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PHARMACOKINETICS IN CHILDREN WITH LIVER DISEASE: CAN *EX VIVO* OBTAINED POPULATION-SPECIFIC CYP450 CHARACTERISTICS BRIDGE THE KNOWLEDGE GAP?

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Voor Mamie

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LIST OF ABBREVIATIONS

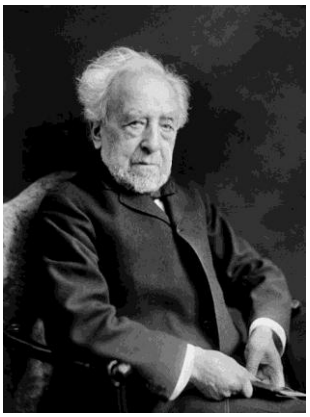
5-PL	Five-parameter logistics function
A1AD	α–1 antitrypsin deficiency
ALF	Acute liver failure
ANOVA	Analysis of Variance
AP	Acetaminophen
AUC	Area under the plasma-concentration time curve
BA	Biliary atresia
BAv	Bioavailability
CAR	Caroli's disease
CF	Cystic Fibrosis
CL	Clearance
CL_H	Hepatic clearance
CL_{int}	Intrinsic clearance
CLP	chlorpropamide
CP	Child-Pugh
CV	Coefficient of Variation
CYP	Cytochrome P450
CZ	Chlorzoxazone
DM	Dextromethorphan
(ds)DNA	(double stranded) Deoxyribonucleic acid
DX	Dextrorphan
ELISA	Enzyme Linked Immunosorbent Assay
EM	Extensive Metabolizer
ER	Extraction Ratio
ESI	Electrospray Ionization
f_u	Fraction unbound drugs
FMO	Flavin-containing monooxygenase
HCZ	6-Hydroxy-chlorzoxazone
HMDZ	1-Hydroxy-midazolam
HME	4'-Hydroxy-mephenytoin
(HP)LC	(High Performance) Liquid Chromatography
HRP	Horseradish peroxidase
HTB	4-Hydroxy-tolbutamide
IM	Intermediate Metabolizer
INR	Internationalized Ratio
IS	Internal Standard
IVIVE	In vitro-in vivo extrapolation
LA	Levallorphan
LOD	Limit of Detection

(L)LOQ	(Lower) Limit of Quantification
MDZ	Midazolam
ME	S-(+)-mephenytoin
MELD	Model for End stage Liver Disease
MF	Matrix factor
MFO	Mixed Function Oxidase
MGB	Minor groove binder
MPPGL	Microsomal protein per gram of liver
MRM	Multiple Reaction Monitoring mode
M&S	Modelling and Simulation
(MS/)MS	(Tandem) Mass Spectrometry
NADPH	Nicotinamide adenine dinucleotide phosphate
NCR	NADPH-cytochrome reductase
NH	Neonatal Hemochromatosis
NTC	No template control
OLT	Orthotopic Liver Transplantation
OME	Omeprazole
PBPK	Physiologically based pharmacokinetics
PBS	4-(1H-pyrazol-1-yl)benzenesulfonyl chloride
PD	Pharmacodynamics
PELD	Paediatric End-stage Liver Disease
PFIC	Progressive Familial Intrahepatic Cholestasis
PH	Phenacetin
PK	Pharmacokinetics
PM	Poor metabolizer
PS	Pyridine-3-sulfonyl chloride hydrochloride
Q	Blood flow
QC	Quality control sample
RE	Relative Error
RSD	Relative Standard Deviation
SD	Standard Deviation
S/N	Signal-to-noise ratio
SNP	Single Nucleotide Polymorphism
SULT	Sulfotransferase
TB	Tolbutamide
TE	Tris-EDTA buffer
TPMT	Thiopurine S-mehyltransferase
UGT	UDP-glucuronyltransferase
UM	Ultra rapid Metabolizers
UPLC	Ultrahigh Performance Liquid Chromatography
VS	Validation sample

INTRODUCTION

'... because the liver is a source of many diseases,
and is a noble organ that serves many organs,
almost all of them: so it suffers, it is not a small
suffering, but a great and manifold one'
- Paracelsus

1 CHILDREN ARE NOT SMALL ADULTS: BETTER MEDICINES FOR CHILDREN



"Paediatrics does not deal with miniature men and women, with reduced doses and the same class of diseases in smaller bodies, but... it has its own independent range and horizon… There is scarcely a tissue or an organ which behaves exactly alike in the different periods of life... There are anomalies and diseases which are encountered in the infant and child only. There are those which are mostly found in children, or with a symptomatology and course peculiar to them."

– Dr Abraham Jacobi

It was over a 100 years ago that Dr Abraham Jacobi (1830-1919) recognized the importance and the need for age-specific pharmacotherapy in his presidential address to the American Paediatric Society [1]. It wasn't nevertheless until 1997 that the first legislative changes to encourage paediatric clinical trials were implemented (FDA Modernization Act, November 1997) [2]. Subsequently, other regulations were adopted in both the United States (the 2002 Best Pharmaceuticals for Children Act; and the 2007 FDA Revitalization Act) and in Europe (Regulation EC No. 1901/2006 on Medicinal Products for Paediatric Use) [3].

Moreover, in 2007, the World Health Organization (WHO) adopted the "Better medicines for children" resolution. This resolution raises the concern on the lack of access to essential medicines of assured quality for children, as well as on the insufficient investment in clinical trials in and development of drugs for children [4]. The potential risk for the public health of children by denying them access to new medicines, or by treating them with drugs not assessed in the same age groups has been generally recognized. Consequently, pharmaceutical companies registering a new drug are obliged by the Regulatory Authorities to submit a Paediatric Investigation Plan (PIP).

In the current clinical practice, drugs are often used off-label in paediatric patients. Drugs are used off-label when they are used outside of regulatory approval. There are different categories of off-label use, based on age, weight, absence of paediatric information (no information at all), lack of paediatric clinical data (lack of evidence of efficacy and safety), contraindicated in children, other indication in children, other route of administration [5]. 50 to 75% of drugs currently used in paediatrics are used off-label [6,7], and this share is even higher in more specific populations, such as neonates or

children with cancer [6]. After a thorough evaluation of the specific situation (indication, alternative drugs, benefit-versus-risk, available evidence), the off-label use can be considered appropriate, if no alternatives are available and the benefits for the patient outweigh the potential risks [8]. Because of the lack of information, each off-label administration can be considered as the performance of an uncontrolled N-of-1 trial [9]. This results in the exposure of the child to possible risks, such as adverse drug reactions, or ineffective treatment.

Recently, there has been a shift in the thinking process of performing clinical trials in children. In the past, children were frantically protected from every possible research, in order to avoid exploiting them as vulnerable subjects [8]. Recently, however, it was accepted to be an ethical obligation to perform, and a child's right to participate in, research that could potentially benefit children's health [6]. Despite the increased intention to perform paediatric clinical trials, many obstacles still have to be overcome in the development and execution of these trials. Firstly, there are the ethical issues, amongst which the informed consent (by surrogate consenter and assent of the child with sufficient intellectual maturity), the inclusion of healthy children, and the risk assessment, in which the risks of not doing research in infants and children should be considered in addition to the direct and immediate risks and benefits to the individual subject [7]. Secondly, the protocol should be age-appropriate, considering inclusion/exclusion, avoidance of excessively ambitious protocols, the use of age-appropriate dose formulations, and realistic sampling procedures. Moreover, centres conducting paediatric clinical trials should dispose of suitable infrastructure and experienced staff [6,7].

In short, efforts should be made to avoid the possible compromise of children's health from off-label or unlicensed drug use. Children deserve the same well-tested drugs as adults, in the correct dose, using an approved route of administration and for the right indication.

2 DEVELOPMENT OF DRUG DISPOSITION AND ACTION IN CHILDREN

2.1 Developmental pharmacodynamics

Drug action can be divided into two processes: (1) pharmacokinetics (PK) ("what the body does to the drug"), and (2) pharmacodynamics (PD) ("what the drug does to the body"). Developmental changes may affect the receptors or therapeutic targets involved in drug action and response, consequently also influencing clinical outcomes [10]. Up until now, there is a paucity on studies on the ontogeny of the interactions between

drugs and receptors, and on the consequence of these interactions [10,11]. The studies that were performed, were reviewed by Mulla in 2010 [10]. Table 1 was adapted from this review to illustrate some possible effects of maturational changes on the PD response.

Alterations in potency or efficacy of a drug may be due to maturational changes in receptor affinity, density or signal transduction, due to complete absence of receptors or mediators, or due to changes further downstream the biochemical pathway [10]. Besides effects on the therapeutic response, the developmental changes may also affect the risk of adverse reactions. For some drugs, the therapeutic window is narrowed or broadened, leading to a more or less likely occurrence of adverse events for a given exposure.

Table 1: Altered PD response in children, as reviewed by Mulla, 2010 [10].

Studied drugs	**Effect in children**	**Developmental difference compared with older children/adults**	**References**
Antidepressants	Lack of effect of tricyclics	Neurodevelopmental delay in noradrenalin system	[12,13]
Antiepileptics	Paradoxical seizures	Excitatory $GABA_A$ receptor instead of inhibitory at young age, and increased receptor density	[12-18]
Opioid analgesics	Increased sensitivity in neonates	Changes in opioid receptor expression	[19-22]
Immunosuppressants	Increased sensitivity	Quantitative and qualitative differences in immune system	[23-28]
Cardiovascular drugs			
Angiotensin receptor antagonists	?	AT_2 receptor highly and transiently expressed in foetal tissues	[29,30]
Drugs prolonging QT interval	Increased propensity for QT interval prolongation	Maturation of myocardial potassium channels	[31]
Oral anticoagulants	Increased sensitivity to effects	Decreased plasma concentration of vitamin K-dependent factors	[32,33]

AT_2= angiotensin receptor, subtype 2; $GABA_A$= gamma aminobutyric acid receptor, subtype A;? indicates not known

2.2 Developmental pharmacokinetics

During gestation and after birth, the physiology of children changes significantly. Many of these developmental changes profoundly affect the responses to medications. In the last decade, many excellent reviews were published describing the relevant changes, such as the reviews by Alcorn and McNamara [34,35], Kearns et al [11], Benedetti et al [36,37], Anderson [38], and Pogessi [39]. Table 2 gives an overview of the main physiological factors that influence the changes in drug disposition during development according to these reviews. A very clear figure displaying some of the items mentioned above was published by Kearns et al [11] (see Figure 1).

