.9% NaCl. Livers were then weighed and flash frozen in liquid nitrogen and stored at $-80^\circ$C until microsomal preparation. All procedures were conducted in accordance with the Canadian Council of Animal Care guidelines for the care and use of laboratory animals, and they were approved by the Animal Care and Supply Committee of the University of Saskatchewan (Saskatoon, SK, Canada).

**Preparation of Hepatic Microsomes.** Hepatic microsomes were prepared as described previously (Elbarbry et al., 2007). Protein concentrations of microsomal preparations were determined in triplicate by the method of Lowry et al. (1951) using bovine serum albumin as a calibration standard.

**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 metabolism formation kinetics for CYP2E1-mediated hydroxylation of CZX and PNP and CYP1A2-mediated phenacetin O-dealkylation and methoxyresorufin O-dealkylation (MROD) activities. Michaelis-Menten parameters $K_M$ and $V_{max}$ for CYP1A2 and CYP2E1 were determined by measuring metabolism formation velocities of CZX and MROD at substrate concentrations of 0 to 1000 μM and 0 to 1000 nM, respectively. With PNP and phenacetin, metabolism formation velocities were measured at saturating probe substrate concentrations (500 and 150 μM, respectively) in age-specific rat hepatic microsomes to provide estimates of $V_{max}$.

**HPLC Analysis.** For CYP2E1, 6-hydroxylation of chlorozoxone in rat hepatic microsomal preparations was quantified using an HPLC analysis as described previously (Elbarbry et al., 2007). For the second probe substrate, PNP hydroxylation (CYP2E1 probe) was assayed as reported previously (Elbarbry et al., 2006). Calibration curves were constructed from known concentrations of 6-OH CZX and 50 μl of the internal standard (0.078 mM umbelliferone) or known concentrations of 4-nitrocatechol and 50 μl of internal standard (6 μg/ml salicylamide) added to heat-inactivated (55°C for 5 minutes) hepatic microsomes. The calibration standards were diluted with 50 mM phosphate buffer, pH 7.4, to achieve calibration standards of 0.31 to 40 μM for 6-OH CZX or 0.1 to 40 μM for 4NC in a total volume of 0.5 ml. Three quality control samples at 0.625 μM (low), 1.25 μM (medium), and 5 μM (high) for 6-OH CZX or 0.5 μM (low), 5 μM (medium), and 20 μM (high) for 4NC were prepared independently of those used for the calibration curves. Coefficients of determination were greater than 0.999 for all calibration curves. Intraday and interday precisions were less than 14%. The accuracy of estimated metabolite concentrations ranged from 91 to 113%.

For CYP1A2, phenacetin O-dealkylation (CYP1A2 probe) was assayed by a published HPLC method (von Moltke et al., 1996) 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 it was delivered at 1.0 ml/min. Calibration curves were constructed from known concentrations of resorufin or acetaminophen, and the internal standard (5 μg/ml caffeine) was 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 0.04 to 2 nM for resorufin and 0.2 to 20 μM for acetaminophen in a total volume of 0.5 ml. Three quality control samples at 0.1 nM (low), 0.4 nM (medium), and 2 nM (high) for resorufin and 0.5 μM (low), 2 μM (medium), and 10 μM (high) for acetaminophen were prepared independently of those used for the
CYP1A2 and CYP2E1 Protein Quantification. Hepatic microsomal proteins were separated on a sodium dodecyl sulfate gel containing 10% polyacrylamide (SDS-polyacrylamide gel electrophoresis) by standard methods. The gel was loaded with rat CYP1A2 purified protein (20, 80, 200, and 500 pmol) or heat-denatured microsomes (0.4 μg of protein) for CYP1A2 and rat CYP2E1 purified protein (20, 50, and 100 pmol) or heat-denatured microsomes (10.5 μg of protein) for CYP2E1, and electrophoresis was conducted for 1.5 to 2 h at 125 V. Proteins were transferred onto a polyvinylidene difluoride membrane (0.2 μm) with a Trans-Blot cell (Bio-Rad) at 25 V for 50 min. The membrane was washed with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T) and subsequently blocked with PBS containing 5% milk with overnight incubation at 4°C. For CYP2E1, the membrane was then incubated overnight at 4°C with sheep anti-rat CYP2E1 polyclonal antibody prepared in PBS solution (1:10000 dilution). For CYP2E1, the membrane was incubated for 3 h at 20°C with rabbit anti-human/rat CYP2E1 polyclonal antibody prepared in the blocking solution (1:750). Following the appropriate incubation period, membranes were washed three times with PBS and once with PBS, and then they were incubated with peroxidase-conjugated antibody prepared in the blocking solution (1:3000) for 2 h at 20°C. The membrane was further washed three times with PBS and once with PBS, and then immunodetection was performed using the Immuno-Star HR kit. To estimate the amount of reacting protein, the blot was scanned with an LKB laser densitometer (GE Healthcare, Piscataway, NJ) using reference samples to calibrate determinations. The results were expressed as absorbance unit per microgram of protein (optical density per microgram of protein).

