PREDICTING PHARMACOKINETIC BEHAVIOR AND DOSE OF SILDENAFIL AND VORICONAZOLE IN NEONATAL AND PEDIATRIC POPULATIONS BY IN VITRO METABOLISM AND PBPK MODELING

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A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Eshelman School of Pharmacy.

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ABSTRACT

Nicole Rachele Zane: Predicting Pharmacokinetic Behavior and Dose of Sildenafil and Voriconazole in Neonatal and Pediatric Populations by In Vitro Metabolism and PBPK Modeling
(Under the direction of Dhiren R. Thakker)

Quantitative and qualitative differences in drug metabolizing enzymes (DMEs) from birth to adulthood affect drug clearance. Yet the effect of these differences on pediatric drug disposition is poorly understood. Moreover, drug disposition relies on both metabolic clearance and age-dependent differences in physiology.

The overall goal of this dissertation was to develop a bottom-up experimental and modeling approach to predict pharmacokinetics (PK) of drugs in pediatric populations. Voriconazole and sildenafil were selected because they are (i) cleared predominantly by oxidative metabolism and (ii) administered to children and premature neonates, respectively.

Hepatic intrinsic clearance from adult and pediatric tissues was elucidated and then incorporated into a physiologically based pharmacokinetic (PBPK) model to predict PK for each drug.

The voriconazole model retrospectively validated this approach by comparing the model’s output against the abundant pediatric clinical data. It showed that the drug was cleared approximately 3-fold faster and bioavailability was nearly half in children ages 2-10 years compared to adults due to contribution differences in CYP2C19 and CYP3A4.

Quantitative proteomic analysis of hepatic tissues revealed higher expression of CYP2C19 protein and higher catalytic efficiency in children compared to adults.

Sildenafil metabolism studies demonstrated the predominant enzymes are CYP3A5 and either CYP3A4 or CYP3A7, which refutes previous reports identifying CYP3A4 and
CYP2C9. Furthermore, these studies revealed that CYP3A7 is a steroid-specific enzyme that generates a unique testosterone metabolite. The expression and functional activity of the three CYP3A isoforms in fetal, pediatric, and adult hepatic tissues was elucidated. This data along with hepatic in vitro sildenafil metabolism was incorporated into a prospective PBPK model for premature neonates. The model revealed how ontogeny affects sildenafil clearance in premature neonates. It also predicted the influence of physiological factors and the potential effect of drug-drug interactions (DDI) on sildenafil PK.

The key outcome was the development of a bottom-up PBPK model in pediatric populations by adapting a validated adult model, which was based on hepatic in vitro metabolism studies and expression of relevant enzymes from hepatic tissue incorporated with physiologic parameters. This innovative approach can be adapted to predict disposition and clinically significant DDI without relying solely on clinical trials.
To my mom and dad for their unending love and support.
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I would also like to thank my entire dissertation committee. I was fortunate to meet Dr. Kim Brouwer while completing my PharmD. She convinced me to come to UNC and accepted the position as chair of my dissertation committee. Her suggestions always helped to push my science to a higher level. I would like to thank Dr. Rick Graham for always being available to discuss my project and professional development as well as providing me opportunities to learn more about modeling and simulation in addition to clinical pharmacology. Dr. Danny Benjamin was integral to the formation of my dissertation project and directed me to great resources and suggestions for external awards. Both Drs. Matthew Laughon and Dennis Williams helped shape my clinical experiences that have broadened my knowledge of pediatric infectious diseases and therapies needed for premature neonates.
I would be remiss if I did not thank every member of the Thakker laboratory including all current and past members. In particular, I would like to thank Dr. Ruth Everett. She has been an incredible inspiration and mentor. Her patience, and even sometimes her tough love, have helped me improve my writing by leaps and bounds in addition to helping me grow as a person. I would also like to thank Drs. Chester Costales, Kevin Han, and Ravindra Alluri for helping me develop my project. Finally, I would like to thank my current lab members Hao Cai, Christine Lee, and Bryan Mackowiak for their scientific advice and friendship.

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<table>
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<th>Description</th>
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<tbody>
<tr>
<td>AUC</td>
<td>Area Under the Curve</td>
</tr>
<tr>
<td>BPCA</td>
<td>Best Pharmaceuticals for Children Act</td>
</tr>
<tr>
<td>CI</td>
<td>Clearance</td>
</tr>
<tr>
<td>Cl&lt;sub&gt;int&lt;/sub&gt;</td>
<td>Intrinsic Clearance</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt;</td>
<td>Peak Plasma Concentration</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of Variation</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>DDI</td>
<td>Drug-Drug Interactions</td>
</tr>
<tr>
<td>DMEs</td>
<td>Drug Metabolizing Enzymes</td>
</tr>
<tr>
<td>EMA</td>
<td>European Medicines Agency</td>
</tr>
<tr>
<td>F&lt;sub&gt;a&lt;/sub&gt;</td>
<td>Fraction Absorbed</td>
</tr>
<tr>
<td>FDA</td>
<td>US Food and Drug Administration</td>
</tr>
<tr>
<td>FMO</td>
<td>Flavin-Containing Monooxygenasae</td>
</tr>
<tr>
<td>F&lt;sub&gt;u&lt;/sub&gt;</td>
<td>Fraction Unbound in the Plasma</td>
</tr>
<tr>
<td>f&lt;sub&gt;u,mic&lt;/sub&gt;</td>
<td>Fraction of Unbound in Microsomal Incubation</td>
</tr>
<tr>
<td>HIM</td>
<td>Human Intestinal Microsomes</td>
</tr>
<tr>
<td>HLM</td>
<td>Human Liver Microsomes</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>IRB</td>
<td>Institutional Review Board</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>K&lt;sub&gt;m&lt;/sub&gt;</td>
<td>Concentration of Half-maximal Velocity (Michaelis-Menten Constant)</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Tandem Liquid Chromatography Mass Spectrometry</td>
</tr>
<tr>
<td>PAH</td>
<td>Pulmonary Arterial Hypertension</td>
</tr>
<tr>
<td>PBPK</td>
<td>Physiologically Based Pharmacokinetic</td>
</tr>
<tr>
<td>PD</td>
<td>Pharmacodynamics</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>PDE-5</td>
<td>Phosphodiesterase 5</td>
</tr>
<tr>
<td>PK</td>
<td>Pharmacokinetics</td>
</tr>
<tr>
<td>PO</td>
<td>Oral</td>
</tr>
<tr>
<td>PREA</td>
<td>Pediatric Research Equity Act</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative Real Time Polymerase Chain Reaction</td>
</tr>
<tr>
<td>$T_{\text{max}}$</td>
<td>Time to Reach $C_{\text{max}}$</td>
</tr>
<tr>
<td>$V_d$</td>
<td>Volume of Distribution</td>
</tr>
<tr>
<td>VPC</td>
<td>Visual Predictive Check</td>
</tr>
<tr>
<td>$V_{\text{max}}$</td>
<td>Maximal Velocity</td>
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HISTORY OF PEDIATRIC DRUG DEVELOPMENT AND ETHICAL DILEMMAS

Throughout the early 19th and 20th centuries, medicinal chemists and charlatans peddled cures for a variety of ailments. Most of these medications lacked efficacy while others were blatantly toxic. Over the years, these toxicities spurred the United States government to pass legislation to regulate the burgeoning pharmaceutical industry. Some of the more tragic events from unregulated medications involved the deaths of children. For instance, Mrs. Winslow’s soothing syrup was marketed as a medication to calm fussy children. However, it contained high levels of alcohol and morphine derivatives and, ultimately, many children passed away after ingesting it. Unfortunately, this was not the last event involving children’s’ deaths. In 1937, a company dispatched over 200 gallons of elixir sulfanilamide, which was administered to children, leading to renal failure in many children who ingested the drug because it contained diethylene glycol. In 1962, thalidomide was approved as a sleeping pill in Europe and caused severe birth defects. All of these tragedies led to amendments in the federal law that would allow the government greater control over the drug development process [1]. These amendments required drugs to be branded and advertised correctly, and also demonstrate safety and efficacy. It is remarkable that despite the relatively high rate of death and retardation in children due to these toxic medications, regulatory rules did not mandate companies to test their products in children until the early 21st century.
However, the conceptualization of children as a special population began in the mid-19th century. Abraham Jacobi, famously known as the “father of American pediatrics”, practiced medicine in New York City in the late 19th century. While the array of medical specialties was not as extensive as it is today, Dr. Jacobi became the premier doctor who treated children. He was the first doctor to open a free pediatric clinic in America and raise awareness of pediatrics by teaching new doctors and becoming one of the first professors of pediatrics [2]. Contrary to the belief that children required smaller doses since they were just smaller people, his teachings stressed the importance of pediatrics as an independent population with different disease etiologies that required unique dosing [3].

Many reasons could be cited for the slow response to national tragedies and Dr. Jacobi’s teachings. One prominent case is the clinical trials conducted at Willowbrook State School in New York, which housed children with moderate to severe mental retardation. Over the span of approximately two decades, these children were enrolled in hepatitis A clinical trials in which some participants were injected with the active hepatitis A virus [4]. Along with other unethical trials such as the exploitation of poor African American men in Tuskegee experiments [5], the Willowbrook studies helped to develop the idea of informed consent and the core values of ethics. A more recent example of an ethically contentious study involved observing the effect of lead on children living in homes with lead paint in Baltimore. This study, conducted by the Kennedy Krieger institute, led to a lawsuit that resulted in an increase in protective measures in pediatric clinical trials in Maryland [6].

Pediatric patients are undoubtedly a vulnerable population. Before approving a pediatric clinical trial, an Institutional Review Board (IRB) must carefully consider the risk-to-benefit ratio, the discomfort of the trial to the child, the volume of collected blood required for the study, and who is authorized to give informed consent for the study. It is now well known that pediatric populations exhibit significant differences in pharmacokinetics (PK). Drugs did not undergo pediatric studies prior to the late 1990s, leaving a significant dearth of dosing
information in newborn and pediatric patients for drugs that have not been approved for this population. Furthermore, it has been observed in clinic that some drugs have higher dose requirements in children versus adults, which could make children vulnerable to under-dosing [7, 8]. As a result, scientific attitudes shifted the belief that not performing pediatric clinical trials is also unethical.

The first regulatory document, the Food and Drug Administration Modernization Act (FDAMA), that involves pediatric research, was passed in 1997. FDAMA encouraged pediatric clinical trials with an economic incentive of an extended 6-month exclusivity on brand name drugs. In 2002, the Best Pharmaceuticals for Children Act was enacted to allow the FDA to request pediatric clinical trials. In 2003, the Pediatric Research Equity Act (PREA) was enacted which stated that pediatric clinical trials were mandated if children were likely to use the new drug [9]. As of November 2014, over 500 labeling changes have been made due to these key pieces of legislation in the United States [10]. Global regulatory bodies, such as the European Medicines Agency (EMA), have followed a similar path to protect children from the events described above [11].

Despite these successes, there are still significant obstacles to performing pediatric clinical trials. Besides ethical concerns, one major challenge is selecting the optimal dose. Many pediatric trials have failed due to narrow dose ranges selected, while successful trials tested a wide range of doses with no overlap in doses [12]. Wider dose ranges also require a larger sample size, which makes it challenging since a large number of pediatric subjects need to be recruited for such studies. To improve the clinical trial outcomes, novel techniques are required for clinical trial design and dose range selection [8]. This introduction will cover traditional methods of dose selection, as well as newer techniques that are being employed to enhance trial design and dose selection in the clinic.
**ALLOMETRIC SCALING TO PREDICT PEDIATRIC PHARMACOKINETICS**

Conventional pediatric dose determinations used allometrically scaled adult doses to establish a pediatric dose. This method was adopted as drug development plans have consistently utilized this method to scale from non-clinical animal models to first-in-human doses. Even though significant differences are observed in the DNA sequence homology of drug metabolizing enzymes (DMEs) between these animal species and adult humans, allometric scaling provided a reasonable prediction [13]. Once the dose was established in human adults, allometry was then used to scale the clearance and dose to pediatrics. Three distinct methods were developed to scale the dose including scaling by body weight, body surface area, and body weight raised to a power function (Table 1.1) [14]. The exponent generally used in the allometric scaling of metabolic rates for children is 0.75 [14].

**Table 1.1 Variations in Equations for Allometric Scaling.**

<table>
<thead>
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<th>Allometric Scaling Method</th>
<th>Equation</th>
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<tr>
<td>Body-weight</td>
<td>$Clearance_p = Clearance_A \times \frac{BW_p}{BW_A}$</td>
</tr>
<tr>
<td>Body surface area</td>
<td>$Clearance_p = Clearance_A \times \frac{BSA_p}{BSA_A}$</td>
</tr>
<tr>
<td>Exponential Scaling</td>
<td>$Clearance_p = Clearance_A \times \left(\frac{BW_p}{BW_A}\right)^x$</td>
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An instance where allometry will be effective is when the drug is administered intravenously and undergoes renal excretion, minimal metabolism, and minimal biliary excretion. Specifically, allometry works well for intravenous dosing in children 12 years of age and older because their physiology is similar to adult physiology and absorption is not involved. However, many drugs do not fit this profile, so allometric scaling has substantial limitations.
It is not unusual for the predicted pediatric drug clearance from allometry to be substantially different than that observed in clinic. Allometric models can either under- or over-predict clearance for drugs depending on the class of the drug, its metabolic route, and the age of the patient. An inverse relationship is observed between the age and size of the child and prediction errors for dose and clearance. In other words, errors in clearance prediction increase as the gestational age or size of the children decreases [15].

In a review that examined the exponential scaling method, a span of values for the exponent applied \((x)\) ranged from 0.75 to 1.0. This review by Mahmood et al. determined that none of these exponents worked well for every drug. Even for the same drug, none of the exponents worked well for all ages that were reviewed, with errors as high as 800% [16].

In a report by Edginton et al., the clearance of 39 drugs in children were predicted by body weight and body surface area scaling using two different types of simulations [17]. The first simulation used a mean adult clearance and the second simulation used a distribution of adult clearances for each drug. The authors concluded that allometric scaling predicted clearance well for children 6 years of age and older. Despite this conclusion, celecoxib, leflunomide, nelfinavir, pantoprazole, quietiapine, and other drugs metabolized by hepatic DMEs exhibited larger prediction errors between observed and predicted clearances.

In a follow-up review, Mahmood et al. investigated the previous method along with five additional methods [18]. Included in this analysis was a maturational model that accounted for maturation of clearance as a function of age. This model was described by the following equation:

\[
Cl = Cl_{\text{std}} \times \left(\frac{BW}{70kg}\right)^{0.75} \times \frac{PMA^n}{PMA^n + (Cl_{50\%})^n}
\]

Where \(Cl_{\text{std}}\) is the average population clearance, PMA is post-menstrual age in weeks, \(n\) is the hill coefficient, and \(Cl_{50\%}\) is the age that normalized clearance at 50% of the maximum value [18]. The results showed that exponential scaling with a fixed exponent of
0.75 had the highest prediction errors. In contrast, the maturational model had an average prediction error of approximately 35% with a range between 7 – 117%. This review clearly indicated that maturational changes need to be accounted for when predicting clearance and doses for pediatric patients. A limitation of this analysis was that only six drugs were considered. It is difficult to extrapolate this model to a wider array of drugs, since in the review by Edginton et al., larger predictive errors were seen with drugs that are metabolized by hepatic DMEs.

Overall, allometric scaling assumes that all organs and enzyme function increase proportionally with body size and, therefore, with age. Notably, all of these methods fail to include hepatic or intestinal enzyme ontogeny, polymorphisms and physiologic developmental changes in renal function, gastrointestinal (GI) function, organ volumes, blood flows, and total body composition.

MATURATION OF PEDIATRIC PHYSIOLOGY

Developmental changes from the fetal period into adulthood include differences in the activity and abundance of DMEs and transporters, changes in GI function, adipose composition, water composition, organ sizes, and organ blood flows. All of these factors affect clearance and distribution of drugs within the body. This section will describe a few of the major age-dependent changes that affect drug PK and its variability. This section will introduce physiological factors prioritized by the magnitude of their effect on pediatric PK.

Clearance

Total body clearance is described by the following equation:

\[ Cl = Cl_{\text{metabolic}} + Cl_{\text{renal}} + Cl_{\text{other}}. \]
This equation establishes that the two main organs of clearance are the liver and the kidneys. Both systems undergo distinct maturation from the fetal period into adulthood. Many traditional drug development processes that utilize standard in vitro and in vivo screening methods and extrapolations do not take these developmental changes into account.

The liver is the main organ of clearance that is responsible for oxidative metabolism. It undergoes significant changes during childhood development. The biliary canals begin to form when the embryo is 18 days old and continue to mature throughout gestation. Bile ducts are not completely formed until four weeks after birth [19]. After birth, the size of the liver continues to grow with age. However, hepatic growth is not proportional to body size. Liver size, as a ratio of liver size to total body mass, reaches a maximum ratio between 1-4 years of age, which is then followed by a gradual decrease (Figure 1.1) [20]. Hepatic DMEs undergo rapid changes from the fetal period into infancy, childhood, adolescence and finally adulthood. The total content of all cytochrome P450 (CYP) enzymes in fetal liver (30-60% compared to adults) increases at birth and approaches adult levels by 10 years of age [21]. However, significant changes in CYP enzymes occur throughout this period.

The development of the renal system also begins during gestation [22]. However, the main function of the kidneys at this stage is to maintain amniotic fluid levels [23]. Kidneys are not truly functional until the last trimester before birth. After birth, the kidneys continue to...
develop until they reach mature filtration values by approximately 1-2 years of age (Figure 1.2) [24]. This maturation function applies to only full term neonates. Glomerular filtration rates of premature neonates is approximately 30% compared to those of full term neonates at 2 weeks of life [25]. The renal function of premature neonates takes years to reach the same milestones as those observed in children born at full term.

**Distribution**

Physiological factors that affect drug distribution also change with age. Body composition changes from the neonatal period into adulthood. Changes in body composition continue as adults enter the geriatric phase. Total blood volume and total fat content increase with age, whereas the reverse is true for total body water [26]. For instance, the total body water (as a percentage of body weight) decreases from over 70% at birth to 50% in adults. The primary changes are in the ratio of extracellular to intracellular fluid compartments. In neonates, body water is split evenly between these two compartments; whereas in adults, the water content is two-fold higher in the intracellular compartment compared to the extracellular compartment [27]. These differences affect the disposition and, ultimately, the clearance of drugs from the body.

Cardiac output increases with childhood growth and development (Figure 1.3) [28]. Despite this proportional increase in cardiac output, the percentage of the cardiac output to individual organs changes with age. Unique developmental patterns are seen with blood flow to each organ. For instance, blood flow to the brain is highest between the ages of two and 10 years of age, and then decreases until adulthood. Liver blood flow increases steadily with a small peak in the first in the first f
few years of life. Differences in blood flows will affect how drugs distribute within the body after both oral and intravenous administration.

Protein binding is another major factor that influences the distribution of drugs. Both albumin and α1-acid glycoprotein are present at a lower levels in premature and full term neonates compared to adults [27]. Neonates and infants on average have 20% and 10% less albumin than adults, respectively [29]. One study, which examined the binding of diazepam in adults and neonates, showed that neonates had an unbound fraction 4-fold higher than adults indicating that the lower concentrations of albumin and plasma proteins significantly affect the binding of drugs in newborns [30]. Another study demonstrated that acetylsalicylic acid had lower binding in neonates compared to adults. Protein binding remained higher in adults even when adult plasma was diluted and bilirubin was added to bring the concentration to neonatal levels [31]. These results demonstrate that neonates have lower binding due to lower plasma protein expression, but also due to lower affinity of plasma proteins for drugs.

**Absorption**

Differences in the GI tract start to occur at the gastric level. In premature and full term neonates, the pH of the stomach is higher. Lower acidity in the stomach enhances the absorption of acid-labile drugs, such as penicillin [32]. Gastric emptying time also decreases with age. Preterm neonates had higher retention in the first few hours after birth compared to term neonates at the same post-natal age [33]. These data indicate that gestational age affects gastric emptying time which does not fully mature until approximately 6-8 months after birth [34].

Absorption of the majority of drug and nutrient occurs in the small intestine. As in the case of the liver, the small intestine begins to form within a few weeks of gestation with the brush border having some functionality by the end of the first trimester [35]. The motility of the small intestine is slower in neonates compared to adults [34]. While it may seem that a
slower rate of peristalsis could increase absorption, another study showed that absorption rates in neonates who were given the GI stimulant, metoclopramide, were slower compared to the absorption rates in infants [36]. While there is a dearth of information about the development of the intestine, it is presumed that the intestine is mature by approximately four months of age [24]. In addition, bile salt concentrations in the intestinal lumen are lower than in adults, which affect the solubility of many drugs [34]. Furthermore, the intestine is the first-line barrier for entry of exogenous compounds into systemic circulation. Little is known about the innate immunity of newborns throughout childhood, so the intestine is vital to protecting children [37].

Absorption and permeability issues are important factors in oral drug delivery to premature infants. Due to developmental delays, premature neonates can experience problems with suckling and feedings. In order to deliver sufficient nutrition to these children, parenteral nutrition or gastrostomy tubes are utilized [38, 37]. These types of feedings can increase the risk of necrotizing enterocolitis [37]. Furthermore, decreases in carrier-mediated absorption and increases in permeability affect the absorption of nutrients and compounds across the intestinal barrier [39]. Overall, the small intestine is underdeveloped in premature neonates, which can affect absorption of drugs differently depending on their physicochemical properties.

It should be noted that there are also differences in skin thickness and body surface area which could affect topically administered medications [24]. However, for the purpose of this research project, oral and intravenous drug delivery were the main focus.

THE ONTOGENY OF DRUG METABOLIZING ENZYMES

Hepatic clearance accounts for the clearance of almost 75% of the top marketed drugs, and about 75% of hepatic clearance is due solely to oxidative metabolism by phase I enzymes [40]. While there are many different families of enzymes and even more isoforms within each family, a few families metabolize a majority of drugs. Contributions of the major
cytochrome families includes 46% by CYP3A, 28% by CYP2C, 12% for CYP2D6, and 9% by CYP1A (Figure 1.4) [40]. Families of enzymes exhibit different maturational profiles [41, 24]. These differences in ontogeny can affect how drugs are metabolized in children, especially when two enzymes with different maturation profiles are involved. Preliminary studies utilized western-blot analysis and differential metabolism in an attempt to quantify the expression of these DMEs. While these methods are prone to generate confounding data due to antibody cross-reactivity and a lack of selectivity and specificity, they generated the first estimation of enzyme expression.

**CYP3A**

The CYP3A family is the most abundant DME in the liver [42]. CYP3A4 mediates oxidative metabolism of many drugs on the market [43]. Despite the significant role it plays toward hepatic drug metabolism in adults, it is virtually absent in fetal tissues where the dominant CYP3A isoform is CYP3A7. Gene expression of CYP3A7 is high throughout fetal gestation, is downregulated at birth, and by approximately 1 year of age, is virtually undetectable. This downregulation of CYP3A7 expression corresponds to a switch from CYP3A7 to the CYP3A4 isoform, which is upregulated over time until it reaches adult expression levels (Figure 1.5) [44]. CYP3A5 is genetically polymorphic and African
Americans are found to express this enzyme at higher levels compared to other ethnicities [45, 41]. Due to the higher variability in CYP3A5 expression, a unique ontogeny profile of this enzyme has yet to be elucidated [46, 45].

Differences in the activities of these isoforms during development contribute to altered PK between adults and children. Midazolam and testosterone are two probes that are recommended for CYP3A activity. *In vitro* and *in vivo* studies have demonstrated a lower metabolic activity of CYP3A7 compared to CYP3A4 [47-49]. It has been reported that metabolic activity of CYP3A7 is greater than 5-fold lower compared to that of CYP3A4 [50]. Furthermore, it is possible that these two isoforms of CYP3A display varying substrate affinities [49]. This difference in affinity and specificity was revealed during CYP3A7-mediated DHEA metabolism during which CYP3A7 converted DHEA to the 16α-OH metabolite preferentially over the 7β-OH metabolite [44]. Overall, CYP3A7 displayed a lower metabolic activity compared to CYP3A5 and CYP3A4 [51].

**CYP2C**

CYP2C9 expression in the fetus is low during the first two trimesters of pregnancy. Expression levels begin to increase gradually during the third trimester, but reach adult levels within a week or two after birth [52]. CYP2C9 activity peaks within the first five months after birth and gradually declines to adult levels [52]. Dosing requirements for phenytoin is 14 mg/kg/day for infants (6 months to 3 years) and decreases to 8 mg/kg/day in children (4 years of age and older). This phenomenon was attributed to a higher $V_{\text{max}}$ [24, 53]. However, enzyme expression data do not explain this higher weight-based dosing requirement in infants compared to children and adults.

CYP2C19 is also expressed at low levels during fetal gestation, but is up-regulated at birth [52]. During the first 5 months of life, CYP2C19 increases linearly in expression and activity. Although there is a positive correlation between age and CYP2C19 expression, there is a significant variability in the expression levels of this enzyme at birth, with
expression as high as adult levels in some neonates soon after birth. Polymorphisms in the CYP2C19 gene code for poor metabolizers (by CYP2C19*2 which is a nonfunctional allele of CYP2C19) and ultra-rapid metabolizers (by CYP2C19*17). Polymorphisms in the CYP2C19 pathway affect clearance of most proton pump inhibitors, clopidogrel, voriconazole, and other drugs [54-56]. This hypervariability in CYP2C19 expression needs to be taken into consideration, in addition to its ontogeny, during pediatric drug development programs.

Despite the positive correlation between age and the expression of CYP2C19, clinical observations have described lower bioavailability of drugs in children (2-12 years of age) compared to adults. These differences in bioavailability have been observed for drugs such as proton pump inhibitors, nelfinavir, and voriconazole [7, 57-62]. In vitro experiments utilizing pediatric liver microsomes established that the contribution of CYP2C19 to voriconazole metabolism was higher in children (2-10 years of age) compared to adults [63]. This discovery led to the hypothesis that higher hepatic clearance accounted for the lower bioavailability of voriconazole in children compared to adults. While this study also examined CYP2C19 protein expression, the variability in the western blots hindered a definitive determination of higher protein expression of this enzyme in children.

**CYP2D6**

The ontogeny of CYP2D6, that was determined by immunochemical inhibition of the enzyme, suggested that neonates had very low expression of CYP2D6 which steadily increased over the first few months of life [64]. Microsomal activity analyses showed that catalytic efficiency increased significantly from newborns into adulthood. These results were further substantiated in another in vitro experiment using the probe substrate dextromethorphan. Results from this experiment revealed that with a 5 µM concentration of fetal human liver microsomes (HLM), there was no detectable formation of dextrorphan, the active metabolite of dextromethorphan. With 50 µM microsomal protein, a 17-fold lower
activity was observed with fetal HLM compared to adult HLM [65]. A full ontogeny profile of CYP2D6 was not determined until dextromethorphan was administered to infants during the first year of life. The ratio of parent-to-metabolite showed that metabolism of dextromethorphan via CYP2D6 was similar from the first few weeks after birth to 12 years of age [66]. Further, these results showed that polymorphisms in CYP2D6 were consistent across these age groups indicating that CYP2D6 undergoes rapid maturation to adult levels within the first month after birth.