Table 2: Summary of the physiological factors changing during development and likely to affect drug therapy (References: [11,34-39]).

Absorption	**Distribution**
Oral administration Gastric pH Gastric emptying time Intestinal motility/transit time Intestinal surface area Secretion and activity of bile salts and pancreatic fluids Enzymes Transporters Intrapulmonary administration Architecture of the lung Ventilator capacity Intramuscular administration Muscular blood flow Efficiency of muscular contractions Percutaneous administration Relative BSA to weight	Plasma proteins Concentration Binding capacities Quality Body composition Relative extracellular and total body water space Adipose stores Regional blood flow Organ perfusion Permeability of cell membranes
Metabolism	**Renal excretion**
Relative liver size Liver microsomal content Phase I and Phase II enzymes ontogeny	Glomerular filtration rate Tubular secretion Renal and intrarenal blood flow

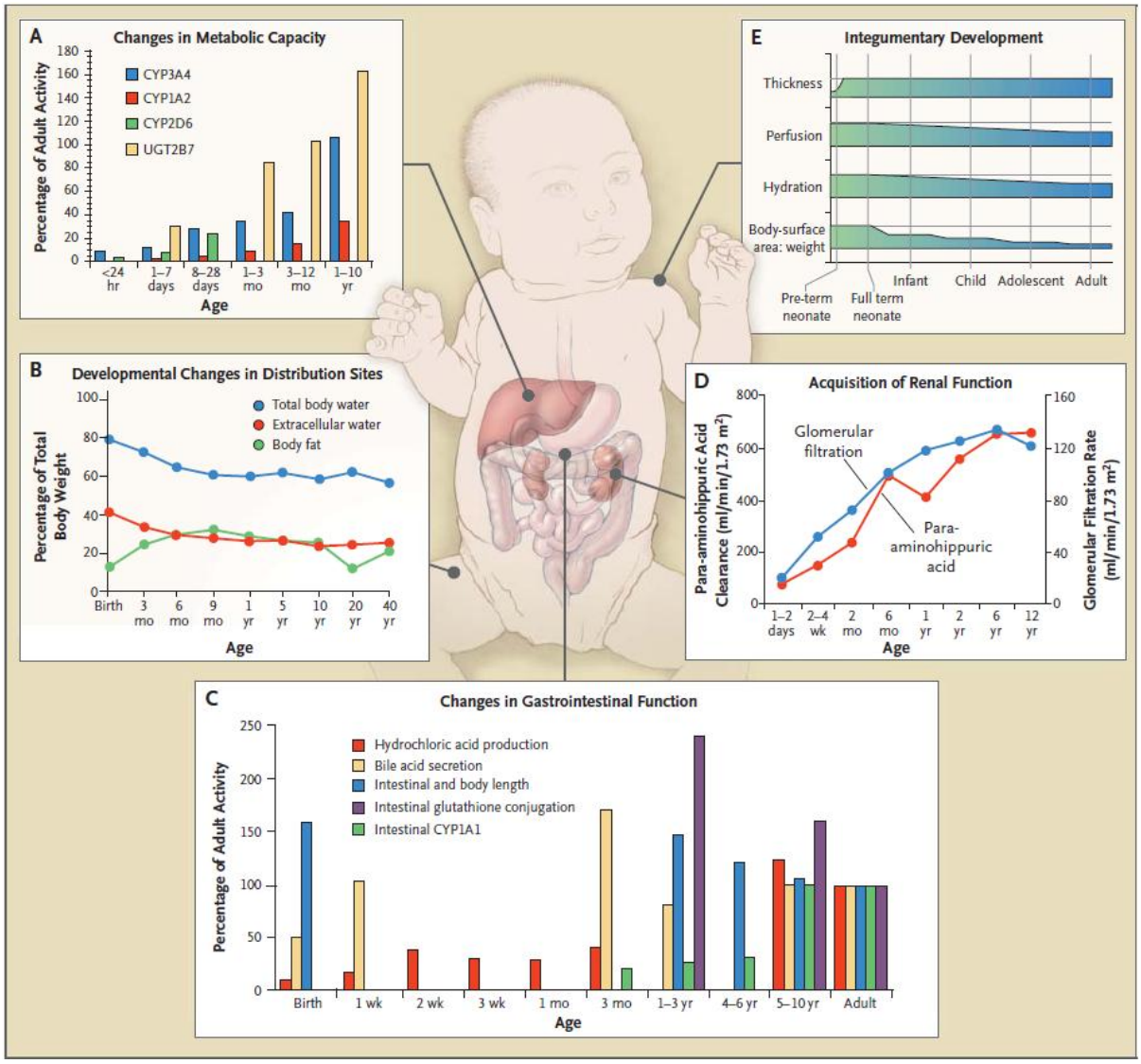


Figure 1: Physiologic changes in multiple organs and organ systems during development are responsible for age-related differences in drug disposition. As reflected by Panel A, the activity of many cytochrome P-450 (CYP) isoforms and a single glucuronosyltransferase (UGT) isoform is markedly diminished during the first two months of life. In addition, the acquisition of adult activity over time is enzyme and isoform specific. Panel B shows age-dependent changes in body composition, which influence the apparent volume of distribution for drugs. Infants in the first six months of life have markedly expanded total-body water and extracellular water, expressed as a percentage of total body weight, as compared with older infants and adults. Panel C shows the age-dependent changes in both the structure and function of the gastrointestinal tract. As with hepatic drug-metabolizing enzymes (Panel A), the activity of cytochrome P450 1A1 (CYP1A1) in the intestine is low during early life. Panel D summarizes the effect of postnatal development on the processes of active tubular secretion — represented by the clearance of para-aminohippuric acid - and the glomerular filtration rate, both of which approximate adult activity by 6 to 12 months of age. Panel E shows age dependence in the thickness, extent of perfusion, and extent of hydration of the skin and the relative size of the skin-surface area (reflected by the ratio of body-surface area to body weight). Although skin thickness is similar in infants and adults, the extent of perfusion and hydration diminishes from infancy to adulthood. (Figure and legend reproduced from Kearns et al, 2003 [11])

2.3 The cytochrome P450 enzyme system

2.3.1 General

The systemic clearance of the top 200 prescribed drugs occurs mainly by metabolism, and predominantly in the liver (see Figure 2). Occasionally, extensive metabolism of a drug occurs in one or more other tissues, such as kidneys, lungs, blood, or the gastro-intestinal wall [40,41]. One of the most important drug-metabolizing enzyme systems in humans is the cytochrome P450 (CYP450) enzyme family [42]. CYP450 enzymes are involved in the oxidative metabolism of a large number of endogenous compounds (such as unsaturated fatty acids, eicosanoids, sterols and steroids, bile acids, retinoids), as well as exogenous compounds (such as drugs, environmental chemicals and pollutants, natural plant products) [43].

A standard nomenclature system was created, in order to avoid confusion in the rapidly growing amount of studies on CYP genes. Since 1996, CYP enzymes are named using 3 characters: (1) a number to indicate the gene family (enzymes with ≥ 40% identity on the amino sequence level belong to the same family), (2) a letter indicating the subfamily (≥ 55% identity), (3) a number indicating the gene [44]. New genes/polymorphisms can be consulted on a website (http://www.cypalleles.ki.se). CYP families 1-3 are involved in 70-80% of all phase I dependent metabolism of clinically used drugs [45-47].

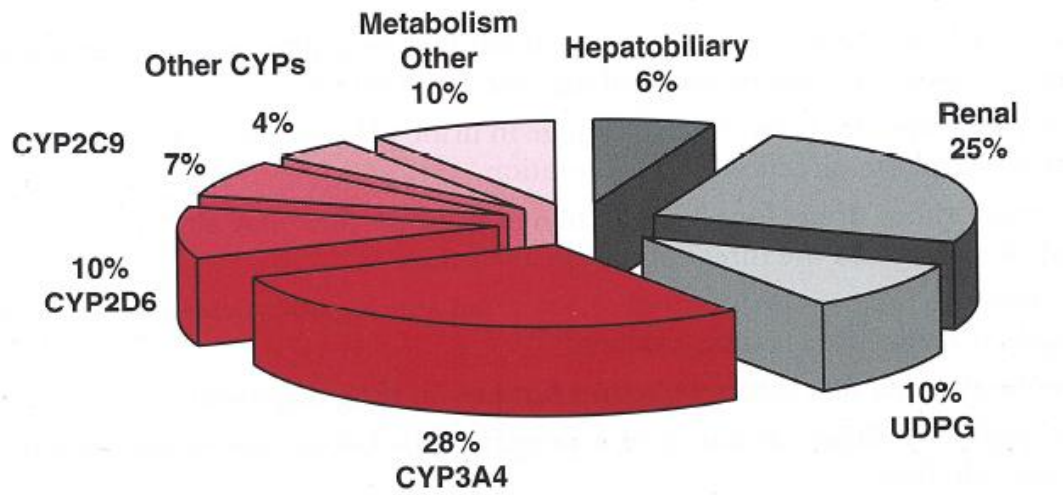


Figure 2: Relative importance of route and mechanism of elimination of the top 200 prescribed drugs. The coloured segments refer to phase I metabolism, the remaining segments refer to either phase II conjugative reactions or excretory processes within the liver and kidney. In addition to the depicted mechanisms, transporter proteins are also important in the elimination of many drugs. (Figure adapted from Williams et al, 2004 [41] and Rowland and Tozer, 2011 [40]).

CYPs are mixed-function oxidases (MFO) primarily situated in hepatic parenchymal cells, but some isoforms are also present in other tissues, such as intestine, lung, kidney, placenta and brain [42,48]. In the parenchymal cells, these hydrophobic enzymes are bound to the membranes of the endoplasmatic reticulum [47,49,50]. Fragmentation of the cells, followed by differential centrifugation leads to the isolation of the microsomal fraction, containing fragments of the endoplasmatic reticulum [49]. Guengerich determined that 96% of the CYPs in the liver can be found in the microsomal fraction [51].