Scaling Factors. Hepatic scaling factors for a particular age [HSF\(_t\)] were estimated from the product of microsomal protein yield [MP\(_t\)] (milligrams of microsomal protein per gram of liver) and liver weight [g] [LW\(_t\)] normalized to body weight (g) [BW\(_t\)] as a function of developmental age, \(t\), according to eq. 1:

\[
\text{HSF}\_t = \text{MP}\_t \times \frac{\text{BW}_{opt}}{\text{BW}_t}
\]

Infant scaling factors at a particular age, \(t\) [ISF\(_t\)] were estimated from the product of HSF\(_t\) and \(V_{\text{max}}\) values at the same age, \(t\) [\(V_{\text{max}}\_t\)] normalized to the product of the respective adult values according to eq. 2:

\[
\text{ISF}\_t = \frac{\text{HSF}_{t,\text{infant}}}{\text{HSF}_{t,\text{adult}}} \times \frac{\text{V}_{\text{max},t,\text{infant}}}{\text{V}_{\text{max},t,\text{adult}}}
\]

Data Analysis. All data within the same age were reported as mean ± S.D. Metabolite formation velocity as a function of substrate concentration was fit to a one-enzyme site Michaelis-Menten equation. The parameters \(\text{V}_{\text{max}}\) and \(K_m\) were estimated by an iterative nonlinear least-squares regression analysis using GraphPad Prism 3.0 (GraphPad Software Inc., San Diego, CA). Significant differences in total microsomal protein content, P450 protein content, and \(V_{\text{max}}\) and \(K_m\) estimates between the different age groups were assessed with one-way analysis of variance with Tukey’s post hoc test for pairwise multiple comparisons using GraphPad Prism. For CXZ, \(K_m\) estimates a Wilcoxon signed rank test was used for pooled data. Pearson correlation coefficients were determined to compare the \(V_{\text{max}}\) values of two different substrates of the P450. The level of significance was set at \(P < 0.05\).

Variation associated with the computed ISF\(_t\) values was represented as a coefficient of variation according to eq. 3, where \(X\) is the infant ISF mean value, \(Y\) is the adult ISF mean value, \(X/Y\) is the ratio of the mean infant ISF to the mean adult ISF values, \(S.D.\) is the standard deviation, and \(n\) is the sample size.

\[
\text{CV}(X/Y) = \sqrt{\frac{S.D.}{X}} + \frac{S.D.}{Y}
\]

Results

HSF as a Function of Developmental Age. To evaluate whether microsomal protein content remains constant with developmental age (the first major assumption of the ontogeny model of P450 enzyme activity), we measured total body and liver weights and liver microsomal protein content (milligrams per gram of liver) in fetal (gestation day 20), neonatal, juvenile, and adult age groups (Table 1). As expected, mean total body and liver weights increased with postnatal age, with the most prominent increases occurring during puberty (PD42–PD63). The age-dependent increase in liver and body weight was associated with marked interindividual variation, which reflects the normal variation in rates and patterns of body and organ growths typically observed in animal species. The average microsomal protein content per gram of liver (MP) was significantly lower than adult levels in fetal and early postnatal age groups. After PD5, MP levels were similar to adult values.