**CYP1A2**

CYP1A2 is an important hepatic enzyme in premature neonates since it is responsible for over 90% of caffeine metabolism. Caffeine is administered to premature neonates as a first-line therapy for apnea and bradycardia [41]. CYP1A2 expression is negligible in the fetal period and undergoes gradual increase. Development of CYP1A2 activity takes anywhere between 4-10 months after birth [41, 67, 24]. Limited data are available on quantitative gene or protein expression of CYP1A2.

**Flavin-containing Monooxygenases**

Flavin-containing monooxygenases (FMO) oxidize nitrogen- and sulfur-containing compounds. As with CYPs, FMOs have different isoforms, including FMO1, FMO2, FMO3, FMO4, and FMO5. Using quantitative real-time polymerase chain reactions (qRT-PCR), gene expression of each of these isoforms was established in various tissues [68]. FMO1, FMO3, FMO4, and FMO5 were located in the liver, with FMO1 and FMO3 being the most highly expressed hepatic isoforms in fetal and adult liver, respectively. FMO1, FMO2, and FMO4 were expressed in the kidney and FMO2 was located primarily in the lung. A 15-fold greater expression of FMO3 was observed in the adult liver compared to the expression of FMO1 in the fetal liver, suggesting that the ontogeny of hepatic FMO isoforms exhibits a similar developmental pattern to that of CYP3A.
Protein expression of hepatic FMO1 and FMO3 was examined in another report (Figure 1.6) [69]. Results from this study corroborated gene expression data that FMO1 is the predominant fetal isoform that is expressed at highest levels during weeks 8-15 of gestation. These levels of enzyme expression decline during fetal development until they are undetectable by the first week after birth. FMO3, the adult hepatic isoform, then replaces FMO1 as the predominant isoform by approximately 1-2 years of age. However, there was only a 3-fold difference between hepatic FMO1 to FMO3 in contrast to the previous report. This dichotomy led to questions about whether the fold-differences between gene and protein expression were due to assay conditions or post-translational modifications. It also raised questions about which assay and report was a better estimate of actual expression.

FMO isoforms exhibit distinct substrate specificity [70]. FMO3 metabolizes trimethylamine at a rate 100-fold greater than FMO1. Similarly, FMO3 also metabolizes benzydamine, another probe of FMO, at a rate 6-fold greater than FMO1 [71]. For most drugs, such as nicotine and cimetidine, FMO3 is thought to be the predominant enzyme due to its higher expression and greater metabolic efficiency for these substrates [72].

**Intestinal Oxidative Metabolism**

The intestine is the first barrier to admission into the systemic circulation that exogenous compounds encounter after oral administration. The small intestine is the site of highest absorption and contains a variety of protective mechanisms. For instance, the intestine has a high level of transporters, both efflux and uptake, to help regulate the passage of compounds [73]. The intestine also has DMEs that regulate the absorption of
medications. CYP3A4 is the most highly expressed oxidative enzyme located within the small intestine followed by CYP2C9 and CYP2C19 [43, 74-78]. In adults, the regulation of intestinal CYP2C enzyme expression was not correlated to hepatic CYP2C regulation [79], which limits the extrapolation of regulation by hepatic DMEs to intestinal DME expression and functional activity in adults.

The ontogeny of DMEs in the small intestine is an area that is in need of significant research. Since the intestine constitutes only one component of first-pass metabolism, it is difficult to assess the ontogeny of all intestinal DMEs using in vivo studies as the data could be confounded by hepatic metabolism and overlapping metabolic routes. However, one small clinical study utilized oral midazolam, a prototypical intestinal CYP3A probe, to study first-pass metabolism and clearance in preterm neonates [47]. The 15 preterm neonates included in the study showed a lower midazolam clearance that was hypothesized to be due to lower CYP3A intestinal activity. The challenges associated with in vitro studies include the collection of healthy intestinal tissue from neonates to children (2-12 years of age). These procurement difficulties present an obstacle to determining the ontogeny of protein expression and functional activity of pediatric intestinal DMEs. However, one study has reported the gene expression of CYP3A in neonates and children using qRT-PCR to [80]. The results suggest that children (1 month to 6 years of age) expressed CYP3A4 and CYP3A5 at higher levels compared to children 6 years of age and older. One limitation in this study was the lack of a comparison to expression of the CYP2C family, which is the next most prominent intestinal DME, and a lack of information on differences in activity in relation to the differences in gene expression. Additional information on the activity and expression of DMEs in the pediatric intestine is needed to confirm the trend of higher levels of CYP3A expression in the small intestine of children, and its implication on pediatric intestinal first-pass metabolism. Due to the limited supply of fresh, healthy pediatric intestinal tissue, an innovative in vitro method must be developed to fill this knowledge gap.
The Ontogeny of Drug Transporters in Pediatric PK

While the major focus of this dissertation was on drugs that are completely cleared by oxidative metabolism, it should be noted that transporters play an equally important role in drug clearance. Additionally, the ontogeny of these drug transporters could be particularly important for drug-drug interactions (DDI) and drug-induced liver injury in neonates and children. Transporter-related changes with maturation have only begun to be studied [81]. A level of complexity associated with the study of the ontogeny, regulation, and function of transporters is their co-expression and overlapping substrate specificity [82, 83]. This overlap leads to redundancy in function. Furthermore, unlike the expression of DMEs, which are mainly confined to the liver and intestine, transporters can be found throughout all organs in the body [73]. This widespread expression of transporters adds another level of intricacy to correlating ontogeny and whole-body PK. Finally, unlike the DMEs, only the transporter proteins that are on cell membrane can modulate the movement of drug molecules in and out of the cell. Hence, quantitation of total transporter protein does not reflect its functional activity. It is expected that the ontogeny of transporters will play as much or greater role in defining drug PK and therapeutic outcomes as DMEs. Therefore, the ontogeny of transporters has not been covered in this section.

CLINICAL TRIAL LIMITATIONS

Despite all the challenges associated with pediatric clinical trials, they are nonetheless being conducted. Many of these trials are conducted without accounting for the ontogeny of the metabolic routes. One prominent example of this was the pediatric clinical trials for voriconazole, which is a known CYP3A4 and CYP2C19 substrate. Over the span of 10 years, four different clinical trials for voriconazole in pediatric patients were conducted. Each trial attempted to explain the hypervariable PK, higher clearance, and lower bioavailability of voriconazole in children (2-12 years of age) compared to adults [57, 58, 60, 61]. However, none of these trials were able to accurately describe why children exhibited...
linear voriconazole PK over a larger dose range whereas adults showed non-linear PK when the drug was administered over a very narrow dose range, even when the doses were normalized to weight.

Pediatric clinical trials have been conducted in an attempt to explain PK variability due to genetic polymorphisms. Clinical trials that investigate a specific genetic polymorphism require a large cohort to prove an effect. To gain approval, pediatric clinical trials need to demonstrate benefit to the child. Therefore, assessing the effect of genetic polymorphisms on drug PK is challenging due to the impracticality of recruiting pediatric patients to investigate the effect of a specific allele-carrier on the PK of drugs. It was also challenging for pediatric clinical trials that attempted to assess genetic polymorphisms to prove the effect of the polymorphism on PK. One specific example is a pediatric study that included CYP2C19*2 poor metabolizers of pantoprazole, a proton-pump inhibitor. This study was able to recruit only two patients with the allele of interest, and therefore, could make no conclusions about the effect of the polymorphism on pediatric PK of pantoprazole [84]. Age and ontogeny can mask the effects of genetic polymorphisms on drug PK, thus adding another layer of complexity to pediatric clinical studies [85]. Therefore, a new method is required where a large number of patients are included in the study to increase the power to assess the effect of genetic polymorphisms on pediatric PK.

**TOP-DOWN OR BOTTOM-UP MODELING**

Clinical trials are costly and challenging and need to meet rigorous requirements to gain approval from IRBs to test drugs in the pediatric population. On the other hand, allometric scaling failed to capture the range of developmental changes in the physiology and ontogeny of enzymes. So, old techniques combined with new computational methods were able to integrate changes in macro- and micro-physiology. Modeling methods have increased in popularity and importance over the past three decades (Figure 1. 7). The figure shows data retrieved from PubMed (http://www.ncbi.nlm.nih.gov/pubmed/) listing all of the
population PK and PBPK models that were published between 1988-2014 [A] and all of the pediatric population PK and PBPK models that were published between 1990-2014 [B].

Two types of whole-body modeling exist: top-down and bottom-up (Figure 1.8). Top-down models analyze clinical PK profiles to understand variability within the population. Of the top-down modeling techniques used, population PK analysis is the more established technique. Population PK analyses derive inter- and intra-patient variability from plasma concentration-time profiles generated for each individual within a clinical trial [86]. The advantage of population PK studies is the ability of the model to utilize sparse samples to generate population estimates of PK parameters. This method succeeds pediatric clinical trials since blood draws are limited due to small blood volumes in children, and therefore only a few plasma samples are usually collected [87]. Unlike allometric scaling, population PK evaluates pediatric plasma-concentration profiles for more than one covariate. These models enable detection of multiple covariates, such as gestational age, weight, or gender, to better understand the causes of PK variability. Population PK analyses are useful to identify covariates and determine effective doses for a variety of medications [88, 89]. Although one notable pediatric
population PK study predicted a dose range for voriconazole in children (2-12 years of age),
a follow-up clinical trial assessing these predictions revealed that even higher voriconazole
doses were needed as the doses determined by the population PK analysis were found to
be inadequate [58, 90].

There are a few significant limitations with population PK studies. First, the size of
the sample population used in the analysis can affect predictions. If a sample cohort is
analyzed, then any random chances in PK variability have a greater effect on the output of
the analysis. This limitation is amplified if the pediatric samples cover a large age range. As
previously mentioned, many maturational changes occur during childhood requiring that
children be binned into a specific age group [91]. Secondly, the PK predictions generated by
the model do not always extrapolate to other age groups [92, 93]. Thus, if the population PK
study only analyzes children within a narrow age range, then the results cannot always be
applied to younger or older patients that are at a different stage of maturation. Finally,
population PK studies cannot determine the cellular mechanisms for differences in PK.

PBPK models are the other PK modeling technique that can be used to generate
population predictions. PBPK models can be split into top-down and bottom-up approaches.
Both PBPK model types require the integration of clearance mechanisms and
physiochemical properties of the drug into the model. These models offer the advantage of
allowing users to test how different input parameters could affect the predicted PK in
simulated patients. This feature provides the ability to understand cellular and drug
characteristics that most affect the model.

The first type of PBPK model is the top-down approach. Similar to population PK
analyses, top-down PBPK models use clinical clearance values to simulate individuals. For
this reason, this type of PBPK model has the same limitations as population PK models.
These models integrate clearance as a whole-organ clearance or a back-extrapolation from
in vivo clearance. These clearance parameters are specific to the age-range studied and do
not necessarily represent younger or older individuals [94]. This limits the ability of the model to extrapolate predicted PK to other pediatric populations. Moreover, top-down PBPK models must also rely on external validation sets. Without these external validation sets, the model predictions are validated based on the same data set that was used to develop the model. An example of the challenge in modeling with \textit{in vivo} clearance is shown by Yang et al. In their PBPK model, two pediatric data sets were discarded due to failure to correctly model methylphenidate in adolescents [95].

The bottom-up method is the second approach that can be used for PBPK modeling. Bottom-up PBPK models rely on \textit{in vitro} data to calculate clearance within the model. This method generates clearance by scaling the \textit{in vitro} data with cellular enzyme expression and ontogeny. These input parameters are incorporated into a model that also includes age-dependent physiological data. Since this type of model utilizes intrinsic clearance by a specific enzyme, changes can be simulated across a wide range of ages. In addition, it can also predict DDI and the fraction of drug metabolized by a specific isoform [96]. This method relies heavily on enzyme expression and the ontogeny of individual enzymes and transporters since \textit{in vitro} data are utilized for generating predicted clearance. Without accurate experimentally derived abundance and ontogeny data, the model will not be able to predict PK parameters. To date, bottom-up pediatric PBPK models have exclusively employed data generated with recombinant enzymes. When data generated with adult HLM were utilized in a bottom-up PBPK model to predict the PK of morphine, the model under-predicted morphine clearance by over 60% in children [97], which clearly demonstrates a need for improving the bottom-up pediatric PBPK models. It has been shown that oxidative enzymes in pediatric tissues can metabolize a drug at a different rate and with a different percent contribution compared to adult tissues [63]. Thus, scaling intrinsic clearance from adult HLM introduces errors that potentially could be overcome by using pediatric HLM.
CURRENT DIRECTIONS AND DISSERTATION PROJECT

In March 2012, the FDA Clinical Advisory Committee concluded that PBPK modeling should be incorporated into pediatric drug development programs [98]. PBPK models are a useful tool to evaluate drug disposition and clearance in adults. Since its acceptance by the FDA, the use of PBPK modeling in pediatric drug development is rising, with specific emphasis on understanding the role of DMEs and transporters in defining the PK of drugs [99-104]. This modeling approach aids in optimizing trial design and dose selection, and permits drawing fewer samples from children, whose smaller blood volumes limit the use of comprehensive serial sampling. It also requires fewer subjects, thereby reducing the cost, and more importantly exposure of this vulnerable population to experimental drugs.

Bottom-up PBPK modeling is the most promising approach to determine pediatric PK without conducting extensive clinical studies involving children. However, previous research suggests that significant refinement is necessary. The overarching strategy developed for this dissertation research has certain unique aspects that have been applied to the bottom-up PBPK modeling for the first time (Figure 1. 9). Since these are the first set of studies being pursued to test the novel approach, the scope of the research was limited to drugs, voriconazole and silidenafil, that are predominantly cleared by oxidative metabolism. The strategy involves characterizing metabolic clearance in an in vitro system from adult tissues. Then a bottom-up PBPK model is constructed and refined using an iterative process until the predictions of the model converge with the observed clinical PK values in the adult population. Once validated, this process is adapted for children using in vitro data generated from pediatric tissues and then a preliminary validation of the pediatric model is made with available pediatric clinical data.
PROJECT RATIONALE AND SPECIFIC AIMS

The objective of this research was to develop a translational technique to evaluate how age-dependent differences in DMEs affect the PK of drugs. The approach involved studying differences in metabolism between adults and specific pediatric populations using hepatic and intestinal tissue, and correlating these in vitro data to in vivo clearance and bioavailability by developing a PBPK model so as to predict PK in target pediatric populations from PK in the adult population.

In order to validate this novel approach, two drugs, voriconazole and sildenafil, were chosen based on their exclusive clearance through oxidative enzymes. First, a retrospective bottom-up PBPK model was created for voriconazole, and the model was validated against clinically observed data on voriconazole from pediatric trials in children 2-12 years of age. After validating this strategy in children, this method was applied prospectively to premature neonates receiving sildenafil to not only determine how the ontogeny of DMEs affects sildenafil PK, but to extend this technique to the neonatal population.

The central hypothesis of the proposed research is that (i) age-dependent changes in the pattern and extent of expression in CYP and FMO enzymes play a pivotal role in determining the clearance and exposure of sildenafil and voriconazole in the neonatal and pediatric populations, respectively; (ii) clearance and bioavailability of drugs cleared predominantly by oxidative metabolism can be predicted in neonates and children using a PBPK model based on relevant in vitro metabolism data.

AIM 1. Characterize the oxidative metabolism of sildenafil by hepatic microsomes from the neonatal population, and determine the expression of relevant oxidative enzymes (e.g. CYP3A, CYP2C, and FMO) in these tissues.

1.1 Determine the rate of sildenafil metabolism by CYP3A, CYP2C, and FMO (intrinsic clearance $V_{\text{max}}/K_m$) by hepatic microsomes in neonatal subjects.

1.2 Assess the relative contribution of individual enzymes to sildenafil clearance.
1.3 Establish CYP3A, 2C, and FMO gene and protein expression in neonatal hepatic samples by qRT-PCR and quantitative mass spectrometric analyses, respectively.

**AIM 2.** Determine the gene and protein expression of CYP3A, CYP2C9, CYP2C19, and FMO enzymes in intestinal tissue, as was done with hepatic tissue in children (2-10 years), and optimize an *in vitro* method to study intestinal intrinsic metabolic clearance of voriconazole.

2.1 Determine CYP3A4, CYP2C, and FMO gene expression in hepatic and intestinal samples by qRT-PCR.

2.2 Quantify protein expression of the aforementioned enzymes in hepatic tissue by quantitative mass spectrometric analyses.

2.3 Optimize an *in vitro* method to measure the rate of voriconazole metabolism utilizing a small sample of pediatric intestinal tissue.

**AIM 3.** Develop PBPK models using *in vitro* data from Aims 1 and 2 to describe the PK behavior of sildenafil and voriconazole in neonates and children ages 2-10, respectively.

3.1 Develop a PBPK model to determine the clearance of sildenafil in neonates based on available clinical data, known and experimentally derived ontogeny of organs and enzymes, as well as *in vitro* metabolism data generated in Aim 1.

3.2 Develop a PBPK model to determine the oral bioavailability of voriconazole in adults and children based on available clinical data and *in vitro* metabolism data generated in Aim 2.

3.3 Validate the results from Aims 3a & b by comparing predicted outcomes to observed pediatric clinical data.
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CHAPTER 2

A Physiologically Based Pharmacokinetic Model for Voriconazole Disposition Predicts
Intestinal First-pass Metabolism in Children

OVERVIEW

Background & Objectives

The effect of ontogeny in drug metabolizing enzymes on pediatric pharmacokinetics
is poorly predicted. Voriconazole, a potent antifungal, is cleared predominantly via oxidative
metabolism and exhibits vastly different pharmacokinetics between adults and children. A
physiologically based pharmacokinetic (PBPK) model was developed integrating hepatic in
vitro metabolism data with physiological parameters to predict pharmacokinetic parameters
of voriconazole in adult and pediatric populations.

Methods

Adult and pediatric PBPK integrated voriconazole physicochemical properties with
hepatic in vitro data into the models. Simulated populations contained 100 patients (10 trials
with 10 patients each). Trial design and dosing was based on published clinical trials.
Simulations yielded PK parameters which were compared against published values and
visual predictive checks were employed to validate models.

Results

All adult models and the pediatric intravenous model predicted pharmacokinetic
parameters that corresponded with observed values within a 20% prediction error, whereas

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and is presented in the style of that journal.
the pediatric oral model predicted an oral bioavailability 2-fold higher than observed ranges. After incorporating intestinal first-pass metabolism into the model, the prediction of oral bioavailability improved substantially, suggesting that voriconazole is subject to intestinal first-pass metabolism in children, but not adults.

**Conclusions**

The PBPK approach utilized in this study suggests a mechanistic reason for differences in bioavailability between adults and children. If verified, this would be the first example of differential first-pass metabolism in children and adults.

**Key Points**

- Previous clinical trials have shown marked differences in voriconazole pharmacokinetic parameters between adults and children without providing a mechanistic basis for these disparities.

- The simulations using the PBPK models suggest that intestinal first-pass metabolism is an important factor that contributes to lower oral bioavailability of voriconazole in children compared to adults.

- This study provides a new paradigm for predicting therapeutic doses in children, in which adult *in vitro* metabolism are related to *in vivo* pharmacokinetics to create and validate a PBPK model and then adapted to utilize pediatric *in vitro* metabolism to predict pediatric pharmacokinetics.

- The most important use of this approach is to develop safe and effective pediatric dosing regimens, despite limited clinical data.
INTRODUCTION

Voriconazole (Vfend®; Pfizer, New York, NY) is a potent triazole antifungal commonly used in patients with life-threatening invasive fungal infections [1]. Since its approval in 2002, studies demonstrated that voriconazole improves survival and produces fewer severe adverse effects compared to amphotericin B, the original standard of care for invasive aspergillus infections [2], making it first-line treatment for patients of all ages. However, voriconazole displays wide variability and significant pharmacokinetic differences in clearance, exposure, and bioavailability between adults and children, with no consensus on pediatric doses that match adult exposures, thereby limiting successful therapy [3-6].

Voriconazole is extensively metabolized by the liver, with only 2% of the parent excreted in urine [7]. Oral bioavailability of voriconazole in adults is ~ 96%, while pediatric bioavailability is 45-66% [4, 6, 7]. Voriconazole exhibits 3-fold higher clearance in pediatric patients compared to adults, and over a narrow dose range of 3-5 mg/kg, it displays non-linear pharmacokinetics in adults but not in children suggesting saturation of metabolic enzymes [6, 7]. Developmental changes in these drug metabolizing enzymes (DMEs) have been implicated in the pharmacokinetic differences between children (aged 2-10) and adults [8-11]. In vitro studies in our laboratory revealed developmental changes in these DMEs showing that two cytochrome P450 (P450) enzymes, CYP3A4 and CYP2C19, and flavin-containing mono-oxygenase (FMO) 3 contribute to 50%, 35% and 15% of voriconazole metabolism in adults, respectively, versus 20%, 50% and 30% in children. Hepatic intrinsic clearance (Clint) scaled to in vivo clearance accounted for 80% of clinically observed values, suggesting that the above differences conferred the increased clearance of voriconazole in children [12].

Although scaling of intrinsic clearance values explains differences in voriconazole clearance between adults and children, it does not provide insights into differences in other pharmacokinetic parameters between these two populations. This study describes a
physiologically based pharmacokinetic (PBPK) model for voriconazole that integrates in vitro hepatic metabolism data, voriconazole physicochemical properties, and physiologic parameters associated with adult and pediatric populations. The models were tested against clinically observed data. The model predictions of pharmacokinetics in children led to a hypothesis that intestinal first-pass metabolism of voriconazole in children would explain its lower bioavailability in children compared to adults. PBPK modeling has proven to be useful in understanding mechanisms underlying the differences in pharmacokinetics of voriconazole between adults and children, and can be applied to predict the influence of drug-drug interactions, genetic polymorphisms, and other factors on voriconazole disposition in children.

MATERIALS AND METHODS

Model Structure

Metabolic assays to generate hepatic in vitro pharmacokinetic parameters, $V_{\text{max}}$ and $K_{m}$, for voriconazole from healthy adult and pediatric tissues were adapted from previously published work [12].

Initial models were created in Berkeley Madonna (version 8.3.18; University of California at Berkeley) to assess linearity. A perfusion-limited model (Supplementary Figure 2.1) incorporated physicochemical properties (Table 2. 1), with tissue distribution compartments based on published data [13]. Age-dependent physiologic characteristics were averaged between males and females [14, 15]. To assess linearity, multiple intravenous infusion doses of 3 to 6 mg/kg were simulated for both adult and pediatric populations and AUC was calculated using Phoenix WinNonlin (version 6.2, Certara). The proportionality of the models was assessed by dividing the AUC of 4-, 5-, and 6 mg/kg doses by AUC of the 3 mg/kg dose, and comparing it to the fold change in dose. Finally, clearance and bioavailability were assessed for these simulations.
Next, a population-based model was developed employing Simcyp (version 12.1; Certara) using a step-wise approach (Figure 2.1). A perfusion-limited model incorporating adult clearance values and physiologic characteristics was created first using adult intravenous infusion dosing of 3-, 4-, and 5 mg/kg infused over 1-hour and dispensed every 12 hours for 7 days. Fasting oral dosing was simulated for 200 mg dispensed every 12 hours for 7 days. Similarly, a pediatric model was created utilizing in vitro $V_{\text{max}}$ and $K_m$ generated from pediatric (2-10 years old) liver microsomes and the Simcyp Paediatric Simulator®. Pediatric intravenous infusion simulated doses of 4- and 6 mg/kg infused every 12 hours for 7 days at a maximal rate of 3 mg/kg/hr and oral dosing of 4-, 6- and 8 mg/kg dispensed every 12 hours for 4 days. Trial designs and dosing were based on published clinical trials and simulated populations contained 100 patients each (10 trials with 10 patients per trial) [5, 16, 17]. Simulations integrated the age ranges and male to female ratios recorded for each respective trial. Physiologic characteristics were predicted applying Method 1 (Poulin and Theil). Method 1 best describe unionized compounds in plasma, such as voriconazole, and predict compound distribution based on tissue-specific parameters [18, 19]. Physicochemical properties and absorption values, along with in vitro data, were combined into a full PBPK model using whole organ metabolic clearance. In vitro voriconazole metabolic rates generated are listed in Table 2.1 along with other pertinent parameters employed in model development [4, 7, 12, 20, 21].

**Model Validation**

Simulations yielded pharmacokinetic parameters which were compared against published values expressed as geometric means. First, models were validated by comparing the predicted pharmacokinetic parameters against observed pharmacokinetic parameters. Given the immense clinical variability in voriconazole pharmacokinetics in both the adult and pediatric population, differences <50% were considered acceptable. If the predicted parameters were within 50% of the observed values, then visual predictive checks were
employed to confirm that the model predicted observed concentrations and standard deviation. Concentration-time profiles from a food effect trial conducted in healthy volunteers and a trial in immunocompromised children were digitized using GetGraph data digitizer version 2.25.0.32. Observed voriconazole concentrations were superimposed on predicted mean and 90% confidence intervals. Lastly, a distributional analysis of hepatic and intestinal CYP enzymes from simulated trials was generated [22].

**Sensitivity Analysis**

Sensitivity analyses, utilized to assess factors that could influence model performance, were performed for gastric emptying time and small intestinal transit time, both of which could significantly influence pharmacokinetic parameters after enteral absorption. Both values were replicated with a 10-fold change in the mean to determine if either parameter significantly affected pertinent pharmacokinetic parameters of AUC and $C_{\text{max}}$.

**RESULTS**

**Simulations to Assess Linearity**

In Berkeley Madonna, non-linear behavior was predicted by the adult model as evidenced by greater than proportional increase in AUC (1.7-, 2.7-, and 4.2-fold) when the doses increased from 3- to 6 mg/kg (1.3-, 1.7-, and 2-fold, respectively). In contrast, the pediatric model displayed linear kinetics with proportional increase in AUCs (1.4-, 1.8-, and 2.3-fold, respectively), (Supplementary Figure 2.2). Predicted clearance was 3.1 and 6.0 mL/min/kg, which were within 25% and 11% of the published values of 2.0 and 6.7 mL/min/kg, respectively. Preliminary analysis of oral bioavailability yielded a prediction of 88% for adults and approximately 100% for children, showing appropriate prediction for the adults but over-prediction for children. Predictions in the adult and pediatric populations, using Berkeley Madonna, utilized average physiologic and *in vitro* parameters, and omitted ranges and standard deviations. Due to this limitation and the unusual preliminary result of
predicting nearly double the observed pediatric oral bioavailability, a population-based approach was deemed essential.