CYPs are part of an electron transport system in the endoplasmatic reticulum, requiring reduced NADPH (NADPH), molecular oxygen, CYP450, NADPH-cytochrome P450 reductase, and phospholipids. The latter is involved in the binding of the drug molecule to the CYP, and coupling the NADPH-CYP450 reductase to the CYP450. The CYP450 is the terminal component of the electron transfer system and acts as both oxygen and substrate binding locus for drugs and endogenous substrates, in conjunction with a flavoprotein reductase, the NADPH-CYP450 reductase [49]. A general scheme of the electron transfer system is represented in Figure 3.

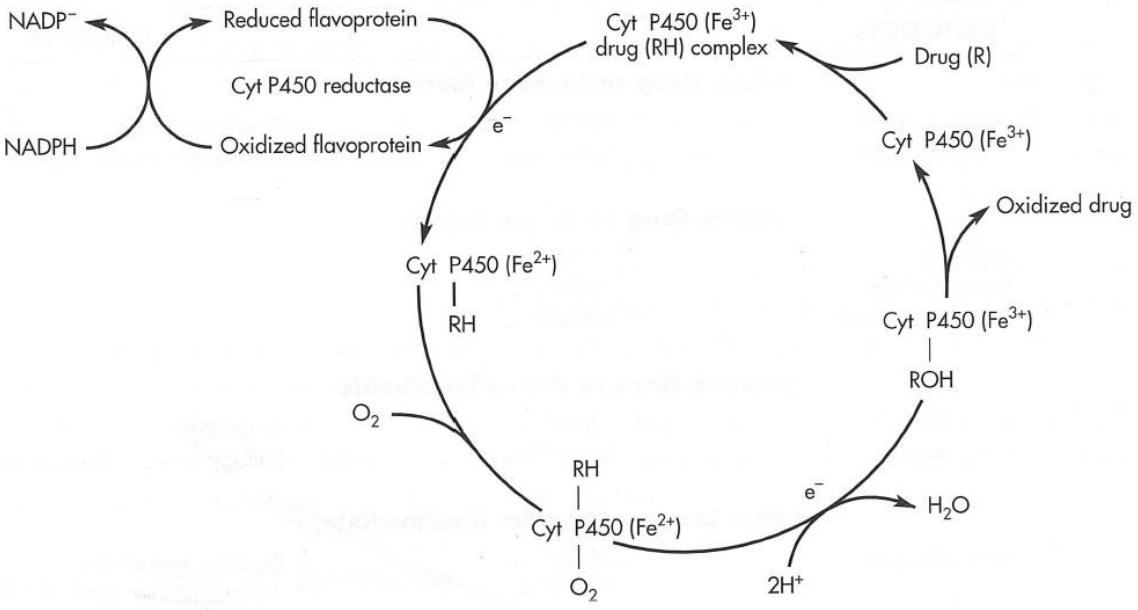


Figure 3: Electron flow pathway in the microsomal drug-oxidizing system. (Figure reproduced from Alvares, 1990 [52] and Shargel, 1999 [49]).

The activity of CYP enzymes is affected by genetics, age, gender, pathological states, environmental influences, intake of drugs, and dietary habits [42,50]. Genetic polymorphisms are found in almost all CYPs amongst the families 1-3, except CYP1A1, 2E1, and 3A4 [47]. These 3 enzymes have an important endogenous function and are

therefore possibly better conserved. The higher genetic variation is seen in genes with gene products that are active in the metabolism of environmental agents. Polymorphisms may lead to abolished, reduced, altered or increased enzyme activity. The clinically most important polymorphisms are seen in CYP2C9, 2C19, and 2D6 [47].

There is no apparent relationship between the abundance of a CYP isoform and its relative importance in drug metabolism [40,47,53]. This is illustrated in two ways in Figure 4. For example, CYP2D6 is responsible for only about 2-3% of the total CYP content, whereas it is responsible for the clearance of 25% of the prescribed drugs.

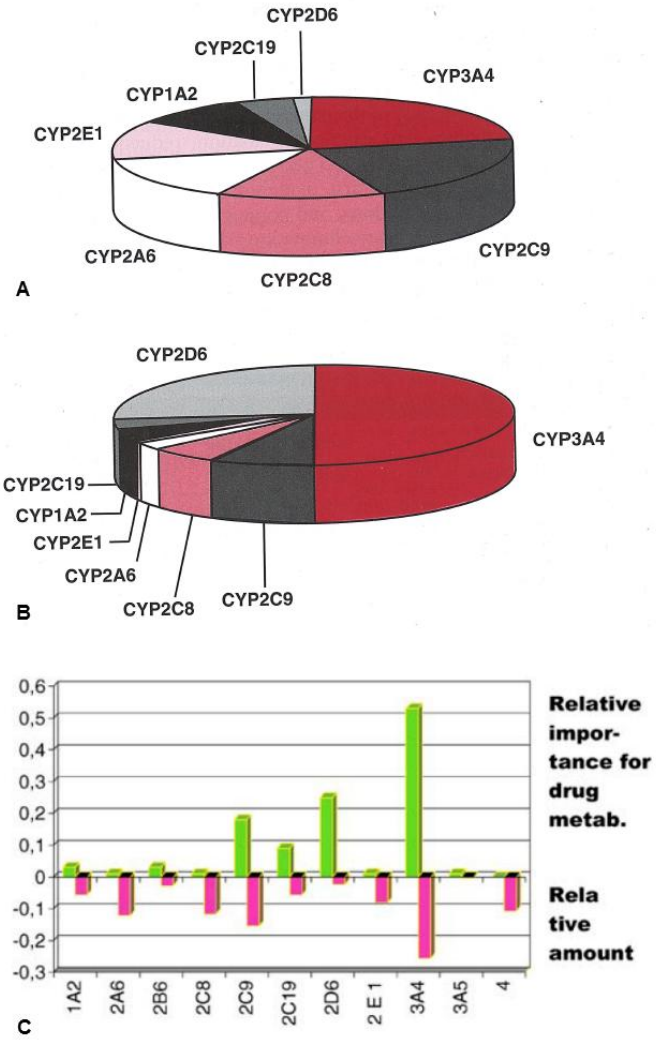


Figure 4: A. Relative abundance of the major hepatic CYP450 enzymes in the human liver. B. Relative contribution of the major CYP450 enzymes in the CYP-mediated clearance of drugs [40,53]. C. Relative amount of the CYPs and relative importance for the metabolism of clinically used drugs [47].

2.3.2 Ontogeny of the CYPs

Developmental changes in drug-metabolizing enzymes make a major contribution to the overall PK differences between adults and children. Knowledge on the ontogeny of these enzyme systems has partially been derived from *in vivo* pharmacokinetic studies in neonates, infant, and older children, in which the plasma clearance of the studied drugs may reflect the ontogeny of one specific enzyme or a combination of enzymes [54]. Additional information on the ontogeny can also be obtained *in vivo* by using the urinary ratio of metabolite/drug or stable isotope labelled probes [55]. These types of *in vivo* studies cope with the same ethical and practical issues as described earlier, such as the inclusion of healthy children, the possible risks, the need for age-appropriate protocols, and realistic sample procedures [6,7].

Another approach to investigate the ontogeny is the use of liver tissue samples to perform *in vitro* experiments. However, this type of research is slowed down due to the low availability of liver tissue over age ranges [54]. This knowledge gap was partially filled by Hines in 2007 [56], who was able to analyze the enzyme contents of 6 key cytochromes P450 in the 240 human liver samples, representing ages from 8 weeks gestation to 18 years. Although oversimplified, these results, combined with information from previously published studies, revealed the apparent existence of three patterns of expression [57], as shown in the comprehensive graph adapted from de Wildt (Figure 5) [54]. The enzymes following the first pattern of ontogeny are characterized by a relatively high expression during foetal life. During postnatal life, these enzymes (e.g. CYP3A7, flavin-containing monooxygenase 1 (FMO1) and sulfotransferase 1A3 (SULT1A3)) are silenced or expressed at low levels. Enzymes from the second group are expressed at relatively constant levels throughout gestation and postnatal life. Representatives of this group are CYP3A5, SULT1A1, and thiopurine S-mehyltransferase (TPMT). The last pattern shows either no expression in the foetus or low levels of expression with the onset in the second or third trimester of pregnancy or within hours to days after birth. The increases in expression are observed within the first 1-2 years of life. A considerable interindividual variability in the postnatal onset and the increase in expression can be seen [56]. This last group of enzymes includes, CYP1A2, 2C9, 2C19, 2D6, 2E1, 3A4, FMO3, and most UDP-glucuronosyltransferases (UGTs). For a detailed description of the ontogeny of the individual isoforms, we refer to the review by de Wildt (2009) [54].