Closer examination of the data showed mean liver weight normalized to total body weight remained relatively constant with postnatal age. The pattern observed in the mean HSF values with age (Fig. 1) mirrored the developmental pattern of microsomal protein content per gram liver (Table 1). HSF values between gestational day 20 and postnatal day 5 remained approximately at 50 to 70% of the adult value. HSF values steadily increased to reach ~2-fold the adult value by PD42. By PD63, mean HSF values were similar to adult values.

\(K_m\) as a Function of Development. To evaluate whether \(K_m\) values remain constant with developmental-age (the second major assumption of the ontogeny model of P450 enzyme activity) we conducted enzyme kinetic studies in rat hepatic microsomes with probe substrates specific for CYP2E1 and CYP1A2, namely, CXZ and methoxyresorufin, respectively. For CYP2E1, limited tissue availability necessitated the use of pooled microsomes (pooled from four animals). We detected no activity in gestation day 20 and PD1 hepatic microsomes, and CXZ apparent \(K_m\) values were significantly lower than adult values (35–40% of adult values) at PD3 and PD5 (data not shown). By PD10, apparent \(K_m\) values were similar to adult values, and they stayed relatively constant thereafter.

For CYP1A2, the apparent \(K_m\) estimates for MROD for PD5 and PD7 were significantly higher than adult values (>2.5-fold adult values) (Fig. 2). No significant differences in apparent \(K_m\) estimates were observed for PD age groups ≥10 days. The data did not allow estimation of \(K_m\) values in fetal, PD1, and PD3 age groups, although activity was detected at low levels at PD3.

### Table 1

<table>
<thead>
<tr>
<th>Age</th>
<th>Total Body Wt</th>
<th>Liver Wt</th>
<th>Microsomal Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetus(_a)</td>
<td>2.69 (0.58)</td>
<td>0.21 (0.055)</td>
<td>5.71 (2.3)</td>
</tr>
<tr>
<td>1 day</td>
<td>7.09 (0.46)</td>
<td>0.32 (0.077)</td>
<td>9.40 (3.3)</td>
</tr>
<tr>
<td>3 days</td>
<td>9.40 (0.82)</td>
<td>0.42 (0.070)</td>
<td>14.7 (3.9)</td>
</tr>
<tr>
<td>5 days</td>
<td>12.9 (1.4)</td>
<td>0.53 (0.096)</td>
<td>15.7 (6.8)</td>
</tr>
<tr>
<td>7 days</td>
<td>17.4 (1.3)</td>
<td>0.63 (0.089)</td>
<td>20.6 (4.0)</td>
</tr>
<tr>
<td>10 days</td>
<td>26.2 (4.3)</td>
<td>0.86 (0.34)</td>
<td>22.3 (4.4)</td>
</tr>
<tr>
<td>14 days</td>
<td>32.7 (3.1)</td>
<td>1.13 (0.24)</td>
<td>27.3 (4.7)</td>
</tr>
<tr>
<td>21 days</td>
<td>39.4 (4.8)</td>
<td>2.62 (0.24)</td>
<td>36.4 (14.1)</td>
</tr>
<tr>
<td>28 days</td>
<td>105 (6.3)</td>
<td>4.93 (0.54)</td>
<td>39.2 (14.0)</td>
</tr>
<tr>
<td>42 days</td>
<td>203 (12.1)</td>
<td>9.39 (0.75)</td>
<td>43.1 (15.3)</td>
</tr>
<tr>
<td>63 days</td>
<td>399 (30.5)</td>
<td>16.2 (2.6)</td>
<td>36.0 (10.9)</td>
</tr>
<tr>
<td>84 days</td>
<td>411 (9.6)</td>
<td>13.8 (0.51)</td>
<td>41.3 (6.9)</td>
</tr>
<tr>
<td>112 days</td>
<td>507 (27.3)</td>
<td>15.0 (1.4)</td>
<td>32.4 (7.3)</td>
</tr>
</tbody>
</table>

\(a\) Fetuses were collected at 20 days gestation from three dams and the fetal livers obtained from a single dam were pooled for microsomal preparation.