**Model Simulations in Healthy Adult Volunteers**

Initial population-based model development utilized standard Simcyp parameters from the healthy adult volunteer simulator. The model was customized for voriconazole (Figure 2. 2) based on specific hepatic *in vitro* metabolism and physicochemical properties (Table 2. 1). The pilot simulations tested both intravenous infusions and oral doses as described in the Methods Section. The output demonstrated a biphasic profile indicating a two-compartment model, consistent with published data [4].

Initially, simulations were used to test the model's capacity to describe voriconazole plasma concentration after multiple intravenous infusion doses in adults. Table 2. 2 lists pharmacokinetic parameters predicted by the model relative to corresponding published values. Model output for intravenous infusions generated a predicted clearance value of 2.4 mL/min/kg (range 1.0-4.8 mL/min/kg), agreeing well with the clinically observed clearance of 2.0 mL/min/kg, a 17% prediction error. Predicted $V_d$ ranged from 3.39-3.65 L/kg, which differed from the observed values of 4.6 L/kg by 20.7-26.3%. The prediction error for AUC and $C_{max}$ in adults increased from 2.6% to 82% and 2.6% to 39.7%, respectively, as the dose decreased from 5 to 3 mg/kg. This is due to the known inability of the model to simulate non-linearity of metabolism. For the 200 mg oral dose in adults, the predicted AUC and $C_{max}$ values corresponded well with observed values (Table 2. 3). The model predicted the mean oral bioavailability of 83% in adults, which agrees well with the clinically observed mean value of 87%. The predicted 90% confidence interval for oral bioavailability was 69-93%, matching the clinically observed range of 75-96% [23-25]. Thus, with the exception of AUC prediction at the low dose of 3 mg/kg, the errors for predictions of all pharmacokinetic parameters were within the accepted limit of <50%, indicating the validity of the model.
After validating the predictions of the pharmacokinetic parameters, the plasma concentrations of the intravenous infusion and oral simulations were overlaid on observed data for visual predictive checks. Figure 2. 3a shows plots of plasma concentrations as a function of time for 4 mg/kg dose, yielding good fit to observed plasma concentration. Despite differences in the visual predictive checks for an oral dose of 200mg, the 90% confidence interval of predicted plasma concentrations encompassed most of the variability (shown as standard deviation) observed in clinical trials in both dosing regimens (Figure 2. 3b). The model prediction of the individual plasma concentrations for oral dosing captures the concentrations at the early time points, but not the concentrations at later time points due to the software’s inability to simulate non-linearity. Despite this, the model accurately predicted the pharmacokinetic parameters, including AUC, $C_{\text{max}}$, and bioavailability, demonstrating that the adult model adequately describes voriconazole in the adult population.

**Initial Model Simulations in the Pediatric Population**

Using a similar approach to the adult model, a pediatric model was created by integrating appropriate *in vitro* data and physiologic parameters. The pharmacokinetic parameters generated by the pediatric intravenous infusion model are listed in Table 2. 2. The model predicted a clearance of 5.0 mL/min/kg (range of 1.3-12.3 mL/min/kg) compared to the clinically observed clearance of 6.7 mL/min/kg, representing a 25% error. The value for $V_d$ in adults was used, as pediatric-specific $V_d$ value is unavailable. The model predicted the values for AUC and $C_{\text{max}}$ that corresponded well to the observed values with an error of 2.5-32.3% and 1.0-22.4%, respectively. Simulated AUC of the 8 mg/kg dose was similar to pharmacokinetic parameters of the adult 4 mg/kg dose.

For the oral doses, the predicted AUC and $C_{\text{max}}$ were overestimated by more than 100% and 30%, respectively (Table 2. 3). Furthermore, the model over predicted the bioavailability by 24-82%, and the values within the 90% confidence interval were 60-94%,
nearly 2-fold higher than observed clinical values. The overestimates for AUC and bioavailability suggest that the initial model does not adequately account for the factors that contribute to poor oral bioavailability, e.g. intestinal permeability and/or first-pass metabolism, during the absorption phase.

The visual predictive checks for the pediatric population are shown in Figure 2. 4a-c represent the multiple intravenous infusions at doses of 4-, 6- and 8 mg/kg, respectively. These profiles show that the simulated mean concentrations for both doses yielded good fit to observed plasma concentrations. Conversely, the calculated profiles for oral 4- and 6 mg/kg doses (Figure 2. 4d and Figure 2. 4f, respectively) over predict plasma concentrations over the entire time period, indicating that the model does not account for all pediatric metabolism and/or absorption barriers, at early or later time points. However, the shapes of the predicted profiles resemble the shape of the profiles for the observed data, suggesting that the absorption parameters are appropriate.

**Incorporation of Intestinal Metabolism Improves Predictions of Oral Bioavailability in children**

Since the oral bioavailability of voriconazole in adults is nearly 90%, it is reasonable to conclude that first-pass metabolism is negligible/absent in this population. Despite accounting for the higher hepatic clearance of voriconazole, the pediatric model was unable to predict lower oral bioavailability of voriconazole compared to adults. Since T_{\text{max}} values were within 20% of the observed data, the absorption rate constant was ruled out as a problem in the model. Therefore, it was hypothesized that the lower oral bioavailability in children is due to intestinal first-pass metabolism. In order to test the hypothesis, a hypothetical intestinal whole organ clearance was incorporated into the pediatric model. A range of Cl_{\text{int}} values, based on hepatic V_{\text{max}} and K_{\text{m}} data from in vitro experiments, were tested. K_{\text{m}} values were retained in each simulation since values from adult and pediatric hepatic microsomal in vitro experiments were similar. The initial unchanged V_{\text{max}} was
subsequently reduced until simulated pharmacokinetic parameters fit the observed ranges (Supplementary Table 2.1).

Incorporating intestinal clearance into the model reduced prediction errors for AUC by ~70% and for $C_{\text{max}}$ by over 50%. Most importantly, the predicted values for oral bioavailability decreased from 82% to 51% for both doses, with a 90% confidence interval range of 27-76%. Thus, the predicted bioavailability better corresponded to the clinically observed bioavailability range of 44-66%. Finally, visual predictive checks of the refined pediatric model, shown in Figure 2.4e & Figure 2.4g, demonstrate that the simulated concentrations for both oral doses better predict the observed concentrations, with most of the observed data superimposing over the predicted values and the 90% confidence intervals incorporating observed variability.

**DISCUSSION**

Despite significant progress in understanding the ontogeny of drug metabolizing enzymes [9-11, 26], our ability to use this knowledge to predict the pharmacokinetic behavior of drugs is far from adequate. Voriconazole is commonly prescribed in vulnerable pediatric populations despite a lack of information on optimal dosing based on clinical evidence. Limited clinical data available for voriconazole disposition in children demonstrate that the drug is cleared ~3-fold more rapidly and has ~50% lower oral bioavailability in children compared to adults, with significant inter- and intra-individual variability. This type of information has led to therapeutic dose monitoring in children to achieve a concentration range of 1-5.5 µg/mL, and upward adjustment of doses from 4 mg/kg (adult dose) to 9 mg/kg to reach this range [27, 28]. Notably, these doses are higher than the recommended doses in regulatory documents for voriconazole. This approach must be replaced with a more rigorous approach to achieve safe and effective dosing for pediatric populations.

The allometric scaling performed by Yanni et al. showed that hepatic microsomal data, obtained using tissues from children (2-10 years of age) and adults, could predict the
striking differences in in vivo clearance of voriconazole between these two populations [12]. This work was able to show that differences in contribution and capacity of the enzymes allow children to metabolize voriconazole more efficiently, leading to the increased clearance. However, this work did not provide an explanation for the differences in the oral bioavailability. The PBPK model developed in the present study represents a further refinement by integrating the ontogeny of DMEs with changing physiologic parameters.

We demonstrate the proof of concept for this approach using voriconazole as a model drug because (i) clinical pharmacokinetic data are available for adult and pediatric populations for intravenous and oral doses, (ii) the pharmacokinetic behavior in children is different from adults with respect to clearance and oral bioavailability, and (iii) the current approaches have not produced a satisfactory dosing strategy for children. In this approach, after confirming the dichotomy of pharmacokinetic behavior between two populations in Berkeley Madonna, Simcyp was utilized due to the availability of both a standardized adult and pediatric populations. Differences in physiology between these two populations were included in Simcyp; therefore only those parameters reflecting age-related changes in DMEs would be considered.

It has been shown previously that voriconazole is predominantly metabolized by CYP3A4, CYP2C19, and FMO3, all of which are highly expressed in the liver and are localized in the endoplasmic reticulum [29-31]. Therefore, metabolism of voriconazole by hepatic microsomes was an appropriate input parameter for the model. Voriconazole is a lipophilic compound of moderate size with no net charge at physiologic pH values, and thus its translocation across cell membranes should occur via passive diffusion without the assistance of transporters. The simulated output of the PBPK model corresponded with previously published pharmacokinetic data in adult subjects, which determined that voriconazole conforms to a two-compartment model [4]. Furthermore, the calculated AUC, $C_{\text{max}}$, and $V_d$ values of intravenous infusions in adult subjects were in agreement with
published clinical data demonstrating that the applied physiologic and metabolic
characteristics could predict clearances and that the liver is the main organ of elimination. In
the multiple oral dosing regimens, pharmacokinetic values were in accordance with those
published in the literature. The model was also able to predict bioavailability in adults [7].

However, the bioavailability of voriconazole predicted by the initial pediatric model
(60-94%) was strikingly different from the published values of 45-66% [5-7], and closer to
the high oral bioavailability observed in adults. Multiple clinical trials and population
pharmacokinetic analyses have not revealed the underlying mechanism that contributes to
the difference in bioavailability between children and adults. Many of these reports
implicated an increased ratio of “body mass to liver volume” in children compared to adults
as a potential reason for differences in clearance and disposition. In apparent agreement
with this hypothesis, the results reported by the Thakker laboratory confirmed that higher
hepatic $V_{\text{max}}$ conferred an increased clearance [12]. Simple allometric scaling of this work
determined that in vitro clearance parameters could accurately determine liver clearance.
Consequently, higher hepatic clearance was suspected to be the reason for the decreased
bioavailability. The initial pediatric model output included only hepatic metabolism of
voriconazole to confirm that an increased $V_{\text{max}}$ conferred higher clearance. The model
demonstrated that clearance in both adult and pediatric population agreed with published
reports, but it was unable to predict bioavailability in children, even after taking into account
the increased hepatic clearance and “liver to body mass” ratio. Since the pharmacokinetic
parameters of the intravenous model and the $T_{\text{max}}$ of the oral models were accurate, this
finding suggested that intestinal first-pass metabolism may contribute to the lower oral
bioavailability of voriconazole in the pediatric population.

One of the first reports to demonstrate that intestinal first-pass metabolism by
CYP3A4 plays a critical role in overall first-pass metabolism was conducted in a study by
Kolars et al. [32]. Prior to this pivotal study, cyclosporine metabolism was widely associated
with the liver, but not the intestine [32]. Their evidence, based on studies with anhepatic patients during liver transplant procedure, definitively demonstrated that the intestine can play a major role in first-pass metabolism. Although Walsh et al. speculated that developmental differences in intestinal enzyme activity could be responsible for the decrease in voriconazole bioavailability [5], experimental or theoretical evidence that intestinal first-pass metabolism of voriconazole reduces oral bioavailability in children is lacking. In order to model the intestinal first-pass metabolism, the apparent $V_{\text{max}}$ and $K_{m}$ generated from adult and pediatric human liver microsomes were considered as a starting point to establish a hypothetical intestinal $C_{l_{\text{int}}}$. It is reasonable to assume that voriconazole would have similar affinity for its metabolizing enzymes expressed in different tissues. Therefore, the $K_{m}$ was fixed, but the $V_{\text{max}}$ was adjusted to find the appropriate rate of metabolism in the intestines to account for the lower observed bioavailability. After incorporating a hypothetical intestinal metabolism into the pediatric PBPK model, the bioavailability of voriconazole was within the range published in the literature, suggesting that the intestine could play an important role in voriconazole metabolism.

The successful modeling of pediatric oral bioavailability that implicates intestinal first pass metabolism requires that the source of higher $V_{\text{max}}$ of children compared to adult be investigated. According to Fakhoury et al., increased CYP3A and P-gp expression in children allude to a marked difference in the “intestinal pie” compared to adult profiles reported by Paine et al. [33, 34]. To our knowledge, pediatric intestinal enzyme content and activity has not been thoroughly studied. So, abundance data from the adult and pediatric simulated populations ($n=400$ /group) were entered into SigmaPlot (version 10.0; Systat Software) and a histogram with 10 bins was created for hepatic and intestinal CYP3A4 and CYP2C19. A distributional analysis was performed to determine if differences in enzyme content could account for pharmacokinetic differences of voriconazole (Figure 2. 5). In both populations, CYP3A4 content was higher than CYP2C19 in intestinal and hepatic tissues,
and hepatic abundance of both enzymes was higher as compared to the intestine, which is consistent with literature reports. This contrasts with the study revealing that (i) CYP2C19 contributes towards a greater percentage of voriconazole metabolism and (ii) a trend that pediatric CYP2C19 content was higher compared to adults [12]. Furthermore, despite limited published data on pediatric intestinal CYP2C19 enzyme expression, the distribution plots showed age-dependent differences in intestinal enzyme expression [33, 35]. Clearly the model and distributional analysis highlight the need to determine both the expression and activity of DMEs in the intestine as a function of age.

Although incorporation of the first-pass intestinal metabolism of voriconazole appears to improve the ability of the model to predict the oral bioavailability of the drug in children, it does not rule out a potential role of physiological factors in bioavailability. As voriconazole is traditionally considered a low extraction compound due to its high bioavailability in adults, blood flow is not expected to cause a large difference in its clearance, which implies that gastric emptying time and small intestinal transit time may be implicated as important parameters affecting rate of drug absorption and systemic exposure in children [36-39]. A sensitivity analysis was conducted to determine if intestinal physiology could influence voriconazole bioavailability in the models. Figure 2. 6 shows no changes in AUC and $C_{\text{max}}$ associated with both a 10-fold increase and decrease in small intestinal transit time (data not shown for gastric emptying time), suggesting that they do not influence absorption.

One limitation was the software’s inability to predict non-linear pharmacokinetic behavior of voriconazole in adults, since $V_{\text{max}}$ and $K_{\text{m}}$ are not input options. This is due to the requirement of Simcyp to employ whole organ clearance instead of enzyme kinetic parameters. Hence the model overestimated concentrations at later time points because of the inability to account for changes in metabolic rate as concentrations decrease over time. However, the adult model was accepted due to the model’s ability to accurately predict observed adult pharmacokinetic parameters within 50% of observed values. For the
pediatric model, visual predictive checks showed greater accuracy than the adult model, which is not surprising as voriconazole displays linear pharmacokinetics in children and non-linear pharmacokinetics in adults over the same dose range.

Lastly, a published population pharmacokinetic model attempted to connect the sparse data from pediatric clinical trials to improve dosing [4]. However in a follow-up clinical trial assessing these dosing recommendations, it was concluded that they were inadequate [3, 40]. The PBPK models presented in this paper are the first to identify a potential underlying cause for the pharmacokinetic differences of voriconazole in children compared to adults.

CONCLUSION

The contribution of this study is to test a novel paradigm in developing a rational strategy for pediatric dosing of drugs extensively cleared by metabolism. By relating \textit{in vitro} metabolism to clinical data, a “bottom-up” approach to finding doses can be validated and then used to prospectively pick a dose or predict drug-drug interactions [41]. A large number of pediatric clinical trials fail due to inadequate dose selection and lack of response. [42]. Modeling has successfully been utilized to determine optimal doses and increase the efficacy of pediatric clinical trials [43, 44]. This study’s algorithm provides an appealing approach to modeling pediatric drug disposition that can improve prediction of pharmacokinetic parameters in children, and thus reduce ineffective therapy and the need for costly and uncomfortable therapeutic drug monitoring.

In addition, these models are the first important step in identifying underlying mechanistic reasons for the pharmacokinetic differences seen between adults and children. The true significance of this work lies in the fact that our modeling approach suggests age-dependent differences in intestinal enzymatic composition with major impact on drug bioavailability. Further, it provides a clear path for future studies to investigate this very interesting possibility that children have a stronger intestinal barrier to oral drug absorption.
REFERENCES


Table 2. 1 Summary of Voriconazole Intrinsic Pharmacokinetic Parameters and Physiochemical Properties Used for Model Development. $F_a$ fraction of voriconazole absorbed in intestines; $V_d$ volume of distribution; $\text{Plasma } f_u$ fraction unbound in the plasma; $f_{u,\text{Mic}}$ fraction unbound in microsomal incubation; $Cl_{int}$ intrinsic clearance; $CV$ coefficient of variation

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Input</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular Weight (g/mol)</td>
<td>349.3 [7]</td>
</tr>
<tr>
<td>Compound Type</td>
<td>Monoprotic Base [20]</td>
</tr>
<tr>
<td>LogP</td>
<td>2.56 [21]</td>
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<td>$pK_a$</td>
<td>1.76 [20]</td>
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<tr>
<td>Absorption Model</td>
<td>1st order</td>
</tr>
<tr>
<td>$F_a$</td>
<td>0.96 (CV: 10%) [20]</td>
</tr>
<tr>
<td>$K_a$ (1/h)</td>
<td>0.849 (CV: 40%) [4]</td>
</tr>
<tr>
<td>Distribution Model</td>
<td>Full PBPK Model</td>
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<tr>
<td>Prediction Model</td>
<td>Poulin and Theil (Method 1)</td>
</tr>
<tr>
<td>$V_d$ (L/kg)</td>
<td>4.6 [7]</td>
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<tr>
<td>Plasma $f_u$</td>
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<tr>
<td>$f_{u,\text{Mic}}$ predicted</td>
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<td>Renal Clearance (L/h)</td>
<td>0.096 [7]</td>
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<td><strong>Elimination: Whole Organ Clearance</strong></td>
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</tr>
<tr>
<td></td>
<td>Adult</td>
</tr>
<tr>
<td>Hepatic $Cl_{int}$ (μL/min/mg)</td>
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</tr>
<tr>
<td>Hepatic CV (%)</td>
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Table 2. 2 Summary of Voriconazole Pharmacokinetic Parameters in the Pediatric and Adult Populations for Multiple Intravenous Infusion Dosing Regimens.

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<th>Population</th>
<th>Dosing (mg/kg)</th>
<th>AUC</th>
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<th>t&lt;sub&gt;max&lt;/sub&gt;</th>
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<tr>
<td></td>
<td></td>
<td>Observed (mg●h/L)</td>
<td>Simulation (mg●h/L)</td>
<td>Error (%)</td>
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<td>3</td>
<td>13.9</td>
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<td>33.2</td>
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<td>Adult</td>
<td>5</td>
<td>43.4</td>
<td>42.2</td>
<td>2.6</td>
</tr>
<tr>
<td>Pediatric</td>
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<td>11.8</td>
<td>15.7</td>
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<td>Pediatric</td>
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<td>22.9</td>
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<tr>
<td>Pediatric</td>
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Table 2.3 Summary of Voriconazole Pharmacokinetic Parameters in the Pediatric and Adult Populations for Multiple Oral Dosing Regimens.

<table>
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<tr>
<th>Population</th>
<th>Dose</th>
<th>AUC</th>
<th>C&lt;sub&gt;max&lt;/sub&gt;</th>
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<tbody>
<tr>
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<td>Observed</td>
<td>Simulation</td>
<td>Error (%)</td>
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<td>Adult</td>
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<td>11.6</td>
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<tr>
<td>Pediatric (+ Intestinal Metabolism)</td>
<td>4 mg/kg</td>
<td>5.2</td>
<td>7.2</td>
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<td>6 mg/kg</td>
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<tr>
<td>Pediatric (+ Intestinal Metabolism)</td>
<td>6 mg/kg</td>
<td>8.4</td>
<td>10.8</td>
<td>29.1</td>
<td>1.8</td>
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Supplementary Table 2. 1 Summary of Voriconazole PK Parameters in the Pediatric Populations for the Multiple Oral Dosing Regimens with Differing Intestinal Whole Organ Clearance Values Compared Against the Observed Data. Observed data was reported by Walsh et al. [5].

<table>
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<th>Dose (mg/kg)</th>
<th>$\text{Cl}_{\text{int}}$ (μL/min/mg)</th>
<th>Observed AUC (mg*h/L)</th>
<th>Simulation AUC (mg*h/L)</th>
<th>Error (%)</th>
<th>Observed $C_{\text{max}}$ (mg/L)</th>
<th>Simulation $C_{\text{max}}$ (mg/L)</th>
<th>Error (%)</th>
<th>Observed $F$ (%)</th>
<th>Simulation $F$ (%)</th>
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Figure 2.1 Experimental Workflow for the Bottom-up Approach to Scaling Voriconazole from Adults to the Pediatric Population.
**Figure 2. 2 Compound and PBPK Model Structure.** The figure includes the compound structure as well as the model structure. The model structure defines all organ compartments and blood flow that were utilized in the simulations. Hashed arrows indicate dosing compartments, including intravenous and oral regimens. Thick black, downwards arrows indicate elimination from kidneys and liver for both the adult and pediatric populations and from the intestines in the pediatric population.
Figure 2. 3 Adult Intravenous and Oral Visual Predictive Checks. Concentration versus time profiles for (A) 4 mg/kg intravenous (infusion) dose and (B) 200 mg oral dose. The squares represent observed data with the associated standard deviation. The solid line represents the simulated mean concentrations as predicted by the PBPK model for adult population, and the dashed lines show the simulated 90% confidence interval. Adult visual predictive checks were based on the dosing regimen reported by Purkins et al. [16, 17]
Figure 2. 4 Pediatric Intravenous and Oral Visual Predictive Checks. Concentration versus time profiles for an intravenous dose of (A) 4 mg/kg, (B) 6 mg/kg, and (C) 8 mg/kg 4 mg/kg oral dose (D) without and (E) with intestinal metabolism (intestinal clearance of 2.7
µL/min/mg), and 6 mg/kg oral dose without (F) and with (G) intestinal metabolism (intestinal clearance of 2.7 µL/min/mg). Squares represent the experimental values with the associated standard deviation reported by Walsh et al. [5]. The solid lines represent the simulated mean and the dashed lines show the simulated 90% confidence interval.
Figure 2. 5 Adult and Pediatric Distributional Analysis of Hepatic and Intestinal CYP3A4 and CYP2C19 Abundance. Adults are represented by the dotted lines and children (2-12 years of age) are represented by the solid lines. Panels represent the abundance of (A) hepatic CYP3A4, (B) hepatic CYP2C19, (C) intestinal CYP3A4, and (D) intestinal CYP2C19.
Figure 2. 6 Sensitivity Analyses for Small Intestinal Transit Time in Adults and Children. These analyses show the effect of alterations in the alpha and beta parameters of the Weibull distribution for adult oral dosing on (A) AUC and (B) $C_{\text{max}}$, and for pediatric oral dosing on (C) AUC and (D) $C_{\text{max}}$. 
CHAPTER 3
Refining In Vitro Techniques to Predict Intestinal First-pass Metabolism in Children

OVERVIEW

Intestinal first-pass metabolism can clear a significant portion of an oral drug before it reaches the liver, which is traditionally considered the organ of first-pass metabolism. However, little is known about how differences in the intestinal expression or activity of drug metabolizing enzymes lead to variability in first pass-metabolism between children and adults. In this study, an in vitro method was developed to investigate age-dependent differences in the expression and functional activity of intestinal oxidative drug metabolizing enzymes using frozen intestinal tissue specimens. Assessment of functional activity of human intestinal microsomes (HIM), prepared from frozen post-mortem tissues, established that a combination of elution and homogenization is required to preserve enzyme function and protein content. Results suggested degradation of cytochrome P450 and flavin-containing monooxygenase (FMO) mRNA/protein as well as functional enzyme activity in HIM from post-mortem tissues over surgical tissues. Thus, intestinal tissue samples begin to degrade rapidly post-mortem. A comparison of gene expression between post-mortem adult and pediatric intestinal tissue samples showed similarity in expression for all enzymes except CYP3A4, which was higher in pediatric samples. This study has significant clinical relevance as in vitro drug metabolism data generated using healthy intestinal tissue samples from pediatric surgical patients can be integrated into a physiologically based

This chapter has been submitted to Drug Metabolism and Disposition and is presented in the style of that journal.
pharmacokinetic (PBPK) model to predict intestinal first-pass metabolism in children. This strategy will be valuable in screening and optimizing new drug candidates for adult and pediatric indications.

INTRODUCTION

Intestinal first-pass metabolism is an important factor that often determines the bioavailability of oral medications. The first drug shown to undergo significant intestinal first-pass oxidative metabolism was cyclosporine, previously thought to be cleared solely by hepatic metabolism [1]. Since then, cytochrome P450-mediated intestinal first-pass metabolism has emerged as an important barrier to oral drug absorption. Spurred by the findings of CYP3A4-mediated cyclosporine intestinal first-pass metabolism, subsequent studies demonstrated that cytochrome P450s in the intestine significantly impact the metabolism of many drugs [2-5], and led to the characterization of intestinal cytochrome P450 expression, which was presented as an adult intestinal “pie” [6]. However, no such intestinal “pie” exists for children despite attempts to describe enzymatic intestinal content in neonatal and pediatric populations [7, 8].

Differences in the oral bioavailability of drugs between adult and pediatric populations have raised questions about the intestinal enzymatic composition and activity between adults and children. One example of this striking dichotomy is voriconazole. Voriconazole oral bioavailability is approximately 90% in adults, suggesting little to no first-pass metabolism, which allows clinicians to prescribe the same voriconazole dose orally or intravenously. Conversely, the bioavailability of voriconazole in children (2-10 years of age) is 44-66%, approximately half of that observed in adults, and its clearance is nearly three times higher than in adults [9]. Voriconazole is cleared predominantly via oxidative metabolism by CYP3A4, CYP2C19, and FMO3 [10, 11]. Physiologically based pharmacokinetic (PBPK) models, developed for adults and children by incorporating known differences in hepatic enzyme content and activity in these two populations, simulated
voriconazole PK after IV dosing in children but were unable to simulate its lower oral bioavailability in children after oral dosing [12]. Therefore, we proposed that voriconazole undergoes intestinal first-pass metabolism in children but not in adults. Clinical differences in voriconazole bioavailability between adults and children warrant consideration of maturational changes in intestinal drug metabolizing enzymes, which may exhibit a different ontogeny to that observed in the liver [7, 13].

Due to ethical constraints, pediatric clinical trials present significant obstacles (such as recruitment, safety, and blood volume sampling among others), which limit the ability of in vivo studies to clinically test the hypothesis that intestinal first-pass metabolism reduces oral bioavailability of voriconazole in children. An alternative approach to test the hypothesis is to refine the “bottom-up” pediatric PBPK model for oral dosing of voriconazole (that was developed using hepatic voriconazole clearance data) by also incorporating intestinal clearance of the drug. To achieve this objective, it is necessary to assess rates of voriconazole metabolism by intestinal enzymes and quantitative assessment of the expression of relevant enzymes.