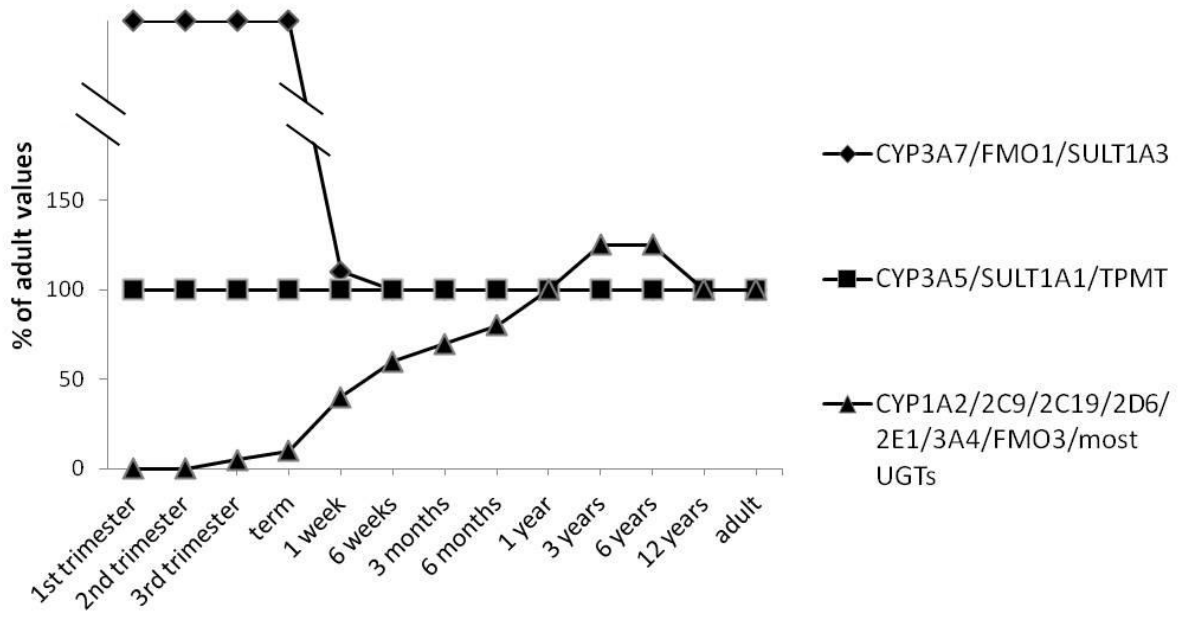


Figure 5: Developmental patterns of drug-metabolizing enzymes *in vitro* (modified from de Wildt, 2011 [54]).

3 LIVER DISEASE

3.1 Liver disease in adults

The management of liver disease consists of several dietary and pharmacological interventions. In some cases, these interventions are insufficient, and orthotopic liver transplantation (OLT) should be considered. The main indications for OLT in adults (see Figure 6) are cirrhosis associated with alcoholism (18%) and with hepatitis C (15%). Other conditions leading to cirrhosis account for 25%, whereas liver cancers account for 13%. Other indications are cholestatic diseases (11%), acute liver failure (9%), mainly due to acute viral hepatitis or drug intoxication, metabolic diseases (6%), and chronic hepatitis (hepatitis B, autoimmune) (3%) [58].

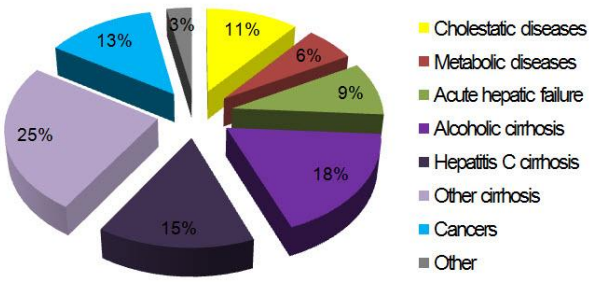


Figure 6: OLT indications in adults.

3.2 Liver disease in infants and children

Liver disease in infants is relatively rare (prevalence of 1/2500 live births in the USA [59]), but is considered a serious cause of morbidity and mortality [60]. In general, liver disease can be classified as cholestatic, hepatocellular or cirrhotic. It may be acute or chronic (when occurring over periods over 6 months) [61].

OLT in children has evolved from a life-saving procedure to an established treatment of many liver diseases [62]. The pattern of indications most frequently leading to OLT differs somewhat between studies. Figure 7 compares the frequencies as reported by Sokal et al. (A) [63] and Adam and Hoti (B and C) [58]. It should be noted, however, that Sokal et al. did not report the age range of the patients, whereas Adam and Hoti clearly divide the population into two groups, i.e. infants from 0-2 years old, and children from 2-15 years old. This could explain the (small) discrepancy between the reported values. However, the main findings are very similar, i.e. cholestatic diseases account for the majority of OLT in children. In adults, the aetiology of liver disease seems to be one of the factors which determine the changes in the activity of CYP enzymes [64]. The substantial differences in the main causes of liver disease between children and adults are an important argument to motivate research in children with liver disease.

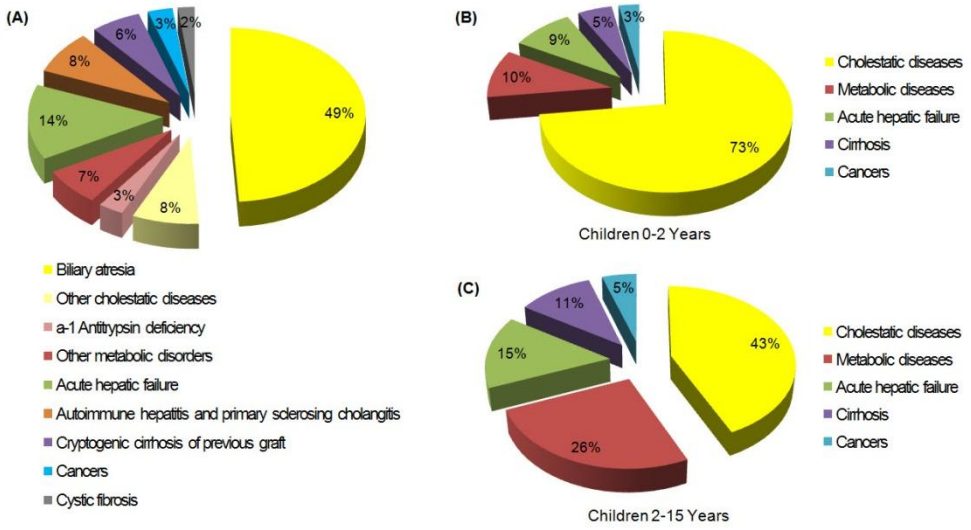


Figure 7: Transplant indications for paediatric OLT, as reported by Sokal et al (A) [63] and Adam and Hoti for children from 0-2 years old (B) and children from 2-15 years old (C) [58].

Chronic cholestatic diseases are responsible for a high number (>50%) of OLT in - especially very young - children (see Figure 7). The main pathology in this group is biliary atresia. This disease, for which the actual cause is not elucidated yet, is characterized by biliary obstruction with unknown origin, leading to secondary biliary cirrhosis [65]. The condition is treated surgically, using the Kasai portoenterostomy, in

order to restore some bile flow [66]. This operation is successful for 33% of the patients, if performed before the age of 90 days [67]. For 67% of the patients, however, this operation is only a bridge to a liver transplantation in the period before adulthood [68]. Another cholestatic disease is progressive familial intrahepatic cholestasis (PFIC), which leads to severe intrahepatic cholestasis with progressive hepatocellular damage. There are 3 types of PFIC, all caused by genetic defects: PFIC-1, also known as Byler disease, with a mutation in the FIC1 gene; PFIC-2, bile salt export pump (BSEP) deficiency; and PFIC-3, multi-drug resistance gene-3 (MDR-3) deficiency. Types 1 and 2 lead to failure of bile acid secretion at the canalicular level [60]. Other cholestatic diseases include Alagille syndrome, total parenteral nutrition-related cholestasis, Caroli disease, and idiopathic neonatal cholestasis [63].

In the category of metabolic diseases, α–1 antitrypsin deficiency (A1AD) is the main indication for OLT. A1AD is a genetic disorder with a highly variable presentation of liver disease. If presented in the neonatal period, it is characterized by jaundice, abdominal distension, poor feeding and weight gain, pruritus, hepatomegaly, and splenomegaly [69]. Other metabolic diseases for which OLT may be indicated are, amongst others, tyrosinemia, urea cycle defects, hyperoxaluria, Wilson's disease, and glycogen storage diseases. Acute liver failure, accounting for 9-15% of OLT in children, often has an unknown aetiology [63]. Possible causes are non A-E viral hepatitis, drug toxicity (acetaminophen, halothane, valproate, aspirin), or autoimmune hepatitis [70]. Liver transplantation has also been proven effective in the treatment of liver tumours. Hepatoblastoma is the most common (>70%) tumour in paediatric OLT [63]. Cystic fibrosis leads to liver disease in 20% of the adolescents, manifesting as compensated cirrhosis [71]. Despite the minimal cholestasis and the satisfactory synthetic function of the liver, the patients are in a "cirrhotic status". Clinical features comprise reduced muscle mass, decreased growth, splenomegaly, abdominal pain, poor appetite, and malabsorption [63], often requiring OLT. In some patients with Alagille syndrome or PFIC, symptoms can be non-life threatening, but can affect the quality of life, due to extensive xanthomas or unmanageable pruritus [63]. In these patients, OLT will not save lives, but will nevertheless spectacularly improve the patient's condition, allowing for a normal life, without limitations in physical, social, and educational activities.

In accordance to the Model for End stage Liver Disease (MELD) score in adults (see 4.1) an allocation system for available organs was developed and implemented to allocate livers to children on the basis of medical need [63]. The Paediatric End-stage Liver Disease (PELD) scoring system uses an algorithm based on the objective and measurable parameters age (< 1 year), serum albumin, serum total bilirubin, INR, and growth failure [72]. In order to avoid discrimination of certain subgroups on the transplant waiting list,

the diagnosis is not incorporated in the calculation. For patients older than 12 years, the MELD score is applied [63]. If the calculated PELD or MELD score is believed to not adequately reflect the pretransplant mortality risk or the severity of the patient's condition (in some conditions, such as tumours, hepatopulmonary syndrome, or metabolic liver disease), the addition of exception points may be requested [63].

3.3 Treatment of liver disease in children

The treatment of chronic liver disease requires a multidisciplinary approach, as well as an intense monitoring of the patient [73]. Figure 8 summarizes the three categories of chronic liver disease, each with their own treatment and management goals, as described by El-Shabrawi and Kamal [73].