Statistically significant from adult (112 days); \(P < 0.05\).
**V**\textsubscript{max} as a Function of Development. To assess whether enzyme activity (\(V\textsubscript{max}\)) is proportional to enzyme level during ontogeny (the third major assumption of the ontogeny model of P450 enzyme activity), we measured CYP1A2 and CYP2E1 activity at saturating probe substrate concentrations in rat hepatic microsomes at different developmental stages, and we compared this activity to immunoquantifiable P450 levels at the same developmental age. MROD activity (Fig. 3A) was first detected at PD3, but it was below the level of quantification of the HPLC assay. PD5 and PD7 hepatic microsomes demonstrated significantly lower MROD activity at 11 and 20% adult activity, respectively. MROD activity increased significantly to reach \(1.75\)-fold the adult values by PD28. However, after weaning (PD28) and during puberty (PD42–PD63) \(V\textsubscript{max}\) 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 (Fig. 1), activity declined after PD28 to the adult value by PD42, unlike the hepatic scaling factor.

We did not detect CZX hydroxylase activity (Fig. 3B) in fetal and PD1 livers. CZX hydroxylase activity was first detected in PD3 livers (70% of the adult value), which increased significantly to reach maximum levels by PD14 (\(1.6\)-fold adult value) and generally declined thereafter to adult values. The marked postnatal increase in \(V\textsubscript{max}\) values for CZX hydroxylase preceded hepatic scaling factor values, where the predominant age-dependent increase occurred between PD14 and PD21 and continued to increase until PD42 (Fig. 1).

Immunoquantifiable CYP1A2 and CYP2E1 exhibited a biphasic pattern of development (Fig. 4). Rapid increases in immunodetectable levels of CYP1A2 and CYP2E1 occurred during the early postnatal period (≤PD7) followed by a slower, more gradual increase to adult values. The rapid rise in immunodetectable CYP1A2 and CYP2E1 in the early postnatal period seemed to parallel increases in MROD and CZX hydroxylase activity. Thereafter, a marked discordance between P450 protein and activity was observed, and \(V\textsubscript{max}\) values did not
correlate (Pearson $r = 0.35$, $P > 0.05$ for CYP1A2; Pearson $r = -0.08$, $P > 0.05$ for CYP2E1) with changes in immunoquantifiable levels observed in the same age group (Fig. 4).

To determine whether enzyme activity ($V_{\text{max}}$) from one substrate will accurately predict the level of activity for all substrates of the enzyme, we correlated the $V_{\text{max}}$ values at each age group from one P450 probe substrate with the second probe substrate (Fig. 5). Figure 5 suggests a strong and significant correlation between the activity of one specific substrate relative to a second specific substrate for both CYP1A2 ($r = 0.68$, $P < 0.05$; data not shown due to low variability in the phenacetin O-dealkylation data relative to the MROD data) and CYP2E1 ($r = 0.69$, $P < 0.05$).

Figure 6 shows the postnatal developmental pattern of CYP1A2 and CYP2E1 when scaled to the whole body. For both P450 enzymes, whole body P450 activity is low at birth and rapidly increases to maximum levels at the end of weaning (PD28). Thereafter, whole body P450 activity declines toward adult values by the end of puberty (PD63).

**Discussion**

The very limited toxicological data in human pediatric populations prompted efforts to advance physiologically based pharmacokinetic models in animal developmental toxicology systems for human pediatric risk assessments (Brent, 2004; Clark et al., 2004). By relating xenobiotic elimination capacity to the time course of P450 maturation, the previously reported ontogeny model of P450 activity (Alcorn and McNamara, 2002b) contributes to such efforts. A valid and robust model can facilitate study design and dosage selection for developmental toxicology studies and identify when age-dependent changes in xenobiotic elimination account for differences in susceptibility to toxicity. To this end, we evaluated the underlying assumptions of the P450 ontogeny model (Alcorn and McNamara, 2002b) in a toxicologically relevant animal system. The assessments in the rat identified important departures from model assumptions, and we discuss their possible underlying causes and overall significance below.