A variety of in vitro techniques have been employed to study intestinal metabolism of drugs, but none has been applied to pediatric tissues. The aim of this study was to apply and refine existing in vitro techniques to study oxidative metabolism by microsomes from pediatric intestinal tissue, so that data can be generated for the development of a “bottom-up” PBPK model that incorporates intestinal first-pass oxidative metabolism.

MATERIALS AND METHODS

Chemicals, Reagents, and Samples.

Voriconazole, voriconazole N-oxide, midazolam, 1'-OH midazolam, diclofenac, and alprazolam were purchased from Sigma-Aldrich (St. Louis, MO). Frozen surgical and post-mortem duodenal and jejunal tissue from adults (> 18 years) and children (2-8 years) were obtained from the NICHD Brain and Tissue Bank for Developmental Disorders (Contract
Discarded surgical jejunal samples were procured from patients undergoing gastric bypass at UNC Hospitals.

**Gene Expression.**

Gene expression was measured by isolating mRNA from tissues (25mg) using a Qiagen RNEasy Kit, and mixed with iScript™ cDNA Synthesis Kit (Biorad) to synthesize cDNA. The cDNA was mixed with iTaq™ Supermix (BioRad) and TaqMan Gene Expression Assay for CYP3A4, CYP2C9, CYP2C19, FMO1, FMO3, and FMO4 (Applied Bio-systems). Enzyme gene expression was measured by quantitative RT-PCR and normalized to 18s rRNA.

**Enterocyte Isolation & Microsome Preparation.**

Elution of enterocytes was performed as previously described [14]. Briefly, 1-2 inches of intestinal samples were incubated at 4°C in Buffer 1 (1.5mM KCl, 96mM NaCl, 27mM sodium citrate dehydrate, 8mM KH$_2$PO$_4$, 5.6mM Na$_2$HPO$_4$). After 30 minutes, the solution was drained and replaced by Buffer 2 (1.5mM EDTA, 3 units/mL heparin, and 0.5mM dithiothreitol) and shaken vigorously at 4°C for 15 minutes. The solution containing enterocytes was collected and this step was repeated 6-7 times. After centrifugation, the pellet was used to isolate human intestinal microsomes (HIM) and protein concentration was measured using a BCA assay [15].

**Functional Assay for Metabolic Activity.**

Human intestinal microsomes were validated using a standard midazolam assay. Adult and pediatric HIM (0.2 mg/mL) were incubated with midazolam or voriconazole (4 or 2 μM, respectively), NADPH (1 mM) and MgCl$_2$ (3 mM) in phosphate buffer (pH 7.4) at 37°C. Aliquots (100 μL) were taken at specified time points up to 15 minutes for midazolam or 2 hours for voriconazole. Aliquots were then quenched with 300 μL ice-cold methanol, containing internal standard (alprazolam 0.02 μg/mL for the midazolam assay or diclofenac 0.3 μM for the voriconazole assay).
Metabolite Analysis.

Samples were analyzed using a LC/MS/MS system consisting of mobile phases (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile with a flow rate of 0.7 mL/min. A Sciex API-4000 triple quadrupole mass spectrometer, coupled with a Zorbax SB-C18 column (2.1 x 50 mm, 5 µm) was used. The samples were ionized using an APCI ion source and, following the injection of 10µL of supernatant, the positive ions were monitored at the following MRM Q1/Q3 transitions (m/z): 342.3→203.2 for 1’OH midazolam, 309.3→281 for alprazolam (IS), 366.1→224.2 N-oxide voriconazole, and 296.2→215.1 diclofenac.

RESULTS AND DISCUSSION

In order to determine the optimal method to isolate enterocytes and create subcellular fractions from a small amount of pediatric intestinal tissue samples, two methods, namely elution and homogenization, were assessed. Since only 1-2 inches of frozen pediatric intestinal tissue samples could be acquired, microsomes that were prepared utilizing the elution and homogenization methods were first evaluated for retention of enzymatic activity. Midazolam was chosen as a probe substrate for validating enzymatic activity due to its extensive metabolism by intestinal microsomes [3]. The yield of microsomal protein obtained by the elution method (approximately 37.5 µg) was much lower compared to the protein yield (approximately 2mg) from homogenization (Inserts i and ii in figure 1A). However, microsomes prepared by elution (Figure 1A) retained significantly higher enzymatic activity than those isolated by homogenization (Figure 1b) (p < 0.01). Next, enterocytes were eluted from a two tissue samples and then after elution the epithelium of tissue samples was homogenized assess any remaining activity in tissues after elution. The residual activity in the HIM prepared by homogenized tissues post-elution was approximately half that of the HIM prepared solely by elution (Figure 1B). Therefore, in order to retain high enzymatic activity of microsomes prepared by the elution method while
simultaneously generating a sufficient quantity of microsomal protein and capture all residual activity, frozen tissue samples were subjected to elution followed by homogenization of the epithelial layer. By combining these two methods, 1-2 inches of tissue produced HIM with a high protein concentration (~20mg/mL) could be obtained and utilized to complete multiple in vitro assays.

Utilizing another set of intestinal tissues, HIM were prepared by the combined method. The formation of 1'-OH midazolam by HIM isolated from adult surgical (ages of donors 66 and 81 years) and post-mortem (age of donor 32 years) intestinal tissue samples was compared to that formed by HIM isolated from pediatric post-mortem (ages 3-6 years) intestinal tissue sample and pooled adult human liver microsomes (HLM; Xenotech, LLC). In general, HIM prepared from surgical tissues retained higher enzymatic activity compared to those prepared from post-mortem adult or pediatric samples. Degradation of intestinal tissue begins within 2 hours post-mortem affecting the enzymatic activity of HIM prepared from post-mortem tissues, whereas surgical intestinal tissue samples retain greater enzymatic activity even when stored for a prolonged period at -80ºC prior to microsomal preparation and functional analysis. Moreover, pediatric HIM yielded lower CYP3A activity compared to HIM prepared from adult intestinal specimens (Figure 2a). HIM prepared from surgical samples had ~5-fold lower activity compared to the activity of pooled HLM, which is comparable to the ~4-fold difference in intrinsic clearance from adult liver and intestinal samples taken during organ procurement surgery [3]; conversely adult post-mortem HIM and all pediatric HIM exhibited 40- to 100-fold lower CYP3A activity. These data suggest that HIM prepared from frozen post mortem intestinal samples, which is the only source of pediatric intestinal samples, have inadequate CYP3A (and likely other CYP) activity for evaluating metabolic activity. The sample with the highest enzymatic activity was used to evaluate voriconazole metabolism in adult intestinal tissue (Figure 2b). Voriconazole was metabolized by HIM, but the rate of metabolism was nearly 20-fold slower than that of
midazolam. This result is expected as voriconazole undergoes little to no first-pass metabolism in adults. Thus, it was clear that the other samples of HIM would not generate meaningful data on the metabolism of voriconazole.

Finally, expression of metabolic enzyme genes in frozen post-mortem intestinal tissues was characterized to gain a better understanding of degradation of the tissue post-mortem. Tissue integrity was assessed by comparing gene expression between post-mortem intestinal samples and surgical tissues obtained from gastric bypass patients, assuming that gene expression is similar between healthy and morbidly obese adults. Our findings showed that all samples (from post-mortem collection and gastric bypass surgeries) exhibited substantial variability in the expression of oxidative enzyme genes, which is expected in intestinal tissues. Despite this high variability, a trend towards higher values for oxidative enzyme gene expression in surgical samples compared to post-mortem samples was observed, indicating substantial degradation of tissue nucleic acids post-mortem (Figure 3). This could be due to the long post-mortem interval (i.e., time from death to tissue collection) or failure to rinse intestinal tissues with phosphatase/esterase inhibitors, both of which could result in tissue degradation by intestinal hydrolytic enzymes. Despite the degradation of tissue, post-mortem samples exhibited an interesting trend towards higher intestinal gene expression of CYP3A4 in children versus adults. While corresponding protein expression cannot be inferred from gene expression data, the trend observed does suggest a possible rationale for the lower bioavailability of voriconazole in children compared to adults. This possibility needs to be further explored since CYP3A4 is the predominant metabolizing enzyme in the intestine, and therefore, higher expression of this enzyme in children could have significant implications on oral drug delivery in the pediatric population.

A variety of in vitro methods have previously been evaluated using pre-clinical and human adult intestinal tissue samples to determine their ability to predict in vivo intestinal metabolism after oral dosing [16]. These studies demonstrated that techniques utilizing the
Ussing-chamber and everted sacs that require the use of intact intestinal tissues (which is unobtainable for the pediatric population) retained the best predictive power. Additionally, other previously reported techniques used to isolate primary intestinal cells used an entire section of the adult intestine (such as the duodenum or jejunum) obtained from surgeries. However, it is challenging to apply this methodology to the pediatric population where tissue samples are small, and availability is limited to scavenging healthy tissue surrounding the excised diseased tissue. Unlike pre-clinical and clinical adult intestinal tissue samples which are collected during surgical procedures, pediatric intestinal tissue specimens used in this study were obtained from deceased patients and frozen within a few hours post-mortem. The technique reported here uses a very small section of intestinal tissue, which could feasibly be collected during pediatric gastrointestinal surgeries or pooled from a few surgeries to provide a reliable method to assess differences in enzyme functionality.

In addition to differences in drug metabolizing enzymes, it is also crucial to define differences in the expression of transporters between pediatric and adult populations. Current regulatory guidance documents for pediatric formulations focus mainly on the ease of use, palatability, and toxicity of excipients, but fail to consider interactions between the active ingredient or excipients and age-dependent enzymatic and transporter expression that affect the pharmacokinetics and, therefore, the efficacy of pediatric oral medications [17]. These data must be obtained using human tissues, as allometric scaling from pre-clinical species to adult humans is ineffective; therefore scaling intrinsic clearance from pre-clinical species or adults does not accurately translate the maturation of drug metabolizing enzymes [18]. While a few studies have attempted to describe the intestinal expression of drug metabolizing enzymes that affect intrinsic clearance, and hence bioavailability in neonatal and pediatric populations [8, 7], this report is the first to compare enzyme expression between children (2-8 years of age) and adults using frozen intestinal samples.
Current techniques used to predict bioavailability and intestinal clearance of drugs in children rely on scaling adult enzyme expression, as previously discussed [12], without considering differences in the contribution of intestinal drug metabolizing enzymes. Clinical trials are not the most desirable approach for investigating the bioavailability of drugs in children, as they are costly and challenging. Thus, the research presented here provides an innovative improvement of existing in vitro methods where a small sample of intestinal tissue (1-2 inches) can be used to isolate HIM using a combination of elution and homogenization techniques. This improved strategy provides a practical approach for studying intestinal drug metabolism in children. If healthy intestinal tissue samples are obtained from pediatric surgical patients, intestinal first-pass metabolism can be predicted in children by integrating in vitro metabolism data into a PBPK model. These in vitro first-pass intestinal metabolism data and modeling predictions are valuable in the screening and optimization of new drug candidates for adult and pediatric indications, and are critical for dosing recommendations in these two populations.
REFERENCES


Figure 3. Formation of 1'-OH Midazolam by HIM that were Prepared Using Elution and Homogenization (Whole Tissue or Intestinal Epithelium Only). Panel A represents formation after each technique and the inserts show the resulting pellet from [i] elution and [ii] homogenization. While the elution method retained 30% more activity ($p < 0.01$), the protein yield was very poor. In panel B, enterocytes were eluted off the tissue and then the tissue was subjected to homogenization to measure the residual activity.
Figure 3. 2 Activity of Adult and Pediatric HIM Prepared by Elution and Homogenization. [A] Metabolite formation by adult and pediatric HIM prepared from surgical and post-mortem tissues by elution and homogenization. HIM exhibited a high degree of variability in metabolic activity. Samples obtained during surgery had higher enzymatic functional activity compared to post-mortem samples. [B] Comparison of the formation of voriconazole N-oxide by HLM and HIM (sample A-IN-04) shows that adult intestinal tissue metabolizes voriconazole, but at a much lower rate than liver tissue.
Figure 3. 3 Intestinal CYP3A, CYP2C, and FMO Gene Expression. Comparison of expression of [A] cytochrome P450 and [B] FMO genes determined in post-mortem pediatric and adult intestinal tissues to surgical intestinal samples obtained from gastric
by-pass patients, normalized to pediatric FMO1. The main graph shows that CYP enzyme expression is much higher compared to FMO enzyme expression. Solid bars represent pediatric intestinal tissue samples, bars with horizontal lines are adult samples, and bars with dots represent surgical jejunal samples obtained from adult gastric by-pass patients. For most enzymes, gastric by-pass samples have a markedly higher expression of intestinal enzyme genes compared to post-mortem samples, indicating a striking degradation of intestinal tissue harvested post-mortem. Interestingly, among post-mortem intestinal tissue samples, there was a trend towards higher expression of CYP3A4 (the predominant intestinal enzyme) in pediatric tissues than in adult intestinal tissue samples.
CHAPTER 4

Characterization of CYP and FMO Families Shows Age-dependent Differences in Expression and Functional Activity

OVERVIEW

Significant age-dependent differences in pharmacokinetic (PK) parameters exist for metabolically cleared medications in a wide range of therapeutic classes. While the ontogeny of drug metabolizing enzymes (DMEs) has been delineated, there remains a lack of knowledge about how differences in the intracellular enzymatic system affect the PK of drugs during maturation. In this dissertation research, bottom-up physiologically based pharmacokinetic (PBPK) models were developed to simulate and predict pharmacokinetic behavior of two drugs, voriconazole and sildenafil, in pediatric and premature neonates, respectively. For the development of these models, it is essential to obtain self-consistent data on the expression of genes and proteins of CYP3A, CYP2C, and FMO families in adult, pediatric, and fetal hepatic tissues as well as the corresponding catalytic activity. Although expression of genes and proteins of the above enzymes have been published previously, this is the first study in which quantitative values of the expression of genes and proteins of these enzymes were correlated with the corresponding functional activity in the same set of samples. Both the CYP3A and FMO families showed a distinct switch from fetal isoforms (CYP3A7, FMO1) to adult isoforms (CYP3A4, FMO3) at birth, while the CYP2C9 enzyme showed a linear maturation from birth into adulthood. In contrast, CYP2C19 expression was higher in pediatric hepatic samples compared to fetal and adult samples. Functional activity of the CYP2C family was linearly correlated with enzyme protein expression in the pediatric
and adult tissues, while both CYP3A and FMO required the inclusion of the expression of the fetal forms before a correlation between protein expression and phenotypic activity could be achieved. CYP3A7 formed a unique and previously unidentified hydroxyl metabolite of testosterone, and showed high catalytic activity, even higher than CYP3A4, when this metabolite was included in the measurement. In contrast, CYP3A7 exhibited poor catalytic activity in metabolizing midazolam. Thus, it is hypothesized that the fetal form of CYP3A, i.e. CYP3A7, is a steroid-specific CYP3A enzyme. Similarly, FMO1, which forms cimetidine sulfoxide with high turnover rate, is likely a steroid-specific FMO since cimetidine exhibits anti-androgenic activity. Finally, the catalytic efficiency of CYP2C19 was greater in the pediatric hepatic tissue than in the adult tissues; this is a surprising finding and suggests that cytochrome P450 enzymes may encounter different micro environment in children versus adults. These data are important to understanding the mechanistic basis of why some medications are cleared faster in children compared to adults, and can be incorporated into PBPK models to enhance the accuracy of PK predictions and, therefore, safety and efficacy in children.

INTRODUCTION

Hepatic clearance accounts for nearly 75% of the top 200 drugs on the market and over three-quarters of hepatic clearance is due to phase I drug metabolizing enzymes (DMEs) [1]. However, the effect of the ontogeny of these DMEs on pharmacokinetics (PK) in children is poorly understood for many of these drugs. In a study comparing the PK of 45 drugs, the medications studied had, on average, a higher clearance and shorter half-life in children 2-12 years of age compared to adults, owing to age-related differences in metabolic clearance [2]. For example, proton pump inhibitors, such as omeprazole and lansoprazole, as well as phenytoin are commonly prescribed in the pediatric population and all have higher clearance in children, necessitating higher weight-based doses [3-6].

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Voriconazole is another prominent example of a drug that was only studied in adult volunteers, but later exhibited markedly different PK in children of ages 2-10. As described in Chapter 2, voriconazole is cleared predominantly by oxidative metabolism through cytochrome P450 (CYP) 3A4, CYP2C19, and flavin-containing monooxygenases (FMO) 3. Differences in voriconazole PK in pediatrics is attributed to the contribution of metabolic enzymes to voriconazole metabolism in children versus adults [7]. Sildenafil is the other main substrate studied in this dissertation. CYP3A and CYP2C9 were specified as the two main metabolizing enzymes responsible for its metabolism [8, 9]. For these reasons, expression and function of the CYP3A, CYP2C, and FMO families were studied.

Early studies examined age-dependent differences in expression of major metabolizing enzymes responsible for medications cleared by oxidative metabolism. For both CYP2C and FMO expression analyses, over 200 fetal, pediatric and adult samples were subjected to Western blotting to determine the ontogeny of enzyme expression [10, 11]. The CYP2C family exhibited low expression in the fetal population, but increased to adult levels after birth (CYP2C9) and after 5 months postnatal age (CYP2C19). A trend towards higher functional activity was observed in samples from children aged 5 months to 10 years compared to samples that were obtained from children 10 years and above [11, 7]. Yet, the authors failed to mention this unique finding, which could not be explained by enzyme expression, leaving questions about differences in factors other than enzyme expression that could affect the activity of the CYP2C family. Furthermore, the quantitation of the CYP2C9 and 2C19 isoforms could have been confounded by cross-reactivity of the antibody with CYP2C8.

The same sample population set also generated an ontogeny profile for FMO, showing that the main hepatic isoforms switched from FMO1 to FMO3 during development. FMO1 is the predominant fetal isoform that is expressed at highest levels during weeks 8-15 of gestation. Expression of FMO1 declines during fetal development until it is undetectable
by the first week after birth. FMO3, the adult hepatic isoform, then replaces FMO1 as the predominant isoform and reaches adult expression levels by approximately 1-2 years of age [10].

Unlike the CYP2C and FMO families, CYP3A expression was measured by differential metabolism of dehydroepiandrosterone (DHEA) as antibodies lacked specificity and selectivity for the individual 3A isoforms. Since CYP3A7 differentially metabolizes DHEA to 16α-OH DHEA and CYP3A4 metabolizes DHEA to 7β-OH DHEA, the ratio of the 16α-OH to the 7β-OH DHEA metabolite was used to assess the contribution of either CYP3A7 or CYP3A4, respectively. A nonlinear multivariate regression analysis was used to determine the protein concentration of each isoform based on the ratio of the 16α-OH to 7β-OH metabolites. However, both these metabolites were also produced by CYP1B1, CYP2C19, and CYP3A5, which could confound the assessment of the expression of CYP3A isoforms.

While these initial studies have improved our understanding of the ontogeny of DMEs, there are still knowledge gaps, which limit current attempts to determine factors that are responsible for differences in drug metabolism across age ranges spanning from birth to adulthood. Without a comprehensive knowledge of the maturational changes in expression and function of DMEs at the cellular level, any dosing or safety predictions will either over- or under-predict PK parameters in children. Ethical concerns and high variability confound clinical trials aimed at investigating the effect of ontogeny of DMEs on the PK of drugs in pediatric populations. Therefore, a more feasible strategy is to study the differences in cellular mechanisms of clearance in pediatric versus adult hepatic tissues, and relate the expression and functional activity differences in an integrated model. The aim of this study was to elucidate the cellular mechanisms that cause differences in metabolic clearance from birth until adulthood. Thus, gene and protein expression, as well as functional activity of CYP3A, CYP2C, and FMO families were evaluated. In light of the deficiencies that existed in determination of protein expression for CYP2C and CYP3A family members, as pointed out
earlier, LC-MS techniques were employed to determine expression of CYP2C, CYP3A, and FMO enzymes.

MATERIALS AND METHODS

Chemicals, Reagents, and Sample Selection

Testosterone, 6-β OH-testosterone, S-mephenytoin, 4’-OH S-mephenytoin, diclofenac, 4’-OH diclofenac, cimetidine, NAPDH, and voriconazole were purchased from Sigma-Aldrich (St. Louis, MO). Cimetidine sulfoxide was purchased from Abcam (Cambridge, MA). Liver tissues from healthy adult (>18 years old), pediatric (aged 5 months-10 years), and fetal (14-29 weeks gestation) donors were obtained from the NICHD Brain and Tissue Bank for Developmental Disorders (Contract #HHSN275200900011C, Ref. No. NO1-HD-9-0011; Baltimore, MD) under an approved UNC-Chapel Hill IRB protocol. Donor information is listed in Table 4.1.

Preparation of Microsomes

Human liver microsomes (HLM) were prepared as previously reported [12]. Briefly, 0.5 mg of hepatic tissue was homogenized in a buffer containing 50 mM Tris-HCl, 1 mM EDTA, and 154 mM KCl with protease inhibitors (pH 7.4). After homogenization, samples were centrifuged at 10,000 x g for 30 minutes, the supernatant was collected, and this step was repeated. The supernatant was mixed with a second buffer containing 100 mM sodium pyrophosphate and 0.1 mM EDTA (pH 7.4) and then centrifuged at 100,000 x g for 70 minutes. This step was repeated one final time. The resulting pellet was then resuspended in a buffer containing 10 mM potassium phosphate, 1 mM EDTA, and 20% glycerol (pH 7.4). Microsomal protein concentration was measured using a BCA assay. HLM were stored at 20 µL aliquots at -80°C.

Gene Expression

Liver tissue samples (25mg) were lysed, sonicated until complete homogenization, and then centrifuged at 10,000 x g for 12 minutes. Phase separation and binding of the
mRNA was completed using a Qiagen RNEasy Kit. RNA was diluted to 500 ng/µL for each sample. The isolated RNA was mixed with iScript™ cDNA Synthesis Kit (Biorad) and incubated at 25°C x 5 min, 42°C x 30 min, and then 85°C x 5 min to synthesize cDNA. The cDNA was mixed with iTaq™ Supermix (BioRad) and TaqMan Gene Expression Assay for CYP3A4, 3A5, 3A7, 2C9, 2C19, FMO1, FMO3 and 18s (Applied Bio-systems). Expression for each enzyme gene was measured (quantitative rt-PCR) and normalized to 18s rRNA.

**Protein Expression**

For enzyme protein quantitation, HLM were subjected to proteomic analysis by mass spectrometry [13]. HLM (30 µg, Xenotech, Lenexa, KS) and recombinant CYP3A4, 3A5, 3A7, 2C9, 2C19, FMO1, FMO3, and FMO5 (BD Gentest, San Jose, CA) was each diluted in 90 µL denaturation solution (44.4 mM NH₄HCO₃, 4.44 mM DTT) and incubated at 60 °C for 1 hour. Iodoacetamide (10 mM) was then added to the solution and kept in the dark for 20 min at room temperature. Next, the solution was incubated for 4 hours at 37 °C with trypsin (1 µg). Samples were spiked with 10 µL of mixed isotope labeled peptides as internal standards and centrifuged at 16,100 x g for 10 min. Separation was achieved using an Acquity UPLC BEH130 C18 column (1.7 µm 2.1 x 100 mm, Waters) at a flow rate of 0.4 mL/min using mobile phases of water containing 0.1% formic acid (A) and ACN containing 0.1% formic acid (B). Protein expression in adult and pediatric liver samples (n=10/group) were assessed by comparing the peak area normalized to the internal standard with known protein content in recombinant enzyme standard curves.

**Functional Assays**

Adult and pediatric HLM (0.1 mg/mL) were incubated with the probe substrate of each respective enzyme (Table 4. 2), NADPH (1 mM) and MgCl₂ (3 mM) in phosphate buffer (pH 7.4) at 37°C for up to 60 min. Aliquots (100 µL) of samples were taken at specified time points, quenched with 300 µL methanol containing internal standard (0.1 µM of either
voriconazole for the CYP assays or phenformin for the FMO assay). Samples were then centrifuged at 10,000g x 10 min and analyzed by LC-MS/MS.

**Metabolite Analysis**

The LC-MS/MS system consisted of mobile phase (A) 0.1% formic acid in water and (B) 0.1% formic acid in methanol with a total flow rate of 0.6 mL/min. A Sciex API-4000 triple quadrupole mass spectrometer was used with a Zorbax SB-C18 column (2.1 x 50 mm, 5 µm). Samples were ionized using an ESI ion source and, following the injection of 10µL of supernatant, positive ions were monitored at the following MRM Q1/Q3 transitions (m/z): 235.2→150.2 for 4’-OH S-mephenytoin, 312.3→230.2 for 4’--OH diclofenac and 350.2→127.2 for voriconazole. For the testosterone assay, samples were ionized using an APCI ion source and monitored at the following MRM Q1/Q3 transitions (m/z): 289.2→97.1 for testosterone, 305.1→269.3 for 6β-OH testosterone and 350.2→127.2 voriconazole.

**Statistical Analysis**

Differences between populations for gene and protein expression as well as differences in metabolite formation were analyzed using a two-way ANOVA. The linearity of maturation profiles and correlation analyses were determined by fitting a linear regression curve and 95% confidence intervals to the model. For all analyses, a p-value less than 0.05 was considered significant.

**RESULTS**

The CYP3A Family

**Gene Expression**

Relative gene expression (Figure 4. 1a) of the three major CYP3A enzymes revealed that CYP3A7 gene was expressed to the highest level in fetal hepatic samples while pediatric samples had a lower, but detectable, level of this gene. Juxtaposing the fetal and pediatric CYP3A7 gene expression, its expression was undetectable in adults. In contrast, the CYP3A4 gene was undetectable in fetal tissue, but its expression increased with age
and was similar between the pediatric and adult populations. Taken together, a clear switch in the expression of the gene for predominant CYP3A isoform is observed from the fetal population (CYP3A7) into adulthood (CYP3A4). Unlike CYP3A4 and CYP3A7, expression of CYP3A5 remained relatively stable across all three groups with no discernable ontogeny profile.

**Protein Expression**

The trends revealed by the relative gene expression corresponded well to protein expression, which was measured by quantitative proteomic analysis using mass spectrometry. As expected, fetal samples had very high expression of CYP3A7 (0.535 nmol/mg) with a corresponding low expression of CYP3A4 (0.0003 nmol/mg). During maturation, CYP3A7 levels decreased in the pediatric and adult populations (0.051- and 0.019 nmol/mg, respectively; Figure 4. 2a). On the other hand, CYP3A4 levels increased from undetectable levels in the fetal period until it reached adult levels (0.069-0.078 nmol/mg) around 1 year of age. Abundance of CYP3A7 was over 10-fold higher and abundance of CYP3A4 was over 200-fold lower in the fetal tissues compared to either the pediatric or adult tissues (p < 0.0001; Figure 4. 2d). This switch from CYP3A7 as the predominant isoform in fetal tissues to CYP3A4 in adult tissues was seen previously in the literature [14]. CYP3A5 remains relatively stable (0.001-0.005 nmol/mg) across all three groups with no statistically significant difference among the three populations.