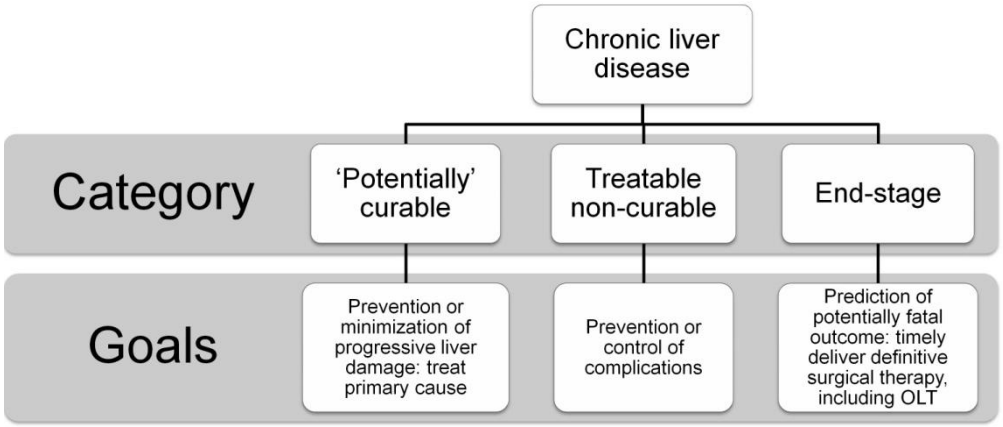


Figure 8: Chronic liver diseases can be classified in three categories. In each category, other treatment and management goals are pursued [73].

The management of liver disease consists mainly of dealing with the organ specific deficiencies, but also requires focus on the prevention or reduction of common complications of hepatic impairment, such as ascites, portal hypertension and associated oesophagogastric varices and bleeding, and pruritus. Moreover, dietary changes are needed, in order to address the substrate and nutrient deficiencies due to impaired absorption or intake, and impaired hepatic homeostasis. Fat malabsorption, for example, leads to fat-soluble vitamins deficiencies, and consequently supplementation of the vitamins A, D, E, and K is essential [74]. As mentioned above, orthotopic liver transplantation (OLT) is considered when the dietary and pharmacological interventions are insufficient.

Whereas acute liver failure only requires short term treatment, most of the other indications for paediatric liver transplantation are chronic diseases, often requiring chronic medication. This is reflected in the results of a medication review in 31 paediatric patients undergoing liver transplantation at Ghent University Hospital or Cliniques Saint-

Luc (Belgium), taking into account the 2 weeks prior to the transplantation. The basic daily therapy of children with liver disease often consists of the use of the same combination of drugs: (1) supplementation of the lipid soluble vitamins A, D, E, and K; (2) rifampicin to mitigate pruritus [75]; (3) a proton pump inhibitor ((es)omeprazole) or H_2-antihistaminic (e.g. ranitidine) to soothe reflux symptoms or dyspepsia; and (4) acetaminophen for pain relief. Other drugs are administered in case of the occurrence of more severe side effects, or to treat liver related problems. An overview of the drugs used in the 31 patients evaluated is depicted in Table 3.

Table 3: Drugs used in 31 paediatric liver transplant patients, up until 2 weeks prior to transplantation.

Most frequently used:
Vitamin A (retinol), D (25-OH Vitamin D), E (α-tocopherol), K (phytonadione)
Rifampicin
(Es)omeprazole, Ranitidine
Acetaminophen
Other
Ursodesoxycholic acid
Amylase, Lipase, Protease
Antilymphocyt IgG
Diuretics
Furosemide, Potassium canrenoate, Spironolactone
Anti-hypertensive drugs
Amlodipine, Propranolol
Antibiotics/Antibacterials
Amoxicillin, Ampicillin, Cefotaxime, Cefuroxime, Ciprofloxacin, Colistin, Meropenem, Penicillamine, Sulfomethoxazole + Trimethoprim, Temocillin, Vancomycin
Antimycotics
Fluconazole, Metronidazole, Nystatin
Domperidone
Somatostatin
Respiratory drugs
Dornase alfa, Ipratropium bromide, Salbutamol (aerosol)
Acetylcysteine
Antihistamines
Dimetindene, Dexchlorpheniramine
Alprostadil
Methylprednisolone
Tranexamic acid

4 PHARMACOKINETICS IN LIVER DISEASE

In patients with liver disease, many changes in drug response can be attributed to pharmacokinetic changes. However, altered therapeutic efficiency can also result from changes in drug receptor binding, in the affinity of a drug for its receptor, or in the intrinsic activity of the receptor [76]. For example, patients with cirrhosis appear to be more sensitive to central adverse effects of morphine [77] and midazolam [78], and to the renal adverse effects of NSAIDs [79]. In contrast, due to a reduced sensitivity to the natriuretic effect of loop diuretics, a higher tubular concentration of diuretics is needed in patients with cirrhosis to excrete the same amount of sodium as a healthy individual [80].

4.1 PK in adults with liver disease

The liver is a key organ in the pharmacokinetics. Not only is it the most important organ for drug biotransformation, other parameters, such as liver blood flow, plasma protein binding, and biliary excretion, which depend on the normal function of the liver, can possibly influence other aspects of pharmacokinetics. It is therefore not surprising that liver disease in adults has been shown to significantly affect the pharmacokinetics and pharmacodynamics of drugs. In order to avoid drug toxicity due to excessive accumulation of a drug, or possibly active metabolites, dose adjustments in these patients may be essential [76,81]. The extent of alterations generally varies according to the severity of the impairment [39]. In adults, the severity of liver failure can be assessed by the Child-Pugh (CP) classification. This score is an empiric compilation of five clinical features of end-stage liver failure: serum levels of bilirubin and albumin, prothrombin time, ascites, and encephalopathy; and divides the patients into mild (class A), moderate (class B) and severe (class C) liver disease (see Table 4) [82-84].

Another classification scheme to assess chronic liver disease is the MELD (Model for End stage Liver Disease) score. This score was originally developed to assess the short-term prognosis of patients with liver cirrhosis undergoing transjugular intrahepatic portosystemic shunt (TIPS) procedure [85]. Afterwards, the MELD score was validated to be used as a prognostic indicator for patients with advanced chronic liver disease [86]. The model uses serum creatinine, total serum bilirubin, INR, and aetiology of cirrhosis to calculate the MELD score. The use of only objective parameters determined with standardized tests, is one of the advantages of this model compared to the Child-Pugh score [87].

Table 4: Child-Pugh classification of the severity of liver disease (adapted from Lucey, 2003 [84] and Ghany and Hoofnagle, 2008 [88])

Variable	**Points**		
	1	**2**	**3**
Hepatic encephalopathy	None	Minimal	Advanced
Ascites	None	Easily controlled	Poorly controlled
Bilirubin (mg/dL)	<2	2-3	>3
Albumin (g/dL)	>3.5	2.8-3.5	<2.8
Prothrombin time (sec prolonged)	<4	4-6	>6
(INR)	(<1.7)	(1.7-2.3)	(>2.3)
Scores are summed to determine Child's class: A = 5-6, B = 7-9, and C = 10-15			

In liver disease, the alterations in drug disposition occur at all levels: absorption, distribution, metabolism and excretion. The pharmacokinetics in patients with hepatic dysfunction has been reviewed in 2008 by Verbeeck [76].

4.1.1 Absorption

Liver disease is often complicated with gastrointestinal dysfunction. Despite the increased intestinal permeability in patients with liver disease, the consequences for intestinal absorption of drugs are not clear [89]. Chronic liver disease mainly affects the bioavailability of orally administered drugs, as a result of reduced presystemic hepatic clearance [76]. Drugs with a high or intermediate hepatic extraction ratio undergo an important presystemic elimination or first-pass effect. A substantial increase in the bio-availability of these orally administered drugs is the result of portosystemic shunts, resulting in a smaller fraction of mesenteric blood flow passing through the liver, and the decreased activity of important drug-metabolizing enzymes [76,90]. The increase in bio-availability, in combination with the reduced systemic clearance (as discussed further), may lead to substantial increases in exposure of flow-limited drugs, necessitating a dose reduction. In contrast, a reduced bio-availability was reported for some lipophilic drugs in patients with cholestasis, due to fat malabsorption [91].

4.1.2 Distribution

The distribution of a drug within the body depends on several parameters, including lipid solubility, and reversible binding to blood cells, plasma proteins, and tissue macromolecules [91]. Only unbound drug is capable of entering and leaving the tissue compartments. In hepatic diseases, the apparent volume of distribution may be affected by changes in protein binding, tissue binding and fluid balance.

Reduced binding of certain drugs to plasma proteins may occur due to reduced synthesis of albumin and α_1-acid glycoprotein, changes in affinity due to conformational changes of the protein, and accumulation of endogenous compounds, such as bilirubin, inhibiting plasma protein binding of certain drugs [92]. Moreover, an increase in the volume of distribution of water-soluble drugs is expected in patients with ascites (i.e. accumulation of fluid in the peritoneal cavity) [91].

4.1.3 Elimination

4.1.3.1 Metabolism

The hepatic intrinsic clearance (CL_{int}) is defined as the ability of the liver, the main organ for drug metabolism, to clear unbound drug in the absence of flow limitations. The CL_{int} depends on the activity of drug metabolizing enzymes and of sinusoidal and canalicular transporters [76]. The impaired metabolism observed in liver disease is the result of a reduction in absolute liver cell mass, a decrease in enzyme activity due to changes in the surviving cells, and a reduced uptake of drug and oxygen due to sinusoidal capillarization [76,80]. The importance of the microsomal MFO system has been discussed earlier. This system, comprising CYP enzymes and NADPH-dependent CYP reductase, requires NADPH and oxygen. Consequently, it is very sensitive to lack of oxygen due to these changes [93,94].

The influence of liver disease on the activity and expression of CYP enzymes has been extensively reviewed by Elbekai et al. [95], Villeneuve and Pichet [64] and Johnson and Thompson [91]. An interesting study was performed by Frye et al, in which the activity of 4 important CYP isoforms (CYP1A2, 2C19, 2D6, and 2E1) was assessed *in vivo* in patients with different aetiologies and severity of liver disease [96]. In general, the activity and protein content of CYP enzymes decrease with increasing disease severity. Additionally, the changes in CYP activity are variable and non-uniform for the different isoforms. These findings lead to a sequential progressive model of hepatic dysfunction, as first suggested by Branch [97], and later optimized by Frye et al [96]. The model is graphically represented in Figure 9. At an early stage of liver disease, the clearance of drugs metabolized by CYP2C19 may be expected to be reduced, whereas the clearance of drugs metabolized by the other studied CYPs (CYP1A2, 2D6 and 2E1) will be relatively normal. At an intermediate level of hepatic dysfunction, the clearances of drugs will be more or less affected by the disease, according to the specific isoform involved in their metabolism. A patient with decompensated end-stage liver disease will have reduced clearances of drugs metabolized by CYP1A2, 2C19, 2D6, and 2E1. A comparable conclusion was drawn by Villeneuve and Pichet [64]. They state that the activity and expression of CYP is reduced in patients with cirrhosis, and that the reduction is not

uniform. CYP1A, 2C19 and 3A seem particularly vulnerable to liver disease, while 2D6, 2C9 and 2E1 appear to be less affected. It is also recognized that the pattern of CYP isoform alteration may vary between liver diseases with different aetiology, as shown in several studies [98-101].