**Hepatic Scaling Factor.** In vitro-in vivo extrapolation of intrinsic clearance data requires knowledge of developmental changes in microsomal protein content, liver weight, and body weight (Carlile et al., 1997; Houston and Carlile, 1997; Obach et al., 1997). In the human P450 ontogeny model, we obtained information on the age-dependent changes in liver and body weight from the literature, but we assumed hepatic microsomal protein content on a per gram liver basis remained constant throughout postnatal development and used a published literature value (Alcorn and McNamara, 2002b). Any age-related changes in hepatic scaling factors, then, became dependent solely on developmental changes in liver weight normalized to body weight.

In this study, rat hepatic microsomal protein content per gram liver increased significantly during the neonatal period, reached a maximum at puberty and declined to adult levels in the early postpubertal stage. The data are consistent with rat hepatic microsomal content described in a limited number of postnatal, prepubertal, and pubertal age groups and adults in the literature (Borlakoglu et al., 1993; Kishi et al., 2005). Postnatal changes in microsomal protein content in the rat suggest a need for similar investigations with human pediatric livers (Johnsrud et al., 2003; Johnson et al., 2006). Interestingly, the age-dependent changes in rat hepatic scaling factors became a function of changes in microsomal protein content rather than liver weight-to-body weight ratio, which stayed relatively constant and...
independent of developmental stage. Although these findings contradict a model assumption, this contradiction does not necessarily weaken its potential value. These results merely suggest the need to measure hepatic microsomal protein content in pediatric livers to incorporate its developmental change into the modeling process to provide predictive estimates of the infant scaling factors.

**Clearance as a Function of Hepatic Metabolic Enzyme Activity.** The ontogeny model makes the limiting assumption that systemic clearance is a function of hepatic metabolic enzyme activity (i.e., the xenobiotic is a low hepatic extraction ratio compound) and uptake and efflux transporters make no contribution to intrinsic clearance. We used probes (Kobayashi et al., 2002) that are known low hepatic extraction ratio compounds, but these probes have not been assessed as transporter substrates. Intrinsic clearance estimates are affected by substrates where membrane transporters act as a rate-limiting process of metabolic clearance (Ito et al., 1998). At present, the model makes no accommodation for such substrates, but it has the potential to incorporate transport processes in the infant scaling factor determinations. Nevertheless, the model can be applied to many xenobiotics as most are low extraction ratio compounds and passive diffusion governs their distribution into the liver.

**Intrinsic Clearance.** The experimental data failed to support two critical assumptions of the ontogeny model (i.e., age-dependent changes in intrinsic clearance reflect changes only in $V_{\text{max}}$ and $K_M$ remains constant throughout development) (Alcorn and McNamara, 2002b). The data suggest a need to incorporate developmental changes in $K_M$ estimates in the model-building process (i.e., model the $V_{\text{max}}/K_M$ ratio) instead of modeling only changes in $V_{\text{max}}$. The incorporation of a new parameter, which undergoes interindividual variability during development, will increase the complexity of the model and possibly result in greater uncertainty in its parameter estimates. However, study design limitations may explain this apparent divergence from model assumptions. Specifically, we question probe substrate specificity in pediatric hepatic microsomal preparations. At best probes are selective (not specific) for the P450 of interest (Kobayashi et al., 2002; Lu et al., 2003), and selectivity is dependent upon substrate concentration (Kato and Yamazoe, 1994). Since different P450 enzymes undergo variable rates and patterns of development (Borlakoglu et al., 1993; Rich and Boobis, 1997; Alcorn and McNamara, 2002a), P450 enzymes that contribute a small percentage to probe metabolism in adult stages may play a larger role during specific pediatric stages. In the study, differences in $K_M$ values may reflect the contribution of other P450 enzymes, and the estimated $K_M$ values will then reflect a hybrid of contributing enzymes. Eadie-Hofstee plots (data not shown), though, gave no evidence of systematic deviation from a single site Michaelis-Menten model at higher substrate concentrations for CZX, but curvilinear plots were observed for MROD, suggesting the contribution of at least a second low-activity enzyme site. Furthermore, nonspecific binding to microsomal protein (Obach, 1997; Obach et al., 1997) and differences in the membrane environment of the endoplasmic reticulum (Dallner et al., 1966) can influence apparent $K_M$ estimates. We cannot ignore the potential for age-dependent changes in such factors. Although the data do not rule out the possibility for developmental changes in the inherent properties of P450s, we failed to find any information regarding changes in P450 enzyme active sites with postnatal maturation. Further investigations are needed to explain these postnatal changes in $K_M$ values. For example, recombinant P450s, singly expressed and mixed in proportion to P450 levels present in hepatic microsomes at different age groups, may facilitate assessment of the contribution of different P450 isoforms to the estimated $K_M$ value at a given age (Tang et al., 2005).