**Functional Activity**

Expression patterns were also examined by measuring enzyme activity with enzyme-specific probe substrates. Initially, testosterone was chosen as the probe substrate to assess functional activity of the CYP3A family in these three populations as well as in recombinant CYP3A4, 3A5, and 3A7 (Figure 4. 3). The rates of formation of testosterone metabolites by fetal, pediatric, and adult HLM samples as well as recombinant CYP3A4, 3A5, and 3A7 were normalized to the total protein in each sample (Figure 4. 3a). Fetal
samples had a high rate of metabolite formation (665.9 pmol/min/mg protein) compared to pediatric and adult samples (256.8 and 278 pmol/min/mg protein, respectively). Recombinant CYP3A4, 3A5, and 3A7 formed metabolite at rates of 1433.6, 375.6, and 199.2 pmol/min/mg protein. However, after normalizing the formation of metabolite to the total amount of CYP3A in each sample, a clear maturation trend was observed across the three populations (1.2, 1.6, and 2.8 pmol/min/pmol 3A, respectively) which correlated well with the formation of metabolite by the recombinant 3A4, 3A5 and 3A7 enzymes (3.9, 3.0, and 1.4 pmol/min/pmol 3A respectively; Figure 4. 3b).

**Detection of a Novel CYP3A7-specific Testosterone Metabolite**

Upon further analysis of the LC-MS/MS chromatograms, a distinct shift in retention time was observed between the major metabolite formed by fetal enzymes and recombinant CYP3A7 compared to the major metabolite formed by adult and pediatric enzymes, and recombinant CYP3A4 and CYP3A5 (Figure 4. 4). Adult/pediatric HLM and recombinant CYP3A4 metabolized testosterone to 6β-OH testosterone (peak at 2.51 min) and to a minor hydroxylated testosterone metabolite (M+16) that eluted at 2.61 min. Interestingly, fetal HLM and recombinant CYP3A7 formed the testosterone metabolite eluting at 2.61 min as the major product, with 6β-OH testosterone (2.51 min) as a minor product. These results indicate that the main metabolite of testosterone formed by CYP3A7 and fetal samples is not 6β-OH testosterone.

**CYP3A7 is a Steroid-specific Enzyme**

Due to the unexpected testosterone metabolite formed by fetal enzymes and CYP3A7, midazolam, another FDA approved CYP3A in vitro probe, was evaluated to assess if a similar result is observed with fetal enzymes and CYP3A7 (Figure 4. 5). In contrast to the testosterone metabolism, 1’OH midazolam formation by fetal samples and recombinant CYP3A7 was very low (0.4 and 0.25 pmol/min/pmol P450). Metabolite formation of 1’OH midazolam increased with age from the pediatric to adult samples (3.9
Recombinant CYP3A4 generated 1’OH midazolam at a lower rate (8.9 pmol/min/pmol P450) compared to recombinant CYP3A5 (28.6 pmol/min/pmol P450), indicating that CYP3A5 is the predominant CYP3A enzyme responsible for midazolam metabolism.

A correlation between enzyme abundance and functional activity was assessed to illustrate the contribution of each isoform to both testosterone and midazolam metabolism (Figure 4. 6). When the rate of testosterone metabolism was compared to total CYP3A4 and CYP3A5 abundance, an inverse correlation was observed. Conversely, when the metabolic rate was compared to the total CYP3A abundance of all three isoforms, a positive correlation was observed indicating that CYP3A7 is a major contributor to testosterone metabolism. However, these results were reversed for midazolam. A positive correlation was attained only when the metabolic rate was compared to the total abundance of CYP3A4 and CYP3A5. No correlation was seen when midazolam metabolite formation was plotted against the abundance of all three isoforms; this is consistent with the fact that CYP3A7 is not involved in midazolam metabolism. These results suggest that CYP3A7 preferentially metabolizes steroids.

The CYP2C Family

Fetal expression of both CYP2C9 and CYP2C19 genes was low to undetectable, with approximately a 100-fold lower expression compared to the pediatric and adult samples. No significant differences were observed for CYP2C9 or CYP2C19 gene expression in adults versus children. However, there was a trend towards higher CYP2C19 expression in the pediatric population compared to the adult population (p < 0.06).

Expression of CYP2C9 protein increased linearly over time ($r^2 = 0.31; p < 0.0001$) and adult level expression was reached in the pediatric population (Figure 4. 2b). Overall, CYP2C9 protein expression corresponded to gene expression (Figure 4. 2e). Fetal tissue
expressed significantly lower levels (0.001 nmol/mg) of CYP2C9 protein compared to pediatric and adult tissues (0.052 and 0.054 nmol/mg, respectively; p< 0.0001).

In contrast to the linear maturation profile of CYP2C9 protein, CYP2C19 protein expression peaked during the pediatric period (Figure 4.2b). The pattern of differences in CYP2C9 gene expression in pediatric samples compared to adult samples was also reflected in protein expression (Figure 4.2e). CYP2C9 protein expression in pediatric tissues was over 2-fold higher compared to adult tissues (0.0093 nmol/mg versus 0.0043 nmol/mg; p<0.05).

To assess how expression affects functional activity, diclofenac and S-mephenytoin were used as probe substrates for CYP2C9 and CYP2C19, respectively. Formation of 4'-hydroxylation of diclofenac by fetal tissue, normalized to total microsomal protein (Figure 4.7a), was over 200-fold lower than by pediatric and adult samples (0.61 versus 240 versus 208 pmol/min/mg protein, respectively). After normalizing metabolite formation to the total CYP2C9 abundance in each sample (Figure 4.7b), fetal samples still had much lower metabolic activity compared to pediatric and adult samples (0.51 versus 4.0 versus 3.6 pmol/mg/pmol CYP2C9 respectively) and there were no significant differences in CYP2C9 activity between adult and pediatric populations.

CYP2C19 displayed differential maturation in activity compared to CYP2C9. Similar to CYP2C9, formation of 4'-hydroxylation of S-mephenytoin by fetal samples was undetectable (Figure 4.8). However, metabolic activity in pediatric samples was higher than that in adult samples when normalized to total microsomal protein (4.0 versus 1.3 pmol/min/mg protein respectively; p = 0.04). After normalizing for the higher expression of CYP2C19 in pediatric samples, a significant difference was still apparent between the metabolite formation rates by the pediatric and adult enzyme (364 versus 199 pmol/min/pmol CYP2C19, respectively; p < 0.003). These results signify that another cellular mechanism, in combination with higher CYP2C19 expression, confers higher metabolic
activity in the pediatric population. Finally, both CYP2C9 and CYP2C19 activity toward their respective probe substrates is significantly correlated with abundance (Figure 4.9).

The FMO Family

The relative gene expression (Figure 4.1c) of both FMO1 and FMO3 revealed that FMO1 had the highest expression in fetal samples while adult and pediatric samples had low to undetectable expression. On the other hand, FMO3 gene expression was low in the fetal tissue and increased to mature levels in the pediatric and adult populations. As seen in the CYP3A family, FMO exhibited a switch from a predominant fetal isoform to an adult isoform after birth.

Similar trends were displayed by the FMO protein expression. As expected, high protein expression of FMO1 was observed in fetal samples (0.007 nmol/mg) with a low expression of FMO3 (0.001 nmol/mg; Figure 4.2c). FMO1 expression was virtually undetectable in pediatric and adult samples (0.00002 nmol/mg in each population), confirming the notion that FMO1 is a fetal isoform. Because of the specificity, and in turn low variability of of the protein expression assay, a significant difference was seen among all three populations as FMO3 increased from 0.001- to 0.02- to 0.037 nmol/mg in the fetal, pediatric, and adult samples, respectively (p < 0.001; Figure 4.2f). A clear ontogeny profile is seen for FMO3 as expression increased linearly from the fetal period into adulthood ($r^2 = 0.52; \ p < 0.0001$).

Finally, functional activity of FMO was assessed using cimetidine as a probe substrate. Formation of cimetidine sulfoxide by recombinant FMO1 was 2-fold higher than by recombinant FMO3. Fetal samples, which had a higher expression of FMO1, generated cimetidine sulfoxide at a rate 2-fold higher than did pediatric and adult samples (p < 0.001; Figure 4.10a). Normalization of functional activity to the abundance of FMO in each sample revealed that FMO activity remained significantly higher (p < 0.0001) in fetal samples (507 pmol/min/pmol FMO) compared to pediatric and adult samples (79 and 74 pmol/min/pmol...
FMO, respectively; Figure 4. 10b). An inverse correlation was observed when functional activity of FMO isoforms was plotted against total FMO1 and FMO3 abundance (Figure 4. 11), indicating that the metabolic activity of FMO3 toward cimetidine is much lower as compared with FMO1.

**DISCUSSION**

It has been recognized for over a decade that expression of DMEs changes, in some cases by more than an order of magnitude, from birth through childhood and adolescence to adulthood. Numerous studies have been published to delineate the age-associated changes in DMEs, and more recently in drug transporters as well as receptors and other mediators of biological effects. However, the expression level of enzyme (and transporter) proteins, determined by Western blot analyses, has been semi-quantitative at best. This work represents the first attempt to undertake a comprehensive study in which expression of DME genes and proteins, as well as phenotypic activity of these enzymes have been determined and related within the same study. Furthermore, the expression of DME proteins, in addition to the expression of corresponding genes, was determined using quantitative proteomic analysis using LC-MS. For this comprehensive analysis of the relationship among gene and protein expression as well as phenotypic activity of DMEs, CYP3A, CYP2C, and FMO family of enzymes were selected because of their known or anticipated role in the clearance of the two drugs studied in this dissertation work, *i.e.* voriconazole and sildenafil.

The gene expression profiles shown in Figure 4. 1a-b correspond well to the protein expression profiles in Figure 4.2d-f, indicating that gene expression can reveal relative protein expression trends among isoforms within the same family. However, absolute quantitation of DMEs is critical, especially for incorporating into a PBPK model, and one cannot rely on gene expression data to predict protein expression as specificity of the primers can affect gene expression results, and factors such as post-translational modifications can alter the final protein expression, as is seen with CYP2C9. Relative gene
expression of CYP2C9 indicates that this enzyme has similar abundance to that of CYP3A7 and potentially higher expression than CYP3A4. Yet protein expression studies show that the CYP3A family has higher expression than CYP2C9, which is in agreement with published reports on hepatic protein content [15].

Initial Western blot analysis and differential metabolism studies provided a reasonable estimate of protein expression [7]. However, the enzyme abundance data reported here are more reliable. Quantitative mass spectrometry-based proteomic analysis provides significant advantages over traditional antibody-based analysis. Only a small amount of sample (30 µg) was used to quantify peptide sequences of multiple enzymes simultaneously. Most importantly, by choosing a peptide sequence unique to an isoform of an enzyme, specificity of the measurement is greatly increased [16]. This improvement over antibody techniques reduces the likelihood of confounding enzyme abundance data due to cross-reactivity of an antibody with a different isoform within the same DME family. This is the first study to report hepatic expression of enzymes in the CYP3A, CYP2C, and FMO families based on quantitative proteomic analysis by LC-MS/MS.

In order to study the CYP3A-mediated functional activity of HLM, testosterone was chosen as a probe substrate, which was quantified using LC-MS/MS. Previous studies and reviews have reported that CYP3A7 has a lower catalytic activity for oxidation of testosterone as determined by testosterone 6β hydroxylation, the reaction catalyzed by CYP3A4 and presumed to be catalyzed by CYP3A7 as well, compared to CYP3A4 with as much as an 18-fold lower metabolite formation [17, 18, 14]. The results presented in this report agree with published data that the CYP3A7 forms 6β-OH testosterone at a much lower rate than does CYP3A4. However, the results also report an exciting new finding that CYP3A7 metabolizes testosterone to a hydroxylated metabolite of testosterone that is distinctly different from 6β-OH testosterone, the major metabolite of CYP3A4 and CYP3A5, as evidenced by its different retention time on LC-MS chromatogram but same mass-to-
charge ratio as 6β-OH testosterone. Thus, the novel hydroxylated testosterone metabolite found in this work is either a stereoisomer or a positional isomer of 6β-OH testosterone; many hydroxylated testosterone metabolites are formed by rat liver microsomes [19], by different Cyp enzymes. The results further show this new metabolite is a major metabolite of CYP3A7 with the-6β-OH testosterone, formed as a minor metabolite. Finally, the results show that fetal HLM form the new metabolite as a major metabolite from testosterone, reflective of CYP3A7 as the major form of CYP3A present in these samples. If one assumes that the mass spectrometric response factor for the new hydroxylated metabolite is similar to that of 6β-OH testosterone, which is a reasonable assumption since both are hydroxylated derivatives of testosterone, then this study, for the first time, provides evidence that fetal hepatic HLM can metabolize testosterone at a higher rate than pediatric or adult liver HLM.

The high abundance of CYP3A7 in fetal tissue, which is approximately 6-fold higher than adult CYP3A4 expression, combined with high catalytic rate of CYP3A7 to metabolize testosterone to the new metabolite, accounts for this high rate of testosterone metabolism by fetal HLM.

In order to determine if this high rate of testosterone metabolism by fetal tissues was a general or substrate-specific effect, the metabolism of a second probe for CYP3A-mediated activity, midazolam, by the same fetal, pediatric, adult, and recombinant CYP3A samples was examined. In contrast to high rate of testosterone metabolism by fetal HLM and recombinant CYP3A7, little to no 1’OH midazolam was formed by these enzymes, and there was no evidence that a different midazolam metabolite was produced by these enzymes. A clear maturation of functional activity was progressively observed with fetal, pediatric and adult samples, which corresponded to the expression and function of CYP3A4 and CYP3A5. These results were confirmed by a correlation analysis where midazolam was significantly correlated with only CYP3A4 and CYP3A5 expression, but testosterone
metabolism required the inclusion of CYP3A7 in the abundance data to show a significant correlation.

As mentioned in the introduction, relative CYP3A4 and CYP3A7 expression were originally estimated by the metabolites formed from DHEA, an endogenous steroid [14]. Analogous to the unique hydroxylated DHEA metabolite formed by CYP3A7, these results demonstrate for the first time that CYP3A7, the fetal form of CYP3A, metabolizes testosterone to a novel hydroxylated metabolite.

Based on these results a new hypothesis is proposed regarding the role of CYP3A7 in human metabolism; that is, CYP3A7 is a steroid-specific cytochrome P450 that is evolved to be expressed in the fetus to metabolically degrade the steroidal hormones of the mother of which the fetus is exposed. The hypothesis is supported by the physiological and hormonal changes associated with pregnancy. During pregnancy the progesterone and estrogen levels increase 3- and 20 fold, respectively, and drop precipitously at birth [20]. The switch from CYP3A7 to CYP3A4 occurs almost immediately after birth, [18], supporting the hypothesis that the physiologic function of CYP3A7 is to metabolically degrade the steroidal hormones from the mother. Furthermore, testosterone levels increase in the first and second trimester, but is significantly higher in women carrying male fetuses [21]. These hormonal changes are handled by the mother as well as the fetus and the placenta. Within the fetus itself, the liver begins forming within the first few weeks of embryonic development and begins functioning by the third month of pregnancy [22, 23]. In addition, the placenta and the fetal membrane surrounding the fetus express CYP3A4, CYP3A5 and CYP3A7. The placenta exhibits a 2-fold higher expression of CYP3A4 compared to fetal membrane, whereas the fetal membrane show higher expression of CYP3A7 and CYP3A5. Homogenates from placentas and fetal membranes established that both these organs had CYP3A functional activity, providing an additional barrier to protect the fetus from circulating compounds [24, 25]. It has also been shown that estradiol and progesterone, two main
circulating hormones during pregnancy, affect the expression and function of hepatic DMEs and it is possible that the metabolites of these steroids produced by either the mother or the fetus could also affect gestational development [26, 27].

As testosterone is a standard probe for CYP3A activity, the significance of these findings will impact the use of both in vitro and in vivo probe assays. These findings will yield a unique method to discriminate testosterone metabolism by CYP3A4 versus CYP3A7. Identification of the CYP3A7-specific testosterone metabolite will lead to an in vivo probe for CYP3A7 activity in fetal, neonatal, and pregnant populations.

The ontogeny of FMO is similar to that of the CYP3A family in that the predominant isoform switches at birth from the fetal FMO1 isoform to the adult FMO3 isoform. Previous studies have examined the differences in FMO isoform activity for a variety of substrates. These studies have shown that FMO3 metabolizes substrates, such as ranitidine, more efficiently than FMO1 [28]. Another substrate of FMO is nicotine, which has a similar metabolic profile between children and adults, but a significantly reduced metabolism in neonates [29]. These published reports indicate that FMO3 potentially has higher activity compared to the fetal isoform FMO1.

To investigate these reports, functional FMO activity of the fetal, pediatric, and adult HLM were assessed using cimetidine as a probe. Cimetidine has been studied in pre-clinical species as well as in human clinical studies, and the evidence provided thus far suggest that FMO3 is the major metabolizing enzyme of cimetidine in adult humans [30, 31]. Interestingly, the results presented in this study show that the rate of cimetidine sulfoxide formation by fetal HLM and FMO1 was approximately 2-fold higher than the rate of cimetidine metabolism by pediatric and adult HLM and by FMO3. Upon closer inspection of cimetidine structure and function, these results indicate that FMO1 may also be a steroid specific fetal isoform as cimetidine has mild antiandrogen properties. Cimetidine has the ability to displace both testosterone and dihydrotestosterone from the androgen receptors.
[32, 33]. Due to this anti-androgen property, it has been studied for its perinatal effects on male rat sex organ development as well as on cancer prevention in humans [34, 35]. As was seen with CYP3A, the switch from FMO1 to FMO3 is linked to parturition rather than with gestational age [36, 10]. Thus, the hypothesis extends to FMO1 as a likely a steroid-specific enzyme, which declines in function and expression after the decrease in pregnancy hormones. Thus, CYP3A7 and FMO1 are fetal DMEs that are an evolutionary response to the surge in steroid hormones during pregnancy.

For the CYP2C families, no fetal isoforms exist for either 2C9 or 2C19, and enzymes exhibit virtually no expression in fetal samples. Between the pediatric and adult populations, CYP2C9 expression was comparable, corresponding to the maturation of the gene and protein expression and, as seen in the correlation analysis, higher abundance was linearly associated with higher activity. In contrast, expression of CYP2C19 protein was higher (nearly 2-fold) in pediatric liver samples compared to adult liver samples. While this trend has been observed previously [7], the sensitivity of the LC-MS/MS proteomic assay decreased variability to provide definitive evidence for a significant difference in 2C19 protein expression between these two populations. This higher expression in children may explain, in part, the higher clearance of voriconazole in children compared to adults, and the previous finding that CYP2C19 accounts for higher percentage of voriconazole metabolism (~50%) in children versus adults [7, 37]. Surprisingly though, when catalytic activity was normalized to CYP2C19 protein expression, the turnover rate of the CYP2C19 probe S-mephenytoin was higher for pediatric HLM compared to adult HLM, suggesting that CYP2C19 exhibits higher catalytic efficiency in children compared to adults. Higher catalytic efficiency of the same enzyme in children compared to adults is a novel finding, and may provide additional explanation for the higher contribution of CYP2C19 toward voriconazole metabolism and its higher clearance in children compared to adults. These results warrant
investigation of other drugs that are oxidatively metabolized by CYP2C19 and that require higher doses in children compared to adults.

The higher catalytic efficiency of CYP2C19 in children compared to adults is surprising. One likely explanation for this may be increased linking between NADPH cytochrome P450 oxidoreductase and CYP2C19. Many studies have been conducted assessing the structure and function of NADPH cytochrome P450 oxidoreductase, including how genetic variations of this enzyme affect drug metabolism of compounds such as steroids and midazolam [38-41]. While many genetic polymorphisms can decrease the function of this enzyme, a few variants can increase the metabolic activity as was shown for both CYP2C19 and CYP1A2 [42]. Another likely factor that can affect catalytic efficiency of a cytochrome P450 enzyme is the lipid composition of the membrane within the endoplasmic reticulum. Since the access of a cytochrome P450 active site to its substrate requires the substrate to traverse the lipid membrane in which the enzyme is embedded, the same cytochrome P450 enzyme may catalyze the same substrate at different efficiency in two different species or two different populations within a species. In addition, different lipid composition also can lead to different level of coupling between a cytochrome P450 enzyme and NADPH cytochrome P450 oxidoreductase, leading to different catalytic efficiency of the P450 enzyme. Thus, the higher catalytic efficiency of CYP2C19 in pediatric HLM compared to adult HLM is an interesting new finding that needs to be further examined to elucidate the molecular or biophysical mechanism underlying the difference.

One limitation of this study is that the sample size is considerably smaller than the 200 samples used in the initial protein expression analyses. However, the post-mortem interval of the 200 samples ranged from 1-41 hours with a median of 17 hours [11] whereas the post-mortem interval of the samples chosen for this research was less than 18 hours with a median of 3 hours. Potential degradation of DMEs during this post-mortem interval could affect functional activity and expression. So despite the small sample size, the short
The post-mortem interval as well as the techniques utilized in this research allowed an increased ability to detect differences in expression between pediatric versus adult samples. It is important to note that the small sample size limits the ability of this work to detect differences in expression or function due to polymorphisms. A few clinically significant polymorphisms have been documented for CYP2C19, CYP2C9, FMO, and CYP3A5 [43-47]. Due to these polymorphisms, the differences in expression of these enzymes reported here require additional analysis to understand the influence of genetic variation on the maturational profile of the DMEs.

The CYP3A and CYP2C families account for 60% of the hepatic enzyme content and metabolize over 70% of the drugs currently on the market [1, 15]. While the FMO family has lower expression, it plays a pivotal role in regulating reactive oxygen species and metabolizing nitrogen- and sulfur-containing compounds [36]. Unique differences in enzyme contributions exist among these three enzyme families during development, and these differences affect the PK of medications administered to children. While changes in the hepatic expression of these proteins and their genes with age as well as the corresponding changes in the catalytic activity have been examined previously, the work presented here is the first study in which the changes in the gene and protein expression as well as in the catalytic activity are measured using the same tissue samples within the same study, with quantitative analysis of protein expression using LC-MS/MS.
REFERENCES


Table 4.1 Individual Human Liver Donors. HLM were prepared from these liver samples. * indicates that information was not provided.

<table>
<thead>
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<th>Gender</th>
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Table 4. 2 Probe Substrates for Enzymatic Activity of Cytochrome P450 and FMO Enzymes. The chemical structures of the substrates and their major metabolite as well as the concentration of the substrates used in phenotypic assay.

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<td>10 µM</td>
<td>6β-OH Testosterone</td>
<td><img src="image" alt="6β-OH Testosterone" /></td>
</tr>
<tr>
<td>CYP3A</td>
<td>Midazolam</td>
<td><img src="image" alt="Midazolam" /></td>
<td>4 µM</td>
<td>1’OH Midazolam</td>
<td><img src="image" alt="1’OH Midazolam" /></td>
</tr>
<tr>
<td>CYP2C19</td>
<td>S-mephenytoin</td>
<td><img src="image" alt="S-mephenytoin" /></td>
<td>10 µM</td>
<td>4’OH S-mephenytoin</td>
<td><img src="image" alt="4’OH S-mephenytoin" /></td>
</tr>
<tr>
<td>CYP2C9</td>
<td>Diclofenac</td>
<td><img src="image" alt="Diclofenac" /></td>
<td>10 µM</td>
<td>4’OH Diclofenac</td>
<td><img src="image" alt="4’OH Diclofenac" /></td>
</tr>
<tr>
<td>FMO</td>
<td>Cimetidine</td>
<td><img src="image" alt="Cimetidine" /></td>
<td>10 µM</td>
<td>Cimetidine Sulfoxide</td>
<td><img src="image" alt="Cimetidine Sulfoxide" /></td>
</tr>
</tbody>
</table>
Figure 4.1 Expression of CYP and FMO Genes in the Fetal, Pediatric and Adult Hepatic Tissues. Gene expression was normalized to the lowest expressed enzyme gene, namely adult FMO1, in order to show relative expression across all enzymes and all three populations. Fetal, pediatric, and adult samples are represented by the blue, yellow, and green bars, respectively. Inserts depict the relative expression of the CYP3A family [A], CYP2C family [B], and the FMO family [C].
Figure 4. 2 Expression of CYP3A, CYP2C, and FMO Proteins in Fetal, Pediatric, and Adult Hepatic Tissues Measured by Quantitative Proteomic Analysis Using LC-MS/MS. Panels depict the expression of the CYP3A family [A], CYP2C family [B], and
the FMO family proteins. [C]. For the CYP3A family, the green, yellow, and blue squares represent CYP3A4, CYP3A5, and CYP3A7 proteins, respectively. For the CYP2C family, the aqua and purple squares represent the CYP2C9 and CYP2C19 proteins, respectively. For the FMO family, the red, orange, and grey squares depict FMO1, FMO3, and FMO5 proteins, respectively. A comparison of mean enzyme protein expression across the fetal, pediatric and adult populations is shown in bar graphs for CYP3A [D], CYP2C [E], and FMO [F]. Fetal samples are depicted by white bars, the pediatric population by grey hatched bars, and the adult population by black bars.
Figure 4. 3 Functional Activity of CYP3A Represented by the Formation of 6β-OH Testosterone in HLM and Recombinant Enzymes. Panel A depicts the metabolite formation normalized to the total microsomal protein. Panel B represents the metabolite formation normalized to the CYP3A expressed in each sample. The horizontal line in each group represents the mean value.
Figure 4. 4 Representative Chromatographs from the LC-MS/MS Analysis of Testosterone Metabolite(s) Formed by CYP3A Enzymes. Each panel shows the chromatograms for testosterone (substrate), 6β-OH testosterone (metabolite), and voriconazole (internal standard). All chromatograms represent metabolite formation at the 30 minute time point for adult HLM [A], recombinant CYP3A4 [B], fetal HLM [C], and recombinant CYP3A7 [D]. The X-axis represents time in minutes and the Y-axis represents abundance of mass to charge corresponding to the indicated analyte.
Figure 4. 5 Functional Activity of CYP3A Represented by the Formation of 1’OH Midazolam from Midazolam. Formation rates were normalized to the CYP3A protein expression in each sample.
Figure 4. 6 Correlation Analysis of Enzyme Expression to Functional Activity.

Formation of 6β-OH testosterone is correlated with the expression of the sum of CYP3A4 and CYP3A5 proteins [A] and expression of the sum of CYP3A4, CYP3A5, and CYP3A7 proteins [B]. Formation of 1′OH midazolam is correlated with the expression of the sum of CYP3A4 and CYP3A5 proteins [C] and expression of the sum of CYP3A4, CYP3A5, and CYP3A7 proteins [D]. The graphs depict the linear correlation (solid line) and the 95th confidence intervals (dotted lines).
Figure 4. 7 CYP2C9 Functional Activity as Shown by the Formation of 4’OH

**Diclofenac.** Formation rates were normalized to the total microsomal protein [A] and the CYP2C9 protein in each sample [B]. Horizontal lines represent the mean values in each panel.
Figure 4. 8 CYP2C19 Functional Activity as Shown by the Formation of 4’OH

**S-mephenytoin.** Formation rates were normalized to the total microsomal protein [A] and the CYP2C19 protein in each sample [B]. Horizontal lines represent the mean values in each panel.
Figure 4.9 Correlation Analysis of CYP2C Enzyme Expression to Functional Activity.

Formation of 4’OH diclofenac is correlated with the expression of CYP2C9 protein [A].

Formation of 4’OH S-mephenytoin is correlated with the expression of CYP2C19 protein [B].

The graphs depict the linear correlation (solid line) and the 95th confidence intervals (dotted lines).
Figure 4. 10 FMO Functional Activity as Shown by the Formation of Cimetidine Sulfoxide. Samples were normalized to total microsomal protein [A] and the total FMO1 and FMO3 protein in each sample [B]. The bars [A] represent values derived from n= 6 and the error bars depict standard deviation. Horizontal lines [B] represent the mean values in each panel.
Figure 4. Correlation Analysis of FMO enzyme expression to functional activity.

Abundance measures the sum of the FMO1 and FMO3 isoforms. The graph depicts the linear correlation (solid line) and the 95th confidence intervals (dotted lines).
CHAPTER 5

Integrating *in vitro* Sildenafil Metabolism into a Physiologically Based Pharmacokinetic (PBPK) Model: Validating a Bottom-up Modeling Approach

OVERVIEW

Sildenafil, a phosphodiesterase-5 inhibitor, is approved for the treatment of pulmonary arterial hypertension in adults. It has become an attractive therapeutic option for premature neonates with pulmonary hypertension due to the lack of effective medications approved for this indication in this population. Characterization of sildenafil pharmacokinetics (PK) is necessary in premature neonates in order to reliably assess the efficacy of this drug in this population. Sildenafil is extensively metabolized by drug metabolizing enzymes (DMEs), with less than 2% of the parent compound being excreted unchanged in the urine. The ontogeny of these DMEs could impact sildenafil metabolism and, therefore, the PK in this vulnerable population. A physiologically based pharmacokinetic (PBPK) model was developed by integrating hepatic *in vitro* sildenafil metabolism data along with physiological parameters to predict the PK parameters of sildenafil in the adult population. Simulated populations contained 100 patients (10 trials with 10 patients each), and the trial design and dosing was based on published clinical trials. Simulations yielded PK parameters which were compared against published values, and visual predictive checks were employed to validate the models. The validated adult PBPK model was then adapted to create a model for premature neonates so as to determine the major factors that contribute to sildenafil PK premature neonates. The adult PBPK models predicted sildenafil PK parameters that corresponded well with observed values within a 1.6
fold-error. A PBPK model for intravenous (IV) infusions in full term neonates accurately predicted sildenafil clearance, and also demonstrated that the fraction of unbound drug affected its volume of distribution ($V_d$) and clearance. The prospective PBPK model for premature neonates suggests that this population exhibits differences in the fraction of unbound sildenafil as well as the fraction of sildenafil absorbed. The PBPK approach employed in this study enables the investigation of physiologic mechanisms that could lead to high variability in sildenafil PK in premature neonates.

**INTRODUCTION**

Sildenafil is a phosphodiesterase-5 (PDE5) inhibitor originally designed as a cardiovascular therapeutic agent. In 2005, seven years after its original approval, sildenafil was finally approved as a therapy for pulmonary arterial hypertension (PAH) in adults. PAH, a group of diseases with a wide range of causes including coronary heart disease and idiopathic PAH, has a high rate of mortality. Sildenafil is an optimal choice for treatment of PAH, as PDE5 is highly specific for cGMP [1]. Although protein expression of PDE5 has been quantified in the cerebellum, heart, pancreas, penile, and lung tissues, only lung and penile tissue demonstrate PDE5 activity [2]. By inhibiting PDE5, cGMP accumulates in the pulmonary smooth muscle cell and inhibits the sarcoplasmic reticulum from releasing calcium thereby inducing vasodilation (Figure 5. 1) [3].

A pivotal randomized control trial conducted in 278 PAH patients confirmed improvement in exercise tolerance, decrease in pulmonary pressure, and improvement in functional status with sildenafil therapy [4]. As cardiac and pulmonary diseases are not restricted to adults, clinicians also began to evaluate the efficacy of sildenafil in the treatment of congenital heart disease and pulmonary hypertension (PH) in children [5-7]. These small scale studies demonstrated that sildenafil was effective in reducing peripheral vascular resistance, time to extubation, time to hospital discharge, and improved exercise tolerance. However, due to the small number of subjects, these studies did not provide
sufficient power to evaluate the effect of sildenafil on morbidity or mortality in children. Thus, to better understand the safety and efficacy of sildenafil treatment in the pediatric population, the STARTS-1 trial, and the follow-up STARTS-2 trial, enrolled over 200 children 1-17 years of age [8, 9]. Results from these trials showed that a moderate dose of sildenafil led to modest improvements in hemodynamics and survival, whereas a high sildenafil dose resulted in increased mortality. Although the authors suggested that further trials are needed to determine optimal sildenafil dosing in children, the FDA issued a black box warning for sildenafil use in children 1-17 years of age.

The STARTS-1 and STARTS-2 trials did not examine the efficacy and safety of sildenafil in pre-term and term neonates, despite the fact that of the more than 80,000 infants born at ≤ 32 weeks of gestational age each year in the United States [10], 40% of neonates, specifically those born at ≤ 28 weeks of gestation, develop bronchopulmonary dysplasia (BPD), which can lead to pulmonary arterial hypertension [11]. Prematurity increases the risk of morbidity and mortality, with problems such as PAH, prolonged hospitalization, developmental delays [12-14] and cognitive [15, 16] and language [17, 18] impairments. The only approved treatment for PAH in neonates is inhaled nitric oxide from which it is difficult to wean these infants as that can lead to rebound hypertension. Other supportive measures that have been used are extracorporeal support, surfactant treatment, and ventilation [19]. Owing to the success in treating adult and pediatric PAH despite the FDA black-box warning, sildenafil is an attractive option in neonates as its off-label use in the premature neonatal population has been shown to improve hemodynamics as determined by ECG, and case reports suggest that sildenafil also improves outcomes in premature infants with BPD and PAH.[20-22]

Optimal sildenafil dosing in premature neonates is unknown and is extrapolated from adult dosing. Sildenafil is cleared predominantly by hepatic oxidative metabolism. During the drug development process, Pfizer identified cytochrome P450 (CYP) 3A4 and CYP2C9 as the
predominant enzymes that convert sildenafil to its main metabolite, N-desmethyl sildenafil (Figure 5.2) [23, 24]. Orally administered sildenafil is subject to extensive first-pass metabolism to the primary metabolite [25]. In healthy adult volunteers, the bioavailability of sildenafil is approximately 41% [26]. Since its elimination and PK rely so heavily on oxidative metabolism, simple allometric scaling of adult sildenafil dosing regimens is inappropriate for neonates, as hepatic DMEs are actively undergoing maturational changes in this population (Figure 4.2).

As was done for prediction of voriconazole PK in children of ages 2 to 10 (Chapter 2) [27], in vitro sildenafil metabolism data obtained with adult human liver microsomes (HLM) were used to create a bottom-up adult PBPK model. Using pediatric in vitro sildenafil metabolism data, the model was then adapted to the premature neonatal population for which clinical data are either sparse or not available. The pediatric PBPK model accurately described sildenafil PK in this population, as well as important physiological factors that affect the PK.

MATERIALS AND METHODS

Chemicals, Reagents, and Sample Selection

Sildenafil, N-desmethyl sildenafil, ketoconazole, quinidine, sulphaphenazole, fluvoxamine, NAPDH, and voriconazole were purchased from Sigma-Aldrich (St. Louis, MO). Pooled HLM were purchased from Xenotech, LLC (Lenexa, KS) and recombinant CYP3A4, 3A5, and 3A7 were purchased from Corning (Corning, NY). Human liver tissues from healthy adult (>18 years old), infant (2-5 months), and fetal (14-39 weeks gestation) donors were obtained from the NICHD Brain and Tissue Bank (Contract #HHSN275200900011C; Baltimore, MD).

Preparation of Microsomes

Human liver microsomes (HLM) were prepared as previously reported [28]. Briefly, 0.5mg of hepatic tissue was homogenized in buffer containing 50 mM Tris-HCl, 1 mM EDTA,
and 154 mM KCl with protease inhibitors (pH 7.4). After homogenization, the samples were centrifuged at 10,000 x g for 30 minutes, and the supernatant collected and centrifuged again. The supernatant from the second centrifugation was mixed with a buffer containing 100mM sodium pyrophosphate and 0.1 mM EDTA (pH 7.4) and then centrifuged at 100,000 x g for 70 minutes. This step was repeated and the resulting pellet was resuspended in a buffer containing 10 mM KPO$_4$, 1 mM EDTA, and 20% glycerol (pH 7.4). Microsomal protein concentration was measured using a BCA assay and HLM were stored in 20 µL aliquots at -80°C.

**Sildenafil Oxidative Metabolism by Human Liver Microsomes**

Infant and fetal HLM (n = 2 each) as well as pooled adult HLM were incubated with sildenafil (10 μM), NADPH (1 mM) and MgCl$_2$ (3 mM) in phosphate buffer (pH 7.4) to assess metabolic linearity over 60 minutes and a range of microsomal protein concentrations of 0.05 mg/mL – 0.4 mg/mL. After establishing the linearity of sildenafil metabolism, HLM were incubated with varying concentrations of sildenafil (0.5 – 250 µM) to determine $V_{\text{max}}$ and $K_{\text{m}}$ for microsomal metabolism in each population. At 15 minutes, 100 µL aliquots of the incubation mixture were quenched with 300 μL ice-cold methanol containing voriconazole (0.1 µM) as the internal standard. Samples were centrifuged and analyzed for N-desmethyl sildenafil formation by LC-MS/MS. Metabolite formation rate was subjected to nonlinear regression analysis (GraphPad Prism version 6; La Jolla, CA) using the Michaelis-Menten equation to determine $V_{\text{max}}$ and $K_{\text{m}}$.

**Role of Cytochrome P450 and Flavin-containing Monooxygenase (FMO) Enzymes in the Formation of N-desmethyl sildenafil**

Involvement of CYP3A, 2C9, 2C19, and 2D6 in sildenafil metabolism was assessed by incubating pooled HLM with sildenafil and inhibitors for CYP3A (0.3 µM ketoconazole), CYP2C9 (30 µM sulphaphenazole), CYP2C19 (3 µM fluvoxamine), and CYP2D6 (5 µM quinidine). The concentrations for the enzyme inhibitors were chosen based on their
potency for enzyme inhibition. In order to determine the contribution of FMO to sildenafil metabolism, pooled HLM were pre-incubated for 5 minutes in a water bath set at 45°C to inactivate FMO activity, and sildenafil metabolism was compared against metabolism by HLM that were not exposed to heat inactivation. In addition, recombinant FMO1 and FMO3 were incubated with sildenafil and monitored for metabolite formation. For all enzyme inhibition studies, N-desmethyl sildenafil formation was measured and the percent inhibition was assessed by comparing individual enzyme activity after inhibition to the activity of control. Finally, recombinant CYP3A enzymes (CYP3A4, CYP3A5, and CYP3A7) were incubated with sildenafil to determine the rate of metabolite formation. Differences in metabolite formation were analyzed using a Kruskal-Wallis non-parametric ANOVA.

**Metabolite Analysis**

The LC-MS/MS system consisted of mobile phase (A) 0.1% formic acid in water and (B) 0.1% formic acid in methanol with a total flow rate of 0.7 mL/min. A Sciex API-4000 triple quadropole mass spectrometer was used with a Zorbax SB-C18 column (2.1 x 50 mm, 5 µm). Samples were ionized using an ESI ion source and, following the injection of 10µL of supernatant, positive ions were monitored at the following MRM Q1/Q3 transitions (m/z): 461.3→283.2 for N-desmethyl sildenafil and 350.2→127.2 voriconazole.

**Model Structure**

A step-wise bottom-up approach (Figure 5. 3) was used to first validate the adult PBPK models and then prospectively determine sildenafil PK parameters in children (aged 0-5 months). Model development employed Simcyp software (version 14; Certara). Intrinsic clearance was calculated from the $V_{max}$ and $K_m$ values generated for fetal, infant, and adult tissues, as described above. Protein expression, determined by quantitative proteomic analysis using LC-MS/MS (Chapter 4), was incorporated into the model in order to accurately reflect fetal enzyme isoforms present in premature and full-term infants. Using a minimal PBPK model structure (Figure 5. 4), the perfusion-limited sildenafil model
incorporated physicochemical properties of the drug, and generated its intrinsic elimination, absorption and distribution parameters (Table 5.1). A single adjusting compartment was added to the model to account for the unexplored second compartment shown in clinical trials. Protein expression data generated in chapter 4 were incorporated into the model. Simulated populations were created from the healthy volunteer and pediatric simulators and contained 100 patients (10 trials with 10 patients in each trial). Adult simulations included both IV (20 mg and 40 mg infused over 40 minutes) and oral (50 mg and 100 mg) dosing regimens. For full term neonates, a clinically-used IV dose of 0.22 mg/h was infused over a mean time of 82.4 hour to simulate the PK profile. Finally, simulations were performed for the premature neonatal population using doses that are administered to patients in the Neonatal Intensive Care Unit at UNC Hospitals.

Model Validation

Simulations for sildenafil disposition in the adult population yielded PK parameters that were compared against published values as well as data submitted in NDA approval documents [29-33]. Full term neonatal infusion doses were validated against a population PK model [34]. Model accuracy was assessed by calculating average fold error of the predicted clearance in simulated individuals plotted as a function of age as defined by the following equation:

\[
AFE = 10^{\frac{1}{n} \sum \log\left(\frac{\text{predicted}}{\text{observed}}\right)} 
\]

(1)

AUC and \(C_{\text{max}}\) were assessed by generating the fold error for all simulated PK parameters using the following equation:

\[
\text{Fold error} = \frac{\text{predicted value}}{\text{observed value}}
\]

(2)

Predicted PK parameters were considered acceptable if they were within a 2-fold error of observed values [35, 36]. For individual plasma sample predictions, a percent error was utilized to determine the difference between observed and predicted using the equation:
Visual predictive checks were employed to confirm that the predictions of the sildenafil plasma concentrations by the model were consistent with the observed plasma concentrations and standard deviation. Concentration-time profiles for adults were taken from published reports as well as NDA submission documents and were digitized using GetGraph data digitizer version 2.25.0.32. Visual predictive checks were performed for full term neonates using data from a population PK study and for premature neonates using data from a phase I clinical trial conducted at UNC Hospitals (data currently unpublished) [34]. Observed sildenafil concentrations were superimposed on profiles containing the simulated mean and the 90% confidence intervals.

**Sensitivity Analysis**

Sensitivity analyses were performed to assess factors that could influence model performance. Specifically, the impact of a single adjusting compartment and fraction unbound, which were expected to affect the PBPK models, was evaluated. The input values were varied with a 10-fold increase and decrease to determine if either parameter significantly affected AUC and $C_{\text{max}}$.

**RESULTS**

**In Vitro Metabolism of Sildenafil by Human Liver Microsomes**

Metabolism of sildenafil by adult HLM was linear up to 15 minutes with microsomal protein concentrations of 0.05 mg/mL to 0.2 mg/mL. With 0.4 mg/mL of microsomal protein, metabolite formation was non-linear at 15 minutes. With fetal and infant HLM, the metabolism of sildenafil was linear up to 30 minutes with little to no metabolite formation under 5 minutes. In order to ensure adequate metabolite formation for quantitation, all assays were incubated for 15 minutes. With adult HLM, as the microsomal protein concentration increased from 0.05 mg/mL, the rate of formation of metabolite per mg protein
decreased (p < 0.001; Figure 5.5). With fetal samples, metabolite formation was only quantifiable at microsomal protein concentrations of 0.1 – 0.2 mg/mL, with no quantifiable metabolite present at a protein concentration of 0.05 mg/mL. Unlike adult HLM, the rate of metabolism (per mg protein) by fetal and infant HLM remained constant with protein concentrations up to 0.2 mg/mL. For all subsequent assays, a microsomal protein concentration of 0.1 mg/mL was used for all three populations, although for the adult HLM, the rate of metabolism per mg protein was slightly lower at 0.1 mg/mL versus 0.05 mg/mL protein.

The concentration-dependence of the rate of N-desmethyl sildenafil formation followed Michaelis-Menten kinetics (Figure 5.6). The apparent $K_m$ for fetal, infant, and adult HLM was similar (10.4 ± 2.7, 41.5 ± 22.3, and 16.4 ± 3.0 µM, respectively). However, a clear trend towards an increase in $V_{max}$ was observed from fetal to adult tissues. $V_{max}$ for adult HLM was 5-fold higher than that observed with fetal samples, and over 2-fold higher than $V_{max}$ obtained with infant tissues (23.5 ± 1.5, 65.8 ± 12.3, and 135.8 ± 7.0 pmol/min/mg protein, respectively; p < 0.01). At high sildenafil concentrations, infant samples displayed over a 100-fold difference in N-desmethyl sildenafil formation between subjects. Eadie-Hofstee plots (data not shown) confirmed the involvement of two enzymes in the metabolism of sildenafil by adult HLM. The intrinsic clearance of sildenafil by these two enzymes differed by 8-fold (187.1 and 22.2 µL/min/mg protein). An Eadie-Hofstee plot for fetal samples demonstrated that only one enzyme was involved in sildenafil metabolism with an intrinsic clearance value of 4.9 µL/min/mg protein.

**Metabolism of Sildenafil by Human Liver Microsomes and Recombinant Enzymes to Assess Contribution of CYP3A and CYP2C9**

In order to confirm the involvement of CYP3A and CYP2C9 enzymes in sildenafil metabolism, chemical inhibitors for these two enzymes, namely ketoconazole (0.3µM) for CYP3A and sulphaphenazole (30µM) for CYP2C9 were used as previously described [37].
Chemical inhibition by ketoconazole resulted in a 90% decrease in metabolite formation ($p < 0.05$), confirming the involvement of CYP3A in sildenafil metabolism. However, no difference in metabolite formation was observed in the presence of sulphaphenazole (Figure 5.7). Therefore, the chemical inhibition scheme was broadened to assess the possible involvement of CYP2C19, CYP2D6, and FMO; i.e. inhibition of CYP2C19 with fluvoxamine (3 µM), CYP2D6 with quinidine (5 µM), and FMO by thermal inactivation. No decrease in metabolic activity of HLM was observed with chemical inhibition of CYP2C19 or heat inactivation of FMO; furthermore, recombinant FMO1 and FMO3 did not form $N$-desmethyl sildenafil. These results clearly demonstrated that CYP2C19 and FMOs do not contribute to sildenafil metabolism. Although CYP2D6-mediated sildenafil metabolism decreased by 20% in the presence of quinididine, this difference was not statistically significant.

Since chemical inhibition of CYP3A decreased metabolite formation by over 90%, the contributions of three isoforms of CYP3A, namely CYP3A4, CYP3A5, and CYP3A7, to sildenafil metabolism were evaluated using recombinant enzymes (Figure 5.8a). All three isoforms formed $N$-desmethyl sildenafil, with CYP3A5 showing a 4-fold and 7-fold higher metabolite formation compared to CYP3A4 and CYP3A7, respectively. Next, metabolism of sildenafil by the three CYP3A isoforms was examined as a function of substrate concentration. CYP3A5 exhibited a higher $V_{\text{max}}$ than either CYP3A4 or CYP3A7 ($77.1 \pm 17.1$, $56.0 \pm 6.5$, and $4.6 \pm 0.6$ pmol/min/pmol of CYP3A; Figure 5.8b), whereas the apparent $K_m$ was similar between CYP3A5 and CYP3A7 ($49.9 \pm 30.0$ versus $44.3 \pm 15.1$ µM). The apparent $K_m$ for CYP3A4 was over 2.5-fold higher than the $K_m$ of the other two isoforms ($118.1$ µM ± 29.0), indicating that CYP3A4 has a low-affinity for sildenafil. Furthermore, intrinsic clearance of sildenafil by CYP3A5 (3.9 µL/min/pmol CYP3A) was 3-fold higher than that observed with CYP3A4, and 14-fold higher than the intrinsic clearance by CYP3A7 (1.2 and 0.1 µL/min/pmol CYP3A). These results suggest that
CYP3A5 has a high-capacity and high-affinity for sildenafil, whereas CYP3A7 is a high-affinity, low-capacity enzyme and CYP3A4 is a low-affinity, high-capacity for this substrate.

Metabolism of sildenafil by adult HLM exhibited biphasic Eadie-Hofstee plots, suggesting involvement of two CYP3A enzymes, CYP3A4 and CYP3A5. To determine the intrinsic clearance of sildenafil by each CYP3A in adult HLM, the intrinsic clearance generated from individual recombinant CYP3A4 and CYP3A5 was multiplied by the mean abundance of the respective CYP3A enzymes in HLM (Chapter 4). This approach allowed a direct comparison between intrinsic clearance of sildenafil by recombinant CYP3A enzymes and its intrinsic clearance by the respective CYP3A enzymes in fetal and adult HLM. The intrinsic clearance of sildenafil by the two enzymes in adult HLM, derived from Eadie-Hofstee plots, was 187.1 and 22.2 µL/min/mg protein, respectively, with approximately eight-fold difference between the two clearances. The intrinsic clearance of sildenafil by recombinant CYP3A4 and CYP3A5 enzymes was 90 and 11.7 µL/min/mg protein, respectively, also showing approximately eight-fold difference. Thus, it is reasonable to conclude that sildenafil clearance by CYP3A4 was 187.1 µL/min/mg protein and by CYP3A5 was 22.2 µL/min/mg protein in HLM. These values were then used in the adult models.

**Adult PBPK Models for Oral and IV Administration of Sildenafil**

Initially, a bottom-up approach using the enzyme kinetics of sildenafil metabolism derived from adult HLM was utilized to test the ability of the PBPK model to describe adult sildenafil plasma concentrations following IV infusion and oral dosing. The model predicted a $V_d$ of 1.4 L/kg (90% confidence interval of 1.1-1.8 L/kg), which corresponds well with published clinical values of 1.2-1.4 L/kg [29, 38]. The AUC for 20- and 40 mg IV infusions was slightly over-predicted with a mean fold error of 1.6 and 1.4, respectively, and the predicted $C_{max}$ for these doses was within a 0.9 and 0.7 fold error, respectively. Model predictions of the AUC and $C_{max}$ for oral doses of 50- and 100 mg doses were accurate, and were within a 1.1-fold and 1.3-fold error for the AUC and $C_{max}$, respectively, as shown in
Figure 5.9. The simulated $T_{\text{max}}$ for oral doses averaged 1h (90% confidence interval of 0.73-1.3 h), which is in agreement with the observed $T_{\text{max}}$ of 1.0-1.5 hours [29, 25]. Finally, the model predicted a mean sildenafil bioavailability of 39% for both oral doses, which is within the range of 38-41% of its reported bioavailability [29, 25, 38], and a predicted 90% confidence interval range of 35% to 40%. The mean IV sildenafil clearance of 17.9 L/h was under-predicted by a small degree, with a mean average fold error of 0.66. The PBPK models predicted an oral clearance of 45.4 L/h for sildenafil, which corresponded with the average oral clearance of 57 L/h, with a mean average fold error of 0.92. The average fold error for adults based on age is shown in Figure 5. 10a. Visual predictive checks for sildenafil IV dosing confirmed that simulated concentration-time profiles contained the mean sildenafil plasma concentration for each time point within the 90% confidence intervals (Figure 5. 11). For sildenafil oral doses, the 90% confidence interval predicted by the model included the concentrations at each time point (Figure 5. 12).

**Neonatal PBPK Models for Oral and Intravenous Sildenafil**

Applying this approach to the neonatal population, data on sildenafil intrinsic clearance by CYP3A7, generated with fetal HLM were incorporated into a PBPK model. The initial neonatal PBPK model predicted a sildenafil clearance of 1.8 L/h, which corresponded well with a clearance of 1.72 L/h in full term neonates that was determined by population PK analysis. Figure 5. 10B shows the average fold error for sildenafil clearance in simulated neonates during the first 7 days of life. Since the population PK study reported that sildenafil is 93.9% protein-bound, the fraction unbound parameter was incorporated into the PBPK model. Following the integration of these changes into the model, the $V_d$ of sildenafil increased from 1.1 L/kg to 1.6 L/kg, resulting in an increase in predicted total systemic clearance of this drug from 1.8 L/h to 2.5 L/h. The simulated means and 90% confidence intervals of the visual predictive checks for full term neonates encompassed 49% of all data points (Figure 5. 13). The change in sildenafil clearance normalized to bodyweight from birth
to adulthood is represented as a graph in Figure 5. 14. Model-predicted sildenafil clearance decreased from the first month of life to 6 months of age, with a 1.3-fold increase in clearance when the fraction unbound was doubled in the first month of life.

In order to determine the effect of the ontogeny of the CYP3A family on sildenafil clearance from birth to adulthood, the fraction of sildenafil metabolized by each CYP3A isoform was estimated and the data are shown as a graph in Figure 5. 15. CYP3A7 accounted for over 89% of sildenafil metabolism within the first month of life, which decreased to less than 1% by adulthood. Conversely, CYP3A4 and CYP3A5 accounted for 8% and <0.1% in the first month of life, respectively, and increased to 92% and 2.8% in adults, respectively.

Finally, a prospective PBPK model for premature neonates was created, and model predictions were compared against clinical data from one of the patients enrolled in a recent phase I clinical trial that has currently enrolled and analyzed concentration-time data for four premature neonates. This patient received oral dexamethasone, a known CYP3A4 inducer, at the end of plasma sample collection, which was used to test if the model could identify a DDI. Four iterations were tested including a decrease in fraction absorbed, a decrease in fraction of unbound sildenafil, a decrease in both fraction unbound and fraction absorbed, and enzyme induction by dexamethasone. Visual predictive checks were conducted to determine if sildenafil concentrations in the sparse samples collected from a neonate born at 25 weeks gestation were within the 90% confidence intervals (Figure 5. 16). The predictions of the first two iterations of the model that incorporated only the adjustments in the fraction of the sildenafil dose absorbed or in the fraction unbound showed that the sildenafil concentrations from three samples were within the 5th percentile of the model. However, the model over-predicted the concentrations of the final sample which was collected after the administration of dexamethasone, by more than 600%. Integrating a decrease in both the fraction of sildenafil absorbed and the fraction of unbound drug improved the model so that
two of the three samples were within a percent error of 3.4 % and 13.8 % of the simulated mean and the third sample was within the 5th percentile.

The final step was to incorporate the potential increase in sildenafil intrinsic clearance by all three CYP3A isoforms due to enzyme induction by dexamethasone administration. This iteration kept the decrease in both the fraction of sildenafil absorbed and the fraction of unbound drug. These adjustments improved model predictions so that all four samples from the patient were contained within the 90% confidence intervals.

**Sensitivity Analyses**

Sensitivity analysis was applied to adjust the input parameters of the fraction unbound as well as components of a single adjusting compartment by 10-fold in both directions (i.e., increase or decrease) to determine how these changes affect AUC, clearance, \( C_{\text{max}} \), and other pertinent PK parameters. Sensitivity indices measure the ratio of changes in PK parameters over the change in input parameters (\( \frac{\Delta \text{PK Parameter}}{\Delta \text{Input Parameter}} \)). The sensitivity analysis of the single adjusting compartment in both neonatal and adult populations showed no significant differences in any PK parameters in either population. However, when the fraction unbound was increased, the \( V_d \) and total systemic clearance increased while AUC and \( C_{\text{max}} \) decreased (Figure 5.17).

**DISCUSSION**

It has been nearly two decades since sildenafil first received FDA approval. Since then, therapeutic indications have expanded from its original approved indication in adult males to include adult females and pediatric/neonates/premature neonates as patients. Despite including the patient population over a wide age range, little information is known about how the ontogeny of DMEs affects sildenafil PK. Since sildenafil is extensively metabolized, it is critical to understand how maturational changes in the predominant sildenafil metabolizing enzymes affect its PK.
Originally, Hyland et al. identified CYP3A4 and CYP2C9 as the two main enzymes responsible for sildenafil metabolism [23]. However, within the same year, Warrington et al. reported that CYP2C9 plays only a minor role in sildenafil metabolism, leaving a significant gap in knowledge that makes it important to elucidate the role of DMEs in sildenafil metabolism and its clearance, their ontogeny, and how they could play a role in DDI. The in vitro work presented in this research corroborated the work by Warrington et al., and implicated CYP3A as the primary enzyme that contributes to over 90% of sildenafil metabolism. Further, the current work expanded on the previous studies, and demonstrated that genetically polymorphic CYP3A5 [39] was also involved in sildenafil metabolism. In fact, studies with recombinant CYP3A enzymes showed that CYP3A5 had higher catalytic activity toward sildenafil than CYP3A4 or CYP3A7. Thus, it was necessary to predict the contribution of CYP3A5 to sildenafil metabolism based on its tissue expression. When the intrinsic clearance of recombinant CYP3A isoforms were normalized to their abundance, it was apparent that CYP3A4 would have greater contribution toward sildenafil metabolism than CYP3A5.

The rate of sildenafil metabolism by fetal, infant, and adult HLM increased with increasing concentrations of the parent drug. Infant HLM, obtained from tissue samples harvested from individuals ranging in age from 2-5 months, displayed a high variability in sildenafil metabolism with some HLM exhibiting enzyme activity similar to that observed in adults, and other infant HLM with activity that was similar to fetal samples. This is not unexpected as CYP3A7 expression decreases and CYP3A4 increases over this age range. Due to this change in enzyme expression and overall enzyme activity, the intrinsic clearance of sildenafil as determined with an Eadie-Hofstee plot could not be resolved. However, because adult HLM express CYP3A4 and CYP3A5 and fetal samples primarily express CYP3A7, Eadie-Hofstee plots were utilized to determine the intrinsic clearance of sildenafil by each CYP3A isoform in HLM. The difference in intrinsic activity between CYP3A4 and
CYP3A5 in HLM was similar to that observed using recombinant enzymes. Interestingly, the intrinsic clearance for recombinant CYP3A7, normalized to enzyme expression, was 10-fold higher than the intrinsic clearance of sildenafil determined with HLM, demonstrating that recombinant systems are not always predictive of tissue enzyme activity. Recombinant systems typically reflect on adult cellular physiology, including protein and oxidoreductase composition, and do not necessarily reflect fetal or pediatric cellular physiology. These concepts were also discussed in Chapter 4.

Incorporating adult CYP3A intrinsic clearance data into the PBPK model produced a reliable adult model for IV and oral dosing. These models provided strong evidence that the bottom-up modeling approach using intrinsic clearance data derived from hepatic HLM could accurately predict the PK parameters of sildenafil. Next, CYP3A7 intrinsic activity was incorporated into a neonatal PBPK model. Model simulations were based on the average sildenafil dose and the duration of sildenafil infusions that were presented in a population PK analysis. By incorporating the known value of sildenafil fraction unbound in adults, the PBPK model accurately predicted sildenafil clearance and accounted for 20% of the variability during the maintenance phase of the IV infusion. Next, the model was altered to incorporate a higher unbound fraction (6.1%) of sildenafil in the neonates. The simulated mean and the 90% confidence intervals derived using the unbound fractions of 3.6% and 6.1%, comprised 49% of all measured plasma samples. Differences in the infusion duration of sildenafil as seen with patient 1 or physiological differences observed in critically ill patients could account for plasma concentration data that were not within the 90% confidence intervals.

The neonatal PBPK model also successfully predicted the fraction of sildenafil metabolized by CYP3A as well as clearance of the drug over the first year of life. In contrast to the published sildenafil population PK report by Mukherjee et al. [34], the neonatal PBPK model developed in the present study predicted that the fraction of sildenafil metabolized by each isoform over the first seven days of life would not be altered. In fact, the only significant
difference predicted by the model in sildenafil clearance was due to the sildenafil fraction unbound. Yet, while clearance and $V_d$ were evaluated and presented, the model did not account for changes in protein binding. Thus, the primary conclusion that rapid maturation of enzymes contributes to increased sildenafil clearance was weakened by the authors’ failure to examine differences in protein binding.

Furthermore, sildenafil clearance data published in their analysis was 1.72 L/h, which converts to 0.51 L/h/kg based on the average weight of the patients enrolled in the study. The IV clearance of this drug in adults is estimated to be approximately 27 L/hr, which is about 0.39 L/h/kg based on an average adult bodyweight of 70 kg. Based on these reported values, sildenafil clearance normalized to bodyweight decreases from the neonatal phase into adulthood, which corroborates the clearance profile presented in this research.

Finally, by adapting the validated adult PBPK model to create a prospective neonatal PBPK model for sildenafil, we were able to confirm that the bottom-up approach could accurately predict CYP3A7-mediated in vivo clearance of the drug. In the premature neonatal model, the patient that was used for the visual predictive checks received 3 mg of sildenafil for over 500 hours before receiving dexamethasone, a known CYP3A inducer. However, in this case, food and medications were delivered via a G-tube which could lower the fraction of drug absorbed as reported previously [40]. Therefore, factors such as age, as well as absorption issues, had to be taken into consideration during the premature neonatal PBPK model development.

It is known that the physiology of the gastrointestinal tract matures with age, and well-documented differences in medication absorption, and therefore PK, exist between neonates and adults. For example, it has been reported that absorption of doxapram is lower in premature neonates compared to adults [41]. The overall effects on drug absorption vary depending on the physicochemical properties of the drug. In an early example of this, acid-labile compounds, such as penicillin, were shown to have greater absorption in
premature and full term neonates compared to infants and children due to the higher gastric pH of the stomach [42]. For highly lipophilic compounds, the decrease in bile salts and slower gastric emptying time during the neonatal phase affect the extent of absorption [43]. Furthermore, food increases the pH in the stomach, thus diminishing the gastric dissolution of weak bases [44]. Studies in adults have shown that food slows down the absorption of sildenafil and also decreases both its $C_{\text{max}}$ and AUC [26]. While it was concluded that in adults these effects would not significantly affect the PK of sildenafil, these changes in sildenafil absorption could have a much larger and more clinically relevant effect in neonates where drug absorption is slower and often erratic [45]. Finally, differences in absorption can be seen when administering medications via a G-tube, in which all other medications and food are also delivered. For these reasons, the integration of a decrease in the fraction of the sildenafil dose absorbed in the premature neonatal model was warranted.

Changes in protein binding associated with age creates a multifaceted concern with drug absorption in neonates. Protein binding is a complex process involving bilirubin, albumin, and other plasma proteins, and has been linked to differences in drug clearance in premature and full term neonates [46-48]. Despite normalization of protein concentrations in the plasma, binding is still lower in neonates compared to adults, indicating that neonatal plasma proteins have a lower affinity for substrates [49]. Increasing unbound fraction of a drug in the plasma increases the $V_d$ of the compound [50], which was predicted for sildenafil by the PBPK model.

When assessing whether protein binding can affect the pharmacokinetic profile of a compound, it is essential to understand the factors that affect clearance. Hepatic clearance ($Cl_\text{Hepatic}$) is influenced primarily by blood flow ($Q_H$) and extraction ratio (E) as shown by equation (4).

$$Cl_\text{Hepatic} = Q_H \times E$$ (4)
Extraction ratio is the efficiency of an organ to clear a compound during one pass and ranges from 0 (no clearance) to 1 (complete clearance). Compounds are empirically labeled as low (≤ 0.3), intermediate (0.3 - 0.7) or high extraction (≥ 0.7), as per the values shown in the parentheses. The extraction ratio is calculated from the systemic unbound fraction ($f_u$), intrinsic clearance ($C_{int}$), and hepatic blood flow ($Q_H$) as shown by equation (5).

$$ E = \frac{f_u \times C_{int}}{Q_H + (f_u \times C_{int})} \quad (5) $$

Integrating equation 5 into equation 4, the final equation (6) for hepatic clearance is:

$$ C_{Hepatic} = Q_H \times \frac{f_u \times C_{int}}{Q_H + (f_u \times C_{int})} \quad (6) $$

In healthy adults, sildenafil is classified as an intermediate to high extraction compound with an extraction ratio between 0.6-0.7. As evidenced by equation 6, only hepatic blood flow influences clearance for high extraction compounds and changes in protein binding do not affect total compound concentrations. Therefore, in adults, changes in protein binding would not yield any differences in sildenafil PK.

However, with intermediate extraction compounds, all three components (protein binding, blood flow, and intrinsic clearance) influence total compound concentration and systemic clearance. It is reasonable to assume that the lower intrinsic clearance in the fetal samples signifies a lower extraction ratio of sildenafil in premature neonates compared to adults, thereby making sildenafil at best an intermediate extraction compound in this population. This concept is important since premature neonates have lower expression of plasma proteins and a decreased affinity of compounds to these proteins.

As shown in the population PK analysis by Mukherjee et al., full term neonates have a higher fraction of unbound sildenafil in plasma as well as higher weight-normalized clearance compared to adults supporting this hypothesis [34]. Similarly, a higher weight-normalized clearance of micafungin is seen in neonates as compared to adults. Micafungin plasma binding was measured in serum taken from adults and neonates. This study found
that the higher fraction of unbound micafungin in neonates was responsible for the higher clearance compared to adults [48]. In addition, age-dependent differences in the extraction ratio have already been presented in this dissertation (Chapter 2). Accordingly, more data are necessary on the extent of sildenafil protein binding in premature and full term neonates. Both the contribution and the affinity to specific plasma proteins need to be elucidated in order to validate any premature or full term neonatal model for sildenafil.

Combining these two physiological parameters improved the prediction of the model to include 75% of the measured plasma samples within 90% confidence intervals of the simulated mean. The only plasma sample that was not encompassed by the model was one that was collected following the administration of four doses of dexamethasone, a known inducer of CYP3A4.

While CYP3A4 induction by a variety of medications has been conclusively shown, little is known about how these inducers affect CYP3A7. Induction of the CYP3A isoforms in fetal hepatocytes demonstrated that binding of ligands to glucocorticoid receptors can induce the gene expression of CYP3A7 [51, 52]. Limited data are available on the extent to which CYP3A7 activity is induced. Studies with CYP3A4 have shown that gene expression does not always correlate with induction of enzyme activity [53, 54]. These studies, as well as initial studies examining the effect of inducers on enzyme activity, revealed that, following induction, the apparent $K_m$ remains unchanged, while the $V_{max}$ can increase by 2- to 8-fold. Assuming an increase in the $V_{max}$ of CYP3A7, similar to that observed with CYP3A4 induction by dexamethasone, the intrinsic clearance by all CYP3A isoforms was doubled. Following the incorporation of this higher intrinsic clearance into the premature neonatal PBPK model, the fit of the model improved to also include the sample with the lowest sildenafil concentration that was collected after four doses of dexamethasone.

One limitation of this research is the inability to specifically inhibit only one CYP3A isoform without affecting the activity of the other isoforms. Due to this limitation, Eadie-
Hofstee plots were required to determine the $V_{\text{max}}$ and $K_m$ of the two enzymes involved in sildenafil metabolism. Despite this limitation, a correlation was observed between the fold-difference in the intrinsic activity of recombinant CYP3A4 and CYP3A5 and the intrinsic clearance of sildenafil by the two enzymes in HLM. Furthermore, the contribution of each isoform could be predicted as enzyme activity and protein expression were incorporated into the PBPK model. Model predictions showed that the fraction of sildenafil metabolized by each isoform was altered in different age groups, suggesting the maturation-related changes in the expression of CYP3A isoforms.

Another challenge with this study was the limited data on the optimal AUC or $C_{\text{max}}$ that is required to reach the target endpoint. Current studies exploring target endpoints and the pharmacodynamics (PD) of sildenafil in premature neonates are ongoing. Until a specific target for the endpoint and further analysis of the PD profile are elucidated, a target dose cannot be reached. However, after validating the ability of the model to predict sildenafil PK and PD, this model can be utilized to assess the dose required to reach the target sildenafil exposure.

The PBPK models presented here demonstrate that a bottom-up approach can be adopted to understand medications that are cleared extensively by oxidative metabolism. This strategy greatly advances our understanding of how the ontogeny of DMEs influences the contribution of enzymes to metabolic clearance of drugs. An in-depth understanding of the expression and activity of each DME can enable us to determine its interaction with physiological factors that play a pivotal role in the variability of drug PK in premature neonates. By identifying factors that affect this variability in PK, future clinical trials assessing sildenafil PK-PD in premature neonates can be improved in order to attain better outcomes.
REFERENCES


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42. Huang NN, High RH. Comparison of serum levels following the administration of oral and parenteral preparations of penicillin to infants and children of various age groups. The Journal of pediatrics. 1953;42(6):657-8.


Table 5. 1 Table Summary of Sildenafil Intrinsic PK Parameters and Physiochemical Properties Used for Model Development. $F_a$ fraction of sildenafil absorbed; SAC single adjusting compartment; Plasma $f_u$ fraction unbound in the plasma; $f_{u,Mic}$ fraction unbound in microsomal incubation; $Cl_{int}$ intrinsic clearance.

<table>
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<th>Parameter</th>
<th>Input</th>
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</thead>
<tbody>
<tr>
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</tr>
<tr>
<td>Compound Type</td>
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</tr>
<tr>
<td>LogP</td>
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</tr>
<tr>
<td>$pK_a$</td>
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</tr>
<tr>
<td>Absorption Model</td>
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<tr>
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</tr>
<tr>
<td>$K_a$ (1/h)</td>
<td>2.58 [55]</td>
</tr>
<tr>
<td>Distribution Model</td>
<td>Minimal PBPK Model</td>
</tr>
<tr>
<td>Prediction Model</td>
<td>Method 1 (Poulin and Theil)</td>
</tr>
<tr>
<td>SAC Q (L/h)</td>
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</tr>
<tr>
<td>Plasma $f_u$</td>
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<tr>
<td>$f_{u,Mic}$ predicted</td>
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<tr>
<td>Renal Clearance (L/h)</td>
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</table>

Elimination: Enzyme Kinetics (HLM)

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<tr>
<th></th>
<th>CYP3A4</th>
<th>CYP3A5</th>
<th>CYP3A7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatic $Cl_{int}$ (μL/min/mg)</td>
<td>187.1</td>
<td>22.2</td>
<td>4.9</td>
</tr>
</tbody>
</table>
Figure 5. 1 Sildenafil Mechanism of Action. Nitric oxide synthases (NOS) produces nitric oxide (NO) which is released to the pulmonary smooth muscle cells and activates guanylate cyclase to produce cGMP, preventing the release of calcium, which induces contractions, from the sarcoplasmic reticulum (SR) thereby allowing for vasodilation.
Figure 5. 2 Metabolism of Sildenafil by Drug Metabolizing Enzymes. Sildenafil is metabolized to the main circulating metabolite, $N$-desmethyl sildenafil. This metabolite is then eliminated into the feces (approximately 70-85% of the dose). An aliphatic hydroxylation of sildenafil occurs and that metabolite is excreted into the urine (approximately 10-15% of the dose)
Figure 5. 3 Sildenafil PBPK Model Workflow. The diagram outlines the bottom-up approach for scaling the \textit{in vitro} intrinsic clearance of sildenafil from adults to full-term and premature neonates as well as the output from the model.
Figure 5. 4 Sildenafil Minimal PBPK Model Utilized in Simcyp. The diagram demonstrates the model structure for the minimal PBPK model, which includes the rate of absorption ($k_a$) from the small intestines into the portal vein. From the portal vein, sildenafil travels with the portal vein blood flow ($Q_{PV}$) to the liver where it can be metabolized. Sildenafil then moves with the hepatic blood flow ($Q_H$) to the systemic compartment from where it can flow back to the liver, the portal vein or into a single adjusting compartment. Intravenous infusions follow similar pathways except for absorption through the small intestine.
Figure 5. 5 Sildenafil Metabolism as a Function of Microsomal Protein Concentration by HLM from Fetal, Infant, and Adult Tissues. Microsomes prepared from adult, fetal, and infant hepatic tissues were incubated in triplicate at microsomal concentrations between 0.05 mg/mL and 0.4 mg/mL. Metabolism by adult, fetal, and infant samples are shown as the mean ± standard deviation in shades of blue, yellow, and purple, respectively.
Figure 5. 6 Concentration-dependent Formation of N-desmethyl Sildenafil by HLM from Fetal, Infant, and Adult Tissues.

Sildenafil at concentrations of 0.5 µM to 250 µM were incubated with fetal [A], infant [B], and adult [C] HLM (n = 6 per group) in triplicate and shown as the mean ± standard deviation for each individual. A nonlinear regression curve was fit to the data using the Michaelis-Menten equation.
Figure 5. Contribution of CYP3A, CYP2C9, CYP2C19, CYP2D6, and FMO1/3 to Sildenafil Metabolism by Human Liver Microsomes. [A] Inhibition of sildenafil metabolism was assessed by incubating pooled HLM (n = 3) with chemical inhibitors of CYP3A (yellow), CYP2C9 (green), CYP2C19 (purple), and CYP2D6 (pink). [B] Contribution of FMO1/3 to sildenafil metabolism was assessed by heat inactivation of microsomes (compare blue bar and the bar with grey vertical lines), and by incubating sildenafil with recombinant FMO1 and FMO3. *In vitro* data are presented as mean ± standard deviation.
Figure 5. 8 Rates of Formation of $N$-desmethyl Sildenafil by Recombinant CYP3A4, CYP3A5, and CYP3A7. [A] Turnover rates of the formation of $N$-desmethyl sildenafil is depicted for CYP3A4 (blue), CYP3A5 (yellow), and CYP3A7 (green). [B] Rates of sildenafil metabolism to $N$-desmethylsildenafil by CYP3A4 (blue squares), CYP3A5 (yellow squares), and CYP3A7 (green squares) as a function of sildenafil concentrations. A nonlinear regression curve was fit to the data using the Michaelis-Menten equation. Data are presented as mean ± standard deviation.
Figure 5. 9 Predictive Accuracy Plots for AUC and $C_{\text{max}}$. The fold error ± standard deviation for AUC (circles) and $C_{\text{max}}$ (squares) of the 100 simulated patients in each dosing simulation are shown for IV infusions of 20- (yellow) and 40 mg (green) as well as oral doses of 50- (blue) and 100 mg (purple) of sildenafil.
Figure 5. 10 Predictive Accuracy Plots for Clearance. Average fold error was calculated for each simulated individual and compared against observed clearance. Adult clearance [A] depicts the average fold error for oral (blue squares) and IV (yellow circles) dosing. For full term neonatal clearance [B] average fold error was compared against typical clearance values for neonates within the first 7 days of life.
Figure 5. 11 Visual Predictive Checks for Adult Intravenous Infusions of Sildenafil.

Concentration-time profiles are shown for the 20 mg [A] and 40 mg [B] doses of sildenafil infused over 40 minutes. For all graphs, the solid black lines represent the simulated mean and the dashed lines represent the 95th and 5th percentiles. Insets represent the concentration-profiles on a log scale.
Figure 5. 12 Visual Predictive Checks for Adult Oral Sildenafil Doses. Concentration-time profiles represent a 50 mg [A] and 100 mg [B] doses of sildenafil. For all graphs, the solid black lines represent the simulated mean and the dashed lines represent the 95th and 5th percentiles. Insets represent the concentration-profiles on a log scale.
Figure 5. 13 Visual Predictive Checks for an Intravenous Infusion in Full Term Neonates. The simulated dose was based on an averaged administered dose from published reports [34]. The simulated concentration-time profiles were generated by varying the $f_u$ from the average adult value ($f_u = 0.036$; black solid line) to the average neonatal value ($f_u = 0.061$; blue solid line). The dashed lines represent the 95th and 5th percentiles.
Figure 5. Predicted Sildenafil Clearance from Birth until Adulthood. Changes in total systemic clearance were predicted using intrinsic clearance values with population ontogeny and expression of CYP3A isoforms. The neonatal group was split into two groups to demonstrate the range in total systemic clearance attributable to differences in protein binding observed in full term neonates [34]. The boxes represent the median clearance and the bars show the minimum and maximum values for clearance.
Figure 5. Ontogeny of the Fraction of Sildenafil Metabolized by CYP3A Isoforms. Pie charts represent the predicted percent metabolized by each CYP3A isoform as well as the fraction excreted renally.
Figure 5. 16 Visual Predictive Checks for Oral Doses to Premature Newborns. Prospective PBPK models were created by
decreasing the fraction absorbed [A], increasing the unbound fraction of sildenafil [B], combining the increase in fraction unbound and the decrease in fraction absorbed [C]. The last iteration of the model included decreases in fraction unbound and fraction absorbed, but also incorporated higher intrinsic clearance for all three CYP3A isoforms [D] due to the administration of oral dexamethasone, indicated by the arrow.
Figure 5. Sensitivity Analysis for Sildenafil Fraction Unbound. Sensitivity analyses were performed to assess the effect of increasing the fraction unbound for neonatal $V_d$ and clearance [A], adult $V_d$ and clearance [B], neonatal AUC and $C_{max}$ [C], and adult AUC and $C_{max}$ [D].
An Introduction to the Historical Context of Pediatric Clinical Trials

Approximately 26% of the worldwide population is under 15 years of age, and thus would be classified as pediatric population [1]. Even in the United States, a country in which most, if not all, pharmaceutical companies obtain regulatory approval for marketing new products, over a quarter of the population is under 18 years of age. Despite this sizable pediatric population in the United States and worldwide, drug development plans focus on adult patients over the age of 18 years. Traditionally, adult doses have been scaled down by normalizing to body weight to approximate the pediatric dose, assuming that distribution and clearance are the same between adults and children, and then adjusting that dose by trial and error until a clinical response is achieved. This watch-and-see method leaves children vulnerable to toxic doses or inadequate efficacy.

Many obstacles have led to this reluctance by the pharmaceutical companies and clinical researchers in identifying safe and effective doses for various pediatric populations during drug development. Issues surrounding pediatric clinical trials arise include risk-to-benefit ratio associated with the clinical investigation, the blood volume available for multiple blood monitoring, the pain associated with blood collection, and the difficulty and ethical issues in obtaining consent. All of these factors increase the difficulty in recruiting pediatric patients into clinical trials. In spite of these significant impediments to pediatric research, safety and efficacy data are still needed for this population. As shown by Benjamin et al., pediatric patients can present significantly different adverse events and safety profiles than their adult counterparts,
and pediatric clinical trials are the only way to obtain these data [2]. Administering medications to children without a complete understanding of their efficacy and safety profile is unethical. Therefore, in 1997 the United States created a legal framework to incentivize pharmaceutical companies that developed clinical evidence for safety and efficacy in pediatric populations of new and marketed products by extending marketing exclusivity periods for approved drugs to these companies. However, it took another six years before the Pediatric Research Equity Act (PREA) was enacted in the United States, and pediatric studies became mandatory for medications that could be used in pediatric populations. These regulatory requirements compelled the research community to develop improved methods to optimize doses for the pediatric populations while minimizing the risk associated with clinical research involving these populations.

**Development of Methods to Improve Pediatric Dosing Selection**

Conventional methods to predict pediatric clearance and dose utilize allometric scaling. When scaling down adult doses based on body weight, allometry could not accurately predict pediatric clearance, and a few different equations (chapter 1) were formulated to address this failure in extrapolation [3, 4]. However, all these scaling variations exhibited high percent error rates, even as high as 800% in some instances. Furthermore, none of these variations consistently predicted clearance better than the others for compounds in different therapeutic areas. Overall, the best allometric equation to scale doses and clearance depended on the age group and compound. This limitation prevented allometric scaling to be used prospectively to predict clearance or doses.

Due to these limitations for predicting pediatric clearance with any reliability, another approach was needed. In the 1930s, a technique incorporating physiological components as well as drug disposition was developed by Toesten Teorell [5], but this method was overly cumbersome and implausible to perform by hand. The availability of high powered computers allowed this physiologically based pharmacokinetic (PBPK) modeling method to be resurrected
and adapted to the healthy adult population as well as “special” populations. Significant progress has been made in PBPK modeling and in March 2012, a FDA Clinical Advisory Committee concluded that PBPK modeling should be incorporated into pediatric drug development programs. However, that vote was split 7 to 6, meaning that PBPK models were still a hotly debated approach and needed further validation in order to be recommended for use in drug development.

In order to validate PBPK modeling, it is necessary to define how to use this type of modeling. Two distinct types of PBPK modeling exist, the top-down and bottom-up approaches. The top-down approach, as it is applied to pediatric populations, uses clearance values from pediatric clinical trials or observations that are combined with physicochemical properties of the compound of interest and age-appropriate physiologic parameters. The utility of the top-down method is limited and difficult to assess due to the limited pediatric clinical data collected. The validation necessitates a circular use of the same data used to create the model. The bottom-up approach utilizes physicochemical properties and physiologic parameters, but derives intrinsic clearance from \textit{in vitro} experiments using human tissues and their subcellular components from target populations. The majority of the initial pediatric PBPK models to date have utilized a top-down approach [6-9]. Gradually an increasing number of bottom-up approaches are being developed and implemented [10-13]. Most of these bottom-up approaches use intrinsic pharmacokinetic parameters derived from recombinant enzymes using allometrically scaled maturation functions to determine whole organ clearance.

One major limitation in using recombinant enzymes is the assumption that an enzyme will function exactly the same in the healthy adults and special populations. These assumptions are not founded in robust experimental evidence. Thus, limitations exist with scaling intrinsic clearance values generated by recombinant enzymes that are similar to allometrically scaling clinical clearance values. For instance, the relative contribution of the three enzymes (CYP3A4, CYP2C19, and FMO3) in metabolic clearance of voriconazole was different when liver
microsomes from adults or children (ages 2 to 10) were used as the source of oxidative enzymes [14]. This study was the first to show comparative metabolism of a drug using pediatric and adult liver tissues reflecting clearance differences between children (2-10 year of age) and adults. Intrinsic clearance values derived with the use of these hepatic tissues from the populations of interest were predictive of hepatic clearance. Such studies demonstrated that cellular differences exist between adults and children, and require a more sophisticated approach than scaling recombinant enzymes can provide.

Thus, it is reasonable to expect that integrating the metabolic data generated with the use of age-specific hepatic tissues into a bottom-up PBPK model, the whole-body voriconazole disposition could be predicted for target populations. However, evidence for success of a bottom-up modeling approach based on in vitro metabolism data generated from tissues is limited in adults, and untested in children. Therefore, the overarching goal of this dissertation was to first develop a bottom-up PBPK model from in vitro metabolism data generated with adult hepatic tissue, and refine/validate the model and its predictions by iteratively using readily available adult clinical data. Upon successful development of an adult PBPK model, a corresponding pediatric PBPK model could then be developed by incorporating the relevant pediatric physiologic parameters, including expression levels of the relevant metabolic enzymes.

**Rationale for Compound Selection**

In order to test that a bottom-up PBPK modeling approach can predict pediatric doses, two compounds were chosen. In the first part of the project, the goal was to test and validate this approach by choosing a drug for which the predictions of the pediatric PBPK model, developed using the above approach, can be substantiated with available pediatric clinical PK data. Voriconazole was selected for this retrospective proof-of-concept phase (Chapter 2). Voriconazole, a potent triazole antifungal used for life-threatening infections in immunocompromised patients, is predominantly cleared via oxidative metabolism by CYP3A4, CYP2C19, and FMO3 in the liver. It displays different PK parameters in adults and children.
(ages 2 to 10) that have been conclusively established in the literature. These differences include nearly 3-fold higher clearance and nearly half the oral bioavailability in children compared to adults, requiring an adjustment to a higher weight-normalized dose in pediatric patients. It is important to note that metabolism of voriconazole by pediatric liver microsomes was approximately 3-fold faster compared to the rate of metabolism by liver microsomes from adult donors [14], indicating that the in vitro system reflected the higher in vivo hepatic clearance. Furthermore, these studies showed developmental changes in voriconazole drug metabolizing enzymes and revealed that two CYP450 enzymes, CYP3A4 and CYP2C19, and flavin-containing mono-oxygenase (FMO) 3 contribute to 50%, 35% and 15% of voriconazole metabolism in adults, respectively, versus 20%, 50% and 30% in children. Since intrinsic clearance between adults and children reflected the 3-fold increase in clearance in children compared to adults, there was now a clear rationale to use in vitro metabolism data to build a pediatric PBPK model. A PBPK model would not only enable prediction of doses in children but also provide the ability to predict changes in whole-body disposition of the drug in response to intrinsic (genetic) and extrinsic (interactions with food or co-administered drugs) factors. Finally, as was shown in Chapter 2, the model could raise important new questions about differences in disposition of the drug in children compared to adults – e.g. the model prediction that voriconazole is subject to intestinal first-pass metabolism in children but not in adults.

After proving the utility of this approach, the second part of this project was to apply this model prospectively to another compound (for which robust clinical data in the target population are not yet available) that is also cleared predominantly by oxidative metabolism so as to extend the applicability of this method to younger children, i.e. premature neonates (Chapter 5). Sildenafil, a phosphodiesterase-5 inhibitor, is under evaluation as a treatment option for pulmonary arterial hypertension, a significant cause of mortality in premature neonates. Orally administered sildenafil is subject to extensive first-pass metabolism, and is cleared predominantly via hepatic metabolism with only 2% of the parent excreted unchanged [15].
Evidence showed that sildenafil was metabolized by CYP3A4 and CYP2C9. Approximately 79% of the metabolism was due to the CYP3A family, 20% by CYP2C9, and CYP2D6 and CYP2C19 together contributing to < 1% [16, 17]. Expression of metabolizing enzymes is often low or absent, or fetal forms (e.g. CYP3A7, FMO1) are expressed in this vulnerable population. Metabolic capacity of these fetal forms, including specificity towards sildenafil, has not been well established, demonstrating why allometric scaling of adult dosing is inappropriate for premature neonates whose enzymes have not fully matured. Furthermore, limited information exists on efficacy and safety of sildenafil at the doses used in the pediatric population. A bottom-up approach could better define the differences, if any, in the enzymes responsible for sildenafil metabolism in the adult and premature neonatal populations, and a pediatric PBPK model could then be adapted from a corresponding (validated) adult model to determine the exposure and clearance of sildenafil in premature neonates, and used to assess sildenafil safety and, eventually, its efficacy.

**Retrospective Pediatric PBPK Model Development for Voriconazole**

For the retrospective proof-of-concept PBPK model, voriconazole in vitro pharmacokinetic parameters, $V_{\text{max}}$ and $K_{\text{m}}$, were generated using healthy adult and pediatric hepatic tissues in the Thakker laboratory [14]. The bottom-up PBPK model incorporated these intrinsic clearance values into a population-based PBPK model using Simcyp (version 12.1; Certara). The PBPK model predicted adult and pediatric voriconazole clearance of 2.4 and 5.0 mL/min/kg, respectively, which was within 20 and 25% of observed values (2.0 and 6.7 mL/min/kg) [14]. The good correspondence of the observed to the simulated clearance is important since in vitro clearance values were generated using hepatic tissue. These results confirm that bottom-up PBPK models created by considering metabolism by relevant tissues in target patient populations incorporate contribution and expression levels of key metabolizing enzymes and allow reliable prediction of clearance. The model also expanded previously published data on differences in voriconazole clearance between adults and children to describe
the whole-body disposition of voriconazole. The PBPK model predicted adult oral bioavailability to be 83% with a 90% confidence interval of 69-93%, which was consistent with the observed bioavailability of 87% (range of 75-96%). For the pediatric population, the simulated bioavailability was predicted to be nearly 2-fold higher than the observed bioavailability in this population. Despite accounting for the higher pediatric clearance of voriconazole by hepatic tissue, the pediatric model was unable to predict lower oral bioavailability of voriconazole compared to adults. This discrepancy pointed to factors affecting absorption and pre-hepatic (intestinal) clearance as likely causes for lower oral bioavailability in children compared to adults. The absorption rate constant was ruled out as a problem in the model since simulated $T_{\text{max}}$ values deviated from observed values by less than 20%. Therefore, intestinal first-pass metabolism was incorporated into the model. Interestingly, this refined model yielded an improved oral bioavailability prediction of 51% with a 90% confidence interval range of 27-76% that corresponds to the clinically observed range of 44-66%. The incorporation of intestinal first-pass metabolism into the pediatric model also improved predictions of AUC and $C_{\text{max}}$, lending further support that this could be the cause of lower bioavailability in children.

The PBPK modeling process raised a very interesting possibility that the oxidative enzymes in the intestine of pediatric population are more effective than those in the intestine of adults in metabolizing compounds as they cross the intestinal epithelium. It was important to eliminate other factors that may influence oral bioavailability of voriconazole preferentially in children in order to subject the hypothesis put forward as a result of the modeling process to rigorous scrutiny. Two likely factors that could contribute to lower bioavailability, gastric emptying time and small intestinal transit time, were tested and rejected after performing a sensitivity analysis that varied these factors by ten-fold in each direction and established that these changes did not yield improvements or variations in relevant pharmacokinetic parameters. Secondly, voriconazole is a highly permeable compound with low solubility, and exhibits high oral bioavailability in adults, which preclude any likely dependence on transporters during
absorption. Additionally, unpublished data from the Thakker laboratory demonstrate that changes in bile salt concentration do not affect voriconazole permeability across human intestinal epithelium. Furthermore, these Ussing-chamber studies also showed that in adult intestinal tissue, voriconazole N-oxide formed during absorptive transport was quantifiable, thus confirming that intestinal oxidative enzymes can metabolize voriconazole. Combining this information with the intestinal enzyme gene expression results, which point to higher expression of CYP3A4 gene in the intestinal tissue from children than in the tissue from adults (Chapter 3) suggests that intestinal first-pass metabolism is the most likely cause of lower bioavailability in children. A population pharmacokinetic model of voriconazole in children suggested that greater hepatic blood flow in children may have contributed to lower oral bioavailability of voriconazole in the pediatric population.[18] However, without measuring blood flow or plasma concentrations in the hepatic vein or portal vein, this statement would be purely speculative. Furthermore, the idea that hepatic blood flow affects voriconazole bioavailability is without merit as voriconazole is a low extraction compound (0.09-0.39); thus hepatic blood flow should have little effect on hepatic clearance and is therefore not a concern for influencing bioavailability. This supposition is further refuted by the voriconazole PBPK model presented in this work since differences in hepatic blood flow between adults and children have already been incorporated in the model.

The PBPK models for adult and pediatric IV infusions predicted voriconazole PK behavior within acceptable limits including 3-fold greater clearance in children compared to adults. This suggests that the model is able to adequately account for higher efficiency of voriconazole metabolism by hepatic enzymes in children. While the adult oral model successfully accounts for nearly 100% oral bioavailability, the pediatric oral model could not simulate voriconazole PK and over-predicted oral bioavailability. This model, taken in the context of the sensitivity analyses and physiological changes, clearly suggests that hepatic metabolism or intestinal absorption/permeability-related parameters do not contribute to poor prediction of oral bioavailability. With this preponderance of evidence, the novel hypothesis
suggesting higher first-pass intestinal metabolism in children, relative to adults, should inspire future studies that would examine intestinal oxidative enzyme composition and expression in pediatric and adult populations. In addition, future studies should examine if the kinetics of voriconazole intestinal metabolism in children is significantly different than in adults because of the possibility that voriconazole in the intestinal lumen of children may achieve significantly different concentrations than in the intestinal lumen of adults. The most obvious follow-up study would be to compare \textit{in vitro} metabolism of voriconazole by microsomes prepared from pediatric and adult intestinal tissues, and determine if the microsomes from pediatric intestinal tissue metabolize the substrate faster than those from adult intestinal tissue. In fact, such a study was conceived and attempted (Chapter 3). However, as discussed in Chapter 3, fresh pediatric intestinal tissue is not available, and the enzymes in the intestinal tissue harvested post-mortem are degraded by proteolytic enzymes to the point that meaningful metabolism data cannot be obtained using the microsomes prepared form such tissue. The studies in Chapter 3 not only demonstrate this, but also provide an improved microsomal preparation method so that only 1-3 in of pediatric intestinal tissue, procured during surgery, can yield sufficient catalytically active microsomal enzymes for future comparative intestinal metabolism studies. Surgical resection procedures in children involving intestinal diseases can yield 1-3 in of healthy intestinal tissue, and thus the methods developed in this work and reported in Chapter 3 open the door for future studies that can test the predictions of the voriconazole oral PBPK model developed for pediatric populations.

Overall, this bottom-up PBPK approach facilitated the understanding of mechanistic reasons for lower bioavailability between adults and pediatric populations, which has not been previously demonstrated. Furthermore, it shows that bottom-up PBPK models are sophisticated enough to not only model age-related differences in metabolizing enzymes, but also provide insights into the mechanisms and sources of pharmacokinetic differences. However, the most important contribution of this retrospective model was to provide a proof-of-concept for the
Defining Cellular Mechanisms of Pediatric Clearance

Clear differences in CYP2C19 and FMO3 mediated voriconazole and probe substrate metabolism by liver microsomes from children and adults were shown in the preliminary in vitro work published by Yanni et al as well as the voriconazole PBPK model [13, 14]. In order to elucidate the differences in expression and function in enzymes, quantitative real-time polymerase chain reaction (qRT-PCR) and quantitative proteomic analysis by mass spectrometry were employed for accurate determination of expression of CYP enzymes in liver and intestinal tissues from children and adults. Expression data were also paired with functional data for a robust analysis of how changes in DMEs’ gene and protein expression affect functional activity of enzymes. It is interesting to note that CYP2C19 is expressed to a greater extent in pediatric samples that in adults. An even more interesting observation was the higher CYP2C19 catalytic activity of hepatic microsomes, normalized to CYP2C19 protein, prepared from pediatric tissues compared to adult tissues. This is a surprising result has not been previously reported, and it suggests that the environment (e.g. lipid membranes of the endoplasmic reticulum) of CYP2C19 protein or its coupling with NADPH cytochrome P450 oxidoreductase is different in the pediatric tissue compared to adult tissue. Thus higher expression of CYP2C19, combined with its higher catalytic efficiency in pediatric tissue, may explain greater contribution of CYP2C19 toward metabolism of voriconazole in children compared to adults.

Hepatic tissues were analyzed for gene and protein expression of the CYP3A isoforms in fetal, pediatric and adult tissues. In addition, gene and protein expression of CYP2C9 and FMO1/3 were also evaluated in these tissues. This is because the second drug examined in this dissertation research was sildenafil, which is known to be cleared by hepatic CYP3A4 and CYP2C9 [16, 17]. Since CYP3A7 is the fetal form of CYP3A and premature neonates are the

proposed pediatric drug development paradigm for compounds predominantly cleared by oxidative metabolism.
target population for studying disposition of sildenafil, information on this enzyme was deemed to be necessary. In addition, the tertiary nitrogens in sildenafil are likely targets for FMO-mediated metabolism; and therefore FMO1 (fetal form) and FMO3 were also examined. The results showed that fetal samples expressed CYP3A7 and FMO1 at the highest levels with little to no expression of CYP3A4, CYP2C9, or CYP2C19. A maturational switch from 3A7 and FMO1 to 3A4 and FMO3, respectively, was evident from these fetal samples compared to pediatric and adult populations. Furthermore, CYP2C9 expression was similar, but CYP2C19 expression was higher in children compared to adults. For the first time, quantitative expression levels of these enzymes in these two populations have been examined.

A complete analysis of cellular activity requires the inclusion of functional activity. This research applied the quantitative protein expression to normalize respective probe substrate metabolism by each enzyme to the corresponding CYP or FMO protein. For measurement of the CYP3A activity, adult and pediatric microsomes, as well as recombinant CYP3A4 and 3A5 were evaluated by analyzing the rate of formation of 6β-OH testosterone (peak at 2.51 min) from testosterone. In addition, a minor metabolite was formed with molecular weight of M+16 (hydroxylated metabolite) that eluted at 2.61 min. Contrary to literature reports, analysis of the metabolites formed by fetal tissues and recombinant CYP3A7 revealed that the metabolite that eluted at 2.61 min was the major product, with 6β-OH testosterone (2.51 min) as a minor product. Since previous studies only considered formation of 6β-OH testosterone by CYP3A7, these studies had concluded that CYP3A7 metabolizes testosterone at rates that are only a small fraction of the rates of testosterone metabolism by CYP3A4. These results, for the first time, provide evidence that fetal tissue can metabolize testosterone at a higher rate than pediatric or adult liver tissue, and that expression of CYP3A7 in fetal tissue accounts for this high rate of testosterone metabolism. Furthermore, testosterone is differentially metabolized by the CYP3A7 isoform to a novel metabolite with a retention time distinct from that of 6β-OH testosterone, the major metabolite formed by CYP3A4 and CYP3A5. For the CYP2C family,
2C9 exhibited comparable activity observed in adult and pediatric populations with negligible activity in fetal tissues, corresponding well to the gene and protein expression.

**Prospectively Applying a Bottom-up PBPK Model to Predict Sildenafil Disposition in Premature Neonates**

Sildenafil, as mentioned previously, was chosen because it is predominantly cleared by oxidative metabolism and because of its proposed use in premature neonates. The two major isoforms that have been implicated in sildenafil metabolism are CYP3A4 and CYP2C9. Preliminary *in vitro* studies in this work indicated that significant discrepancies exist regarding the enzymes responsible for sildenafil metabolism. Contrary to published reports that the primary enzymes involved were CYP3A4 and CYP2C9, these studies showed that sildenafil is metabolized predominantly by CYP3A4 and CYP3A5 in the pediatric and adult tissues and by CYP3A5 and CYP3A7 in the fetal tissue. The switch from CYP3A7 to CYP3A4 within the first few months after birth adds an interesting and novel feature to the development of a PBPK model of sildenafil in premature newborns when compared to a pediatric or adult PBPK model for sildenafil.

The same approach that was used to develop the PBPK model for disposition of voriconazole in children was adopted for designing the model to predict sildenafil disposition in premature neonates. The adult PBPK sildenafil models (IV and oral) validated the use of a bottom-up approach utilizing intrinsic clearance generated from hepatic tissues, with CYP3A4 and CYP3A5 as major contributors to metabolism. Using this approach and the intrinsic clearance values, the full term neonatal models for sildenafil disposition after IV and oral administration were developed. Simulations were validated against the sparse clinical data available in the literature. Integration of the *in vitro* metabolism of sildenafil by liver tissues from neonates into the PBPK model yielded important new information on the fraction of sildenafil metabolized by CYP3A7. These predictions show that the high expression of the CYP3A7 enzyme in neonates overcomes its lower intrinsic clearance compared to CYP3A4 and
CYP3A5. Modeling the expression and activity generated an ontogeny pattern for clearance and the fraction of sildenafil metabolized by each isoform. In future studies these predictions can be tested using testosterone as a probe since CYP3A7 and CYP3A4/5 form different metabolites. Finally, after determining the ontogeny of enzymes, the model was used prospectively to predict PK and the physiological factors most likely to influence this profile in premature neonates, which were confirmed by the visual predictive checks from patients enrolled in a phase I trial at UNC Hospitals.

Most importantly, this section of the dissertation confirms that bottom-up PBPK modeling, using tissues from relevant populations, increases and refines the knowledge regarding the major metabolizing enzymes involved in metabolic clearance, and how they function in different populations. A wide array of medications administered to the premature neonatal population could potentially influence the expression and function of the CYP3A family. Therefore, understanding the impact of metabolic differences on the disposition of a drug, like sildenafil, caused by differential expression and/or catalytic function of major metabolizing enzymes (i.e. CYP3A7 versus CYP3A4) between adults and premature neonates can play a pivotal role in determining drug-drug interactions caused by inhibition or induction.

**Physiological Relevance of CYP3A7 – The Fetal Form of CYP3A Family**

In the process of developing an understanding of the impact of the expression of CYP3A7 instead of CYP3A4 in premature neonates on sildenafil disposition, the studies performed to elucidate the expression and catalytic activity of the CYP3A7 protein revealed a very interesting property of CYP3A7. These studies showed that CYP3A7 is a steroid-specific P450 that has much higher metabolic efficiency for testosterone than reported previously [19-21]. Relating this information to the existing knowledge that there is a precipitous decline of CYP3A7 expression over the first month after birth with concomitant rise in the expression of CYP3A4, the results provide an interesting insight into an important question: why does an isoform of CYP3A, which has low catalytic activity for other CYP3A4 substrates but high
efficiency to metabolize a steroid, exhibit high expression in the fetus and then switch off at birth? It is hypothesized that CYP3A7 is expressed in the fetus predominantly to metabolize high concentrations of the steroidal hormones that the fetus is exposed to from mother’s blood. At birth, there is a sudden decline in the steroidal hormone in the baby’s blood, and thus CYP3A7 is switched off while CYP3A4, an enzyme with a broad substrate specificity, is switched on.

**Novelty of Approach and Future Directions**

The paradigm for predicting pediatric doses proposed and supported by this research provides a new approach to modeling pediatric drug disposition that may improve prediction of pharmacokinetic parameters in children and thus dosing, and reduce ineffective or unsafe therapy and the need for costly and uncomfortable therapeutic drug monitoring. While the concept of bottom-up PBPK modeling of pediatric drug disposition has been previously utilized, the true novelty and significance of the approach developed in this work lies in the fact that these models are built on *in vitro* data from pediatric tissues that inherently include expression and catalytic efficiency of the relevant enzymes involved in the metabolic clearance of the drug being considered. This information is further refined by understanding the relative contributions of these enzymes in the metabolic clearance of the drug. For each of the two drugs studied in this work, a role of a new enzyme, FMO3 for voriconazole and CYP3A5 instead of CYP2C9 for sildenafil, was uncovered that was not identified even after extensive metabolism work performed by the company to meet the regulatory requirement for a New Drug Application. This work highlights the importance of mechanistic cellular and molecular studies prior to building a bottom-up PBPK model. These results demonstrate that use of expressed enzymes to build the bottom-up model would miss important and relevant information about how these enzymes interact in the tissue to affect the overall metabolic clearance of drugs. Such an approach provides *in vitro – in vivo* correlation and avoids the creation of a model that succeeds as random chance. The true significance of this work lies in the fact that the modeling approach will
improve PK predictions before initiating a pediatric clinical trial since it can be used to understand the disposition of metabolically cleared drugs as well as improve the knowledge of age-related differences in cellular mechanisms of clearance.

When considering the bottom-up PBPK modeling approach as an in vitro-in vivo extrapolation (IVIVE) technique, it is important to note that predicting PK behavior of a metabolically cleared drug from in vitro metabolism is not always successful, even in adults where we have substantial knowledge of physiology. While minimal IVIVE methods work sometimes, it has been shown time and again that this seemingly simple technique does not work at all times. For instance, using human liver microsomes to identify intrinsic pharmacokinetic parameters overestimated \( K_m \) and \( V_{\text{max}} \) of phenytoin due to the lack of consideration of the effect of albumin binding; predictions were improved with addition of bovine serum albumin and fatty acid-free human serum albumin to the incubations [22]. This type of limitation is overcome in PBPK models as albumin concentrations and fraction unbound can be incorporated into the model. If corrections for nonspecific binding in the assay are not taken into the account, as they are in PBPK models, then clearance values can be over-predicted [23]. Additionally, simple IVIVE methods fail to incorporate the variability in populations because they use the average results to predict population clearance [24]. Therefore, using a simple IVIVE approach to determine clearances in the pediatric population would be challenging and unreliable. Applying this proposed bottom-up PBPK model can improve pediatric predictions by combining physiological components with the understanding of the cellular and molecular basis for differences in in vitro metabolic behavior of pediatric and adult tissues.

For the bottom-up model to be truly successful, the results in this dissertation show that a few key steps need to be taken (Figure 6.1). First and foremost, enzymes involved in major metabolic transformation must be identified. After identifying the predominant enzymes, the kinetic parameters for each enzyme and enzyme expression should be characterized in the adult population in order to create an adult in vitro-in vivo relationship. The next step is to
characterize the kinetic parameters and measure quantitative expression of these enzymes in the pediatric tissues to evaluate the contribution and efficiency of each enzyme to metabolism of the compound. Relating these results to the adult in vitro-in vivo relationship will help define the differences between the adult and pediatric populations. Finally, these cellular mechanisms, functional data, and expression can be incorporated into the bottom-up PBPK model to predict clinical pharmacokinetic parameters in each population.

Applying these steps will allow this approach to be expanded to different areas of research. For instance, while the work shown in this dissertation focused solely on metabolically cleared compounds, it is a viable technique for compounds absorbed, distributed or cleared via transporters. Currently, the ontogeny of transporters is poorly defined. As with this work, there is a strong possibility that transporters can function differently in pediatric patients compared to adults. In order to apply this method, quantitative expression of the transporters and their functional activities across the age ranges of interest will be required. As mentioned previously, improvement in experimental techniques will be required in order to quantitate the expression and function of transporters that are localized on the cell membrane as these are the only pharmacokinetically- and pharmacodynamically-active transporters for trafficking drugs between cell and systemic circulation. After quantifying these data, this information can then be incorporated into a bottom-up PBPK model to assess the effects of the maturation of transporters on pharmacokinetics, and eventually, pharmacodynamics.

However, this proposed bottom-up approach does not come without some limitations. Techniques to study hepatic metabolism and transport in children have evolved to allow rigorous metabolism studies as well as transport studies. Cryogenically preserved fetal hepatocytes are now available for purchase [25]. However, intestinal tissue samples of high quality for metabolic and/or transporter studies are difficult to obtain for children under the age of 12 years old. While sufficient amounts of healthy intestinal tissue could be procured during surgeries, particularly in light of the enhanced techniques developed in this work for isolation of
subcellular fractions, the number of children undergoing gastrointestinal surgery is limited. The problem associated with limited supply is further amplified due to the need to bin children into developmental age groups unlike with adults. Taken together, these factors contribute to poor outcomes in developing intrinsic clearance parameters. Yet, research on the differences in cellular systems is increasing due to the realization of the importance of basic biological input parameters for metabolism and transporters in the development of computational models. As was seen with hepatic tissue, intestinal tissue procurement is increasing as academia, the medical community, and the pharmaceutical industry respond to these scientific needs [26]. Furthermore, a large sample size is not always necessary. Population models add these specific input parameters into a larger system comprising physiological factors and simulate individuals with combinations and variability in these components, thereby decreasing the effect of the error of one component on all predictions. As science progresses and more samples become available, the predictions will continue to improve. Despite these limitations, this bottom-up method still represents the best technique for predicting pediatric PK.

The aim of this research was to develop a modeling method to aid in predicting pediatric dosing and DDI. The research presented in this dissertation provides a reliable method to build a comprehensive PBPK model in order to understand the metabolic processes governing pediatric PK. However, this work highlights crucial gaps in our knowledge that need to be filled in order to improve current PBPK models. In order to improve the ability of the model to predict pediatric dosing and DDI, it is essential to understand the ontogeny of gastrointestinal enzymes and how this affects their activity and expression during development. In addition, more information is required in premature neonates to define the differences in binding proteins as well as how prematurity affects the development of organs and drug distribution. By adding these important physiological ontogeny data, the bottom-up PBPK model will be able to accurately predict dosing and DDI driven by PK differences between adults and children.
The potential of these models is not limited solely to understanding age-dependent PK differences in the general pediatric population. With the addition of data describing the relationship between genetic polymorphisms and ontogeny, the physiological changes associated with specific pediatric disease states, and the differences in distribution and ontogeny of pharmacodynamic targets between adult and pediatric patients, these models can evolve to become a tool used by clinicians. Ultimately, this line of investigation will expand the models to improve individual pediatric therapy at the bedside.
Figure 6.1 Proposed Paradigm in Prediction of Pediatric Drug Disposition and its Use in Pediatric Drug Development.
REFERENCES