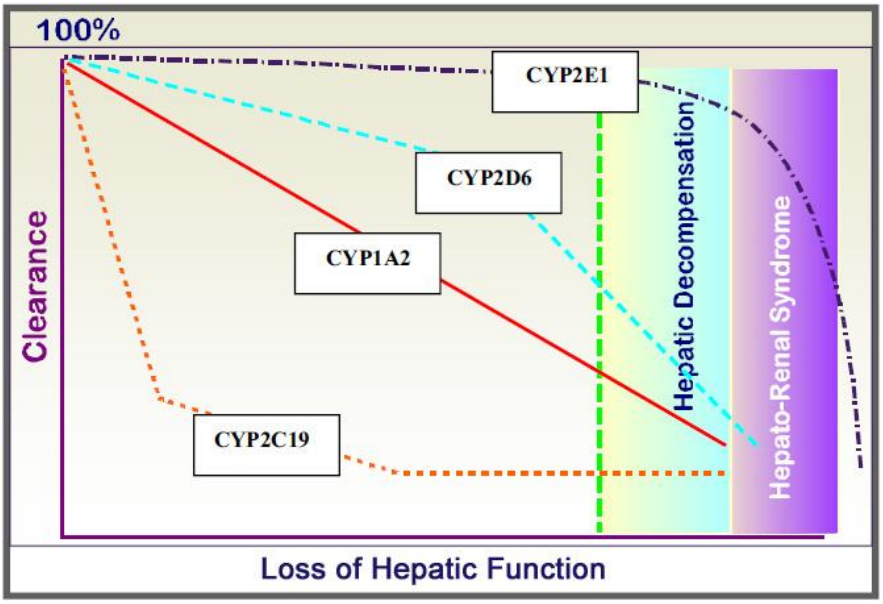


Figure 9: The sequential progressive model of hepatic dysfunction as described by Branch [97] and Frye et al [96].

In order to predict the effect on the clearance of a particular drug in an individual patient, 3 factors should be taken into account: (1) which enzyme(s) is (are) involved in the metabolism of the drug?, (2) what is the sensitivity of the enzyme(s) to liver disease?, and (3) how severe is the liver disease in the patient?

Assessment of the CYP activity can be done both *in vivo* and *in vitro*. When data obtained by using selective *in vivo* probes are compared to *in vitro* data, corrections have to be made to account for concomitant changes due to liver disease [102]. Firstly, there is a decrease in liver size with increasing severity of hepatic impairment [103]. Secondly, as described earlier, there is an increase in the fraction of unbound drug due to reduced plasma protein binding. Finally, the renal function decreases with deteriorating liver function (see further). When these considerations are taken into account, Johnson et al concluded there is a reasonable agreement between the currently available *in vivo* and *in vitro* data on the CYP expression and activity in patients with cirrhosis [102].

Conjugation reactions (phase II metabolism) were long believed to be affected to a lesser extent by cirrhosis. However, older studies appear to have used only patients with mild and moderate liver disease [104]. More recent studies included patients with advanced cirrhosis and showed impaired glucuronidation of some drugs, such as morphine,

lormetazepam, zidovudine, and mycophenolate mofetil [105-108]. Moreover, in accordance with the CYP isoforms, a differential effect on the various UGT isoforms appears to be present in patients with liver diseases [109].

4.1.3.2 Biliary excretion

Substances that are eliminated by biliary excretion may encounter reduced clearance due to extra hepatic or intrahepatic cholestasis. Additionally, biliary obstruction may lead to hepatocellular damage with consequent impairment of metabolic drug clearance. For example, the activity of CYP2C and CYP2E1 was shown to be reduced in patients with end-stage liver cirrhosis with cholestasis [98].

4.1.3.3 Renal excretion

A common complication of advanced hepatic disease is an impaired renal function. The hepatorenal syndrome is defined as unexplained progressive renal failure occurring in patients with chronic liver disease in the absence of clinical, laboratory, or anatomical evidence of other known causes of renal failure [76]. Consequently, dosage modifications may also be indicated for renally cleared drugs in patients with advanced chronic liver disease. An additional pitfall is the assessment of renal function. The estimation of the creatinine clearance based on serum creatinine measurements, as in the Cockroft-Gault method, is often inaccurate due to reduced muscle mass and impaired metabolism from creatin to creatinine in these patients [110]. Actual measurement of creatinine clearance also often overestimates the true glomerular filtration rate [111]. A more accurate marker seems to be the serum cystatin C level, as this marker is not influenced by muscle wasting [110].

4.2 Dose alterations in adults with liver disease

The effect of liver disease on drug metabolism is very complex. Additionally, the term liver disease comprises a wide variety of aetiologies and pathophysiological changes in liver function [90]. A lot of research in the past decades stresses the need for dose optimization in patients with liver disease. In contrast to the use of creatinine clearance or serum cystatin C for dose adjustments in renal impairment, there is no naturally occurring marker that can be used to estimate the hepatic clearance of drugs [112]. Although the Child-Pugh and the MELD score are valuable tools in the assessment of the severity of the liver disease, they lack the sensitivity to quantitate the specific ability of the liver to metabolize individual drugs. Up until now, most of the tests using exogenous substances (such as indocyanine green, sorbitol or erythromycin) used for the quantification of liver function or liver metabolism are too invasive or time consuming, and thus also have limited clinical value. Moreover, they are not able to predict the complex metabolism of drugs accurately [113].

In 2003 and 2005, the FDA and the EMA, respectively, published recommendations on the assessment of the PK of drugs used in patients with impaired hepatic function [114,115]. They recommend to perform a study in patients in the following situations: (1) if the hepatic metabolism and/or excretion accounts for a substantial portion (>20% of absorbed drug) of the elimination of a parent drug or active metabolite, (2) if the drug is eliminated to a lesser extent, but is a drug with a narrow therapeutic range, and (3) if the metabolism of the drugs is unknown, it should be considered as extensively metabolized. A full study should be performed in patients of all 3 Child-Pugh (CP) classes, and with at least n=6 in each study arm. If studies are performed only in patients with moderate liver disease (CP B), the findings can be used in patients with mild impairment, but the drug should be contraindicated in severe dysfunction.

These recommendations by the FDA apply for newly registered drugs, whereas for older drugs, the PK, response and toxicity in patients with liver disease was often not studied. Moreover, most of the studies describing dose adjustment in patients with liver disease did not include patients with CP class C (severe), whereas the differences in PK often associate with severity of liver disease, and the results can consequently not be extrapolated [76,116]. Consequently, there are still very limited drugs with specific dosage recommendations based on the CP scores [117].

In 2012, Periáñez-Párraga et al. [112] published a review containing dose adjustment recommendations in patients with hepatic impairment, based on the WHO list of medicines to avoid or to use with care in patients with liver disease [118], and supplemented by therapeutic novelties. This review, however, does not contain information on all drugs used in patients with liver disease. For the missing drugs, the general recommendations as published by Delco et al [113], Verbeeck [76] and Klotz [119] should be followed. These recommendations are based on flow-dependence, first-pass extraction ratio, hydrophilicity, liver metabolism extent, and plasma protein binding [116].

4.2.1 Hepatic clearance

The hepatic extraction ratio (ER) is the proportion of the drug that is removed as the drug passes through the liver. It is defined as follows:

$$ER = (C_{in} - C_{out})/C_{in} \qquad \text{(Eqn 1)}$$

where C_{in} is the concentration entering the liver, and C_{out} the concentration leaving the liver. The ER can vary from 0 (no extraction) to 1 (complete extraction). The 'well stirred' or 'venous equilibrium' model is a mathematical model relating hepatic clearance (CL_H) to

hepatic blood flow (Q), the fraction of unbound drug concentration (fu), and the intrinsic clearance of the unbound drug (CL_{int}) (Equation 2) [40].

$$CL_H\ (L/h) = Q\ (L/h) \times ER = Q\ (L/h) \times \frac{f_u \times CL_{int}}{Q + f_u \times CL_{int}} \qquad \text{(Eqn 2)}$$

This model is used to categorize drugs according to their ER, and subsequently deduce dose requirements under different clinical circumstances [91].

4.2.2 High ER drugs (ER >0.7)

In high ER drugs, clearance is very effective and consequently the $f_u \times CL_{int}$ factor dominates the denominator, following which Q becomes negligible. As $f_u \times CL_{int}$ then cancels out, the well stirred model as shown in equation 2 can be simplified into $CL_H \approx Q$. Changes in f_u or CL_{int} become less important [91].

Due to the reduced blood flow in liver disease and the formation of portosystemic shunts, there is an increase in bio-availability after oral administration, necessitating a dose reduction of both the initial and the maintenance dose [76,113]. The extent of reduction is difficult to determine, as blood flow and portosystemic shunts in a patient are usually not known. Delco et al. therefore propose a conservative approach, in which a bio-availability of 100% is assumed in patients with liver disease. Equation 3 can be used to calculate the initial dose and the first maintenance:

$$\text{Reduced dose} = \text{normal dose} \times BAv/\ 100 \qquad \text{(Eqn 3)}$$

The BAv is the bio-availability of the drug in a healthy person [113]. The maintenance dose should then be determined, taking into account the desired pharmacological effects, as well as the possible toxicity in the patient.

After systemic (e.g. intravenous) administration of a high ER drug, a decreased clearance is expected if the hepatic blood flow is reduced [76]. The initial dose should be the normal dose, whereas the maintenance dose should be determined according to the hepatic clearance of the drug. As this is reflected by blood flow, clinical tests such as Doppler measurements could be used to determine this parameter, but up until now, no clinical studies are available to confirm this methodology [113].

4.2.3 Low ER drugs (<0.3)

The equation of the well stirred model can be rewritten to $CL_H \approx f_u \times CL_{int}$ for low ER drugs, as the $f_u \times CL_{int}$ factor in the denominator becomes negligible, and the hepatic blood flow can consequently be eliminated [91].

The low ER drugs should be divided into two groups: drugs with a high binding to plasma proteins (≥ 90%), and drugs with a low plasma protein binding (< 90%). In the latter group (low binding), the clearance mainly depends on the intrinsic clearance of the liver. As described earlier, the clearance depends on the functional status of the liver, as well as on the pathways involved in the elimination of the drug. As protein binding is low, only small fluctuations in the unbound drug fraction will occur in patients with liver disease, consequently having only little or no impact on the clearance [76,113]. For these drugs, a normal initial dose can be administered to patients with liver disease. As for the maintenance dose, the following recommendations are formulated by Delco et al. [113] if no studies with dose requirements are available: (a) in patients with CP score A, administer 50% of the dose, (b) in patients with CP score B, use 25%, (c) and in patients with CP score C, only use drugs that were proven safe in a clinical trial, that have a PK that is not affected by liver disease, or for which therapeutic drug monitoring is available.

The clearance of low ER drugs with a high plasma protein binding does not only depend on the CL_{int}. Decreases in plasma protein binding, as seen in liver disease, result in an increased fraction unbound of the drug, and consequently in a more rapid metabolism of the drug [113]. However, when only the total concentration of the drug is considered, no changes in clearance are observed. Therefore, it is necessary to assess the unbound concentration of the drug, as the total concentration can be within the normal range [76].

Klotz added the recommendation to adjust both the initial and maintenance dose if a low ER drug has a narrow therapeutic range [119].

4.2.4 Intermediate ER drugs (0.3 – 0.7)

Some drugs can be classified as intermediate ER drugs. In the assessment of drug clearance, all factors of the well stirred model matter, i.e. Q, f_u and CL_{int}. Therefore, it is very difficult to predict the clinical outcomes of a drug used in patients with liver cirrhosis [91,113].

4.2.5 Additional considerations

A major pitfall in classifying drugs according to the previously described method (ER) is the scarce availability of reliable data on the hepatic extraction ratio of drugs [113].

When administering drugs to patients with liver disease some additional considerations should be taken into account [76]. For drugs that are partially excreted unchanged by the kidneys, dosing is even more complex, as patients with advanced liver disease often show concomitant renal insufficiency, or the hepato-renal syndrome. Drugs with a narrow therapeutic range should be administered with extreme caution to patients with liver

disease. Extreme caution is also recommended when any drug is administered to a patient with severe liver disease (CP score C). An increased loading dose might be required for hydrophilic drugs, as an increased volume of distribution may occur in patients with oedema or ascites, common complications of liver dysfunction.

4.2.6 Summary

The general recommendations as described above are summarized in Table 5. The review by Periáñez-Párraga et al. [112] contains an extensive list of dose recommendations for 186 drugs when used in patients with liver disease. Dose adjustments are described for drugs used in oncology therapy, anti-infective agents (antibiotics, anti-tuberculosis drugs, antifungal and anti-retroviral agents), cardiovascular therapy and other pharmacological groups.

Table 5: Dose recommendations in patients with hepatic impairment (Table adapted from Periáñez-Párraga [112])

Hepatic extraction ratio	**Protein binding**	**Dose recommendations**
High (>0.7)	Any	LD and first MD =
		ND x BAv / 100
Intermediate (0.3 – 0.7)		LD: start in low range of normal
		MD:
		CP-A: 50% of ND
		CP-B: 25% of ND
		CP-C: drug monitoring
Low (<0.3)	≥ 90%	Drug monitoring (unbound concentrations)
	< 90%	LD: normal
		MD:
		CP-A: 50% of ND
		CP-B: 25% of ND
		CP-C: drug monitoring
LD = loading dose; MD = maintenance dose; ND = normal dose; BAv = bioavailability in healthy person; CP = Child-Pugh score		

5 SAFER DRUG USE IN CHILDREN WITH LIVER DISEASE: THE (POTENTIAL) ROLE OF PBPK MODELLING

As described earlier (1.1), most drugs in children are used off-label [6,7]. This can be attributed to the many ethical and practical pitfalls in performing clinical trials in children, such as the difficult inclusion of healthy subjects, the informed consent by a surrogate consenter, and the need for an age-appropriate protocol including appropriate formulations and realistic sampling procedures [6,7]. Due to the scarce information on drug behaviour in children, dose regimens for children are currently often scaled from adult doses, using body weight, height and age. These simplified dosing approaches do not take into account the known differences in PK response in children. Additionally, there are differences between the age groups ((pre)term newborns, infants, toddlers, children, and adolescent) in rates of organ maturation, blood flow, body composition, and ontogeny of enzymes and transporters [5,11,120]. Moreover, many complex absorption and disposition processes are not captured by these "simple" scaling methods [120]. Despite the acknowledged vulnerability of the paediatric population, the social awareness for the necessity of paediatric clinical trials has grown [120]. Nevertheless, the same restraints will keep on hampering the conduction of these trials.

5.1 Modelling and simulation: tools to meet the current needs?

In order to minimize the ethical and practical issues, modelling and simulation (M&S) strategies have gained a lot of interest in drug development. M&S could become the 3rd approach in addressing a therapeutic problem, next to theoretical reasoning and hypothesis building, and actual clinical experimentation [121]. Modelling is defined as the description of the behaviour of a system or process by a set of mathematical expressions, whereas simulations are the application of the mathematical model to explore situations that have not been investigated experimentally [121]. Modelling and simulation are applied in many stages of (paediatric) drug development and are a useful tool to enhance the information gain and the efficiency of the decision-making process [121,122]. There are two types of M&S, useful in every drug development process, but illustrated here for their application in paediatric studies. *A posteriori* techniques are applied to analyze PK and PK-PD data from paediatric studies, especially using population approaches [120]. As such, maximum information can be derived from limited data. *A priori* techniques, such as Physiologically Based Pharmacokinetic (PBPK) models, are based on adult data, supplemented with PK and PD information based on preclinical species, *in vitro* experiments, paediatric data from pilot studies, and data from studies in older paediatric patients. The development and application of these models have evolved extensively in the past decade. In 2003, Nestorov published a comprehensive review on

the general structure and development of such models [123]. Several software tools are available for PBPK modelling, such as SimCYP®, GastroPlus™ and PK-Sim® [124]. Due to the unambiguous usefulness of PBPK modelling in paediatric drug design, many excellent reviews on the progresses in the field were published to keep the interested researchers up to date [120-122,125-130].

5.2 Properties of a PBPK model

PBPK models are multi-scale models containing both system-specific (patient-specific) and drug-specific properties. Relevant physiological, physicochemical and biochemical processes that determine the PK behaviour of a drug are mathematically described [122]. The *in silico* system consists of a multi-compartmental model in which each compartment represents a specific organ or physiological space. The compartments are interconnected by a blood-circulation loop and each have their specific mass-balance equation expressing the appearance and exit of the drug through the compartment [120]. Figure 10 shows the general structure of PBPK models. Other relevant physiological, pharmacogenetic, biochemical and thermodynamic information is also incorporated. Moreover, due to the possibility to add system specific information, the information on the known maturation processes can be integrated. All the system specific properties are independent of the evaluated drug [120].

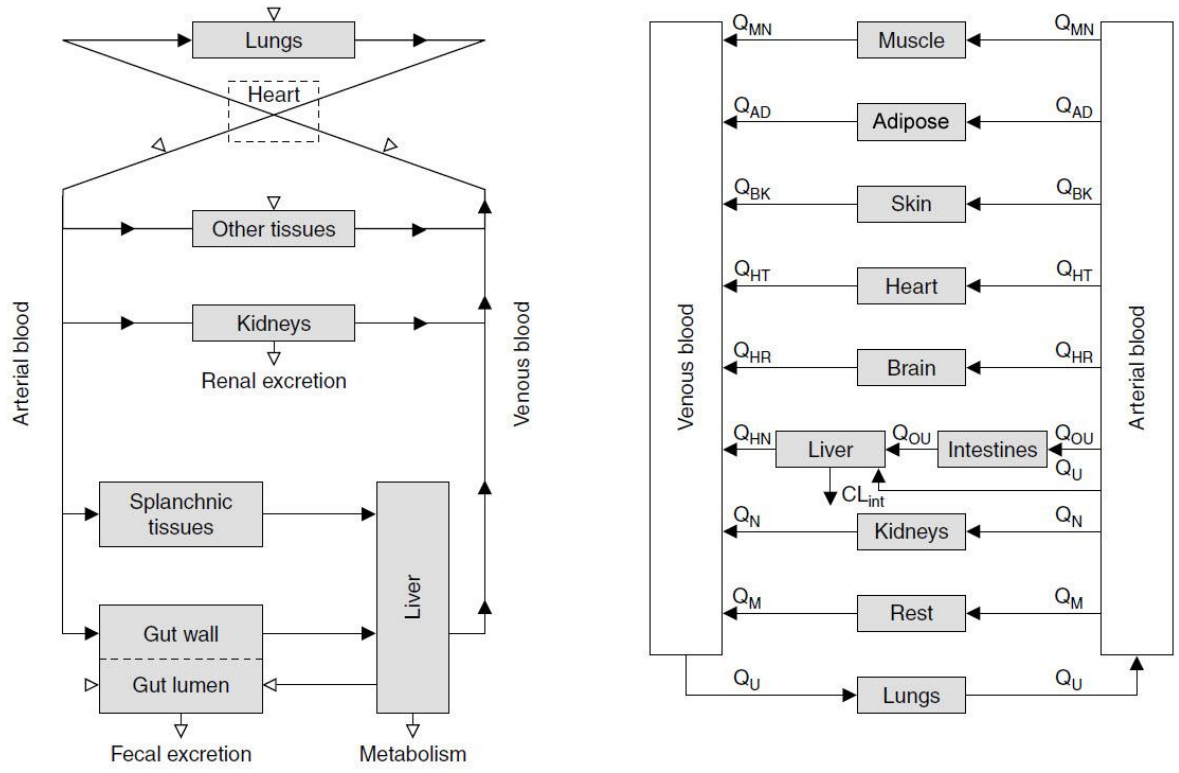


Figure 10: The left panel shows the realistic physiological pathways of drug absorption, distribution and elimination. PBPK models aim to map the complex drug transport scheme into a multi-compartment model (right panel). (Figure reproduced from Nestorov [123])

The general development of a PBPK model can be divided into 5 steps, as described by Khalil and Läer [122]. At first, the general model structure needs to be specified, dependent on the model's purpose, and physicochemical and pharmacological properties of the modelled drug. Moreover, PBPK models can be partial-body, including only certain body systems or tissues, or whole-body, when almost all body tissues are included. Subsequently, the model of each particular organ or tissue needs to be specified. The next step consists of the derivation of the mass-balance model equations. Then, the physiological and compound dependent model parameters are defined or estimated. Parameters characterizing the anatomical structure or physiological processes of the studied species are incorporated. These parameters, such as organ or tissue volumes, cardiac outputs, tissue composition, surface area, pH values, transit times for the gastro-intestinal tract, are known to vary not only among species and subjects, but also among age and altered physiological or pathological state [122]. The parameters that can be used as input for the model, can be obtained from different sources, i.e. *in vitro* experiments and *in vivo* studies [131]. Besides the system parameters, compound parameters are necessary to build the model. *In vitro* experiments, extrapolation from *in vivo* values from animals to adults, or estimations using specific algorithms may provide information on permeability, lipophilicity, ionization, plasma fraction unbound, amongst other relevant physicochemical properties. Finally, PK parameters are estimated or simulations are performed after coupling the entire system, using one of the available software systems. The output of a model is a set of concentration-time curves illustrating the behaviour of the drug in blood/plasma or other relevant organs.

Due to the incorporation of system-specific information, PBPK models allow the description of the PK of a substance based on a physiological reality [125]. Because of the integrated inter-individual variability in physiological and drug-related parameters, parameters and concentration-time profiles can be predicted realistically for an entire population. Moreover, a specific target population can be studied/modelled when specificities linked to particular organs and to a given population are used to build the model. For example, reasonable estimates of PK parameters of drugs in children can be provided after the integration of physiological age-related changes during childhood, as well as changes in protein binding, maturation of renal function, and the ontogeny of CYPs and transporters [131].

5.3 Applications of PBPK models

In general, PBPK models can be used to describe and/or predict drug PK in certain individuals, or under certain physiological or pathological conditions [122]. They can already be used early in drug development: through the anticipation of PK in animals, unnecessary animal testing can be reduced and a significant amount of time can be

saved. In the subsequent steps of development, they can help to extend the information on the drug, by exploring the effects of food, aging, rest, physical exertion and gender differences on the PK [122].

Many studies, however, focus on the specific use of PBPK models in paediatric drug development. Simulation-based trial design may help in guiding paediatric clinical trials. After the integration of maturation and growth processes, dose-exposure and exposure-response relationships can be predicted [121]. Based on this information, more informative clinical trials can be designed, as optimal sampling times, number of subjects and number of samples per subject may be selected more accurately [131]. Also, this may facilitate the first-time-in-paediatrics dose selection [132], a critical point in paediatric drug development [120].

The clearance of drugs in children has already been successfully predicted by PBPK models [133]. For this purpose, a generic whole-body Paediatric PBPK model was published by Edginton et al. [134], and this model was successfully used to scale PK from adults to children. The cause(s) of altered PK in children compared to adults can be uncovered through 'what if' scenarios using the PBPK model. Recently, a paediatric PBPK model was evaluated by performing simulations to replicate previously published paediatric clinical trials using this approach [135]. By changing the model parameters, a reduction in hepatic blood flow of 30% lead to acceptable predictions of the observed clinical values. The use of these what if scenarios may also support future trial design, or even guide individual drug treatments [122]. By suggesting age-specific dosing, clinical trials may become more "confirmatory" rather than "exploratory". Consequently, time, effort, and the number of trials needed to be performed in children can potentially be reduced.

The models may also provide support in risk assessment. They can for example predict or simulate possible drug-drug interactions [135-137]. They can also predict the PK in several (critical) situations, such as pregnancy, surgery, or organ impairment, as different physiological and pathological conditions may lead to altered PK. The use of PBPK modelling to predict the effect of liver cirrhosis was already explored by Edginton and Willmann [138]. Quantitative measures of the physiological changes in liver cirrhosis, such as organ blood flows, cardiac index, plasma binding proteins, hematocrit, functional liver volume, hepatic enzyme activity, and glomerular filtration rate, were used to build a PBPK model. The model of Edginton et al. successfully accounted for the altered physiology, and consequently the altered PK in the disease. It has been put forward as a building block for creating a generic/global whole-body PBPK model for the progressive disease of liver cirrhosis, which may facilitate future simulations and prediction of other drugs' PK [122]. The model has already been extended to orally

administered drugs by Johnson et al. [102]. It should however be noted that the parameters used in these models were all based on data obtained in adults.

The ultimate goal of all these applications is to provide better and safer drug use in children. The use of M&S in clinical situations may provide clinicians with a greater confidence in their decisions on dosing drugs in children. Moreover, it may support them in optimizing the overall medication use in paediatric patients and providing personalized medicine in children [121].

It should be noted that children are not the only special population for which extra attention is needed. As Johnson et al. [139] and la Casa Alberighi et al. [140] state, PBPK modelling can be useful at "the extremes of age", as clinical trials are not only difficult in children, but also in frail older people.

5.4 PBPK models: tools to meet the current needs in paediatric drug use?

PBPK models rely on accurate and consistent information on physiological, biochemical and physicochemical processes [122]. Some of these processes are poorly characterized, such as the abundance of transporters or the absorption process in newborns and infants. Due to information gaps, a model may fail in optimally describing the PK. As long as these gaps remain, the uncertainty on the data used to build the model should be stated. Furthermore, future research should aim at identifying and reducing the areas of greatest uncertainty [121]. One of the focal points should be the gathering of disease-related factors that could possibly influence drug PK or PD in order to develop disease-specific models [141]. However, despite the current limitations, PBPK models could offer a solution to reduce the ethical and practical hurdles encountered in the design and execution of clinical trials in children.

It should be noted that another promising approach to obtain *in vivo* pharmacokinetic information in children, is a microdosing study. The main advantage of this technique is the absence of a therapeutic effect or adverse events, as only very small amounts of the drug are administered. However, some typical practical hurdles of clinical studies in children remain, such as sample volume. Moreover, this technology requires very sensitive analytical techniques for the analysis of the samples.

6 CONCLUSION

The use of medication in children is seldom based on extensive scientific research. There is an urgent need for a more solid basis to make decisions concerning which drug to administer or the dose regimens and related risks. Due to the large differences between adults and children, a simple extrapolation from adult data is not possible. Therefore,

more clinical trials in children are required. As these trials are characterized by many ethical and practical issues, other resources, such as physiologically based pharmacokinetic models may provide important support.

The use of disease-specific PBPK models may allow the prediction of the PK in a specific patient population. The optimization of these models requires population specific information on various aspects of drug disposition. Liver disease was shown to have a huge impact on the PK of drugs in adults. Amongst others, large changes in CYP activity were described. In paediatric patients, however, there is only sparse information. In order to take the first step in optimizing a PBPK model in paediatric patients with liver disease, information on the CYP activity could be very valuable. Of course, not only the CYPs will be affected. In order to build an optimal model, many other parameters, such as absorption, should be evaluated. Nevertheless, every piece of information that fills the knowledge gap is helpful in the optimization of PBPK models, and as such provides extra support to tackle the current needs in paediatric drug use.

REFERENCES

[1] *American Pediatrics: The Social Dynamics of Professionalism, 1880-1980*. University of California Press. 1988.

[2] Steinbrook R. Testing medications in children. *N Engl J Med* 2002; 347(18):1462-1470.

[3] Hines RN, Sargent D, Autrup H, Birnbaum LS, Brent RL, Doerrer NG *et al.* Approaches for Assessing Risks to Sensitive Populations: Lessons Learned from Evaluating Risks in the Pediatric Population. *Toxicol Sci* 2010; 113(1):4-26.

[4] WHO. http://www.who.int/childmedicines/en/. 2007.

[5] Kimland E, Odlind V. Off-Label Drug Use in Pediatric Patients. *Clin Pharmacol Ther* 2012.

[6] Rocchi F, Tomasi P. The development of medicines for children: Part of a series on Pediatric Pharmacology, guest edited by Gianvincenzo Zuccotti, Emilio Clementi, and Massimo Molteni. *Pharmacol Res* 2011; 64(3):169-175.

[7] Kauffman RE. Clinical Trials in Children: Problems and Pitfalls. *Pediatric Drugs* 2000; 2(6):411-418.

[8] Yamashiro Y, Martin J, Gazarian M, Kling S, Nakamura H, Matsui A *et al.* Drug Development: The Use of Unlicensed/Off-label Medicines in Pediatrics. *J Pediatr Gastroenterol Nutr* 2012; 55(5):506-510.

[9] Orenstein SR, Hassall E. Infants and proton pump inhibitors: Tribulations, no trials. *J Pediatr Gastroenterol Nutr* 2007; 45(4):395-398.

[10] Mulla H. Understanding Developmental Pharmacodynamics: Importance for Drug Development and Clinical Practice. *Pediatric Drugs* 2010; 12(4):223-233.

[11] Kearns GL, Abdel-Rahman SM, Alander SW, Blowey DL, Leeder JS, Kauffman RE. Developmental pharmacology - Drug disposition, action, and therapy in infants and children. *N Engl J Med* 2003; 349(12):1157-1167.

[12] Bylund DB, Reed AL. Childhood and adolescent depression: Why do children and adults