The data on the age-dependent changes in CYP2E1 and CYP1A2 activity are consistent with the few reports of rat P450 ontogeny (Jahn et al., 1993; Wu and Cederbaum, 1993; Rich and Boobis, 1997; Iba et al., 2000; Anand et al., 2006). However, the observed lack of correlation between developmental increases in P450 activity and immunoreactive protein levels violates an important model assumption. Posttranslational modifications with development that markedly influence enzyme activity may explain the apparent discordance. Alternatively, other P450 enzymes whose development precedes CYP2E1 and CYP1A2 maturation may contribute significantly to probe metabolism at particular postnatal age stages. Finally, inadequate antibody specificity (i.e., cross-reactivity with other P450 enzymes) may also contribute to this lack of congruence. For these reasons, P450 immunoreactive protein levels may not necessarily reflect enzyme activity (Johnsrud et al., 2003). Interestingly, immunohistochemical analysis revealed homogeneous expression of CYP1A2 and CYP2E1 in the hepatic acinus in fetal and early postnatal livers with increasing preferential localization of CYP1A2 and CYP2E1 expression in perivenous hepatocytes with postnatal development (Elbarbry et al., 2007). With intrasinusoidal gradients in oxygen tension and substrate supply, age-dependent changes in intrahepatic P450 expression patterns will likely have some influence on P450 regulation and activity. Further investigations into antibody specificity, involvement of alternative P450 enzymes in probe substrate metabolism and the role of developmental changes in intrahepatic P450 expression patterns are necessary to explain the lack of correlation between probe substrate activity ($V_{\text{max}}$ estimates) and immunonquantifiable P450.

Finally, we observed that the activity of two different probe substrates for each P450 covaried as a function of age, thus satisfying an important model assumption. However, activity was generally detected earlier with the less specific P450 substrate, which suggests the involvement of additional P450 enzymes. Although the data do not necessarily refute an implicit assumption of the ontogeny model, namely, the infant shares the same metabolic pathways as the adult, it does suggest that the relative contribution of different pathways involved in the metabolism of a particular substrate may have varied importance during development. This will influence both qualitative and quantitative predictions, and the model will need to accommodate...
the potential for P450s to have different contributions at different stages of development.

Conclusion

The data identified several important inconsistencies with the underlying assumptions of the P450 ontogeny model. The observed age-related changes in hepatic microsomal protein content suggest a need to incorporate its developmental change into the modeling process. Inconstant $K_M$ values during early postnatal development may reflect questionable probe substrate specificity during P450 ontogeny rather than age-dependent changes in the inherent properties of substrate-P450 interactions. Further investigations are needed to determine the reason for age-dependent changes in the apparent $K_M$ values. Consistent with model assumptions, the apparent $V_{max}$ estimates increased postnatally to reach a maximum before or during weaning, and the activity of two separate substrates for a given P450 covaried as a function of age. The lack of correlation between age-dependent changes in $V_{max}$ estimates and immunoreactive P450 requires further investigation. Future studies are planned to 1) explain the incongruence between the experimental data and several model assumptions and 2) to develop a model of rat P450 ontogeny that provides reasonable predictions of in vivo intrinsic clearance of P450 probe substrates.

References


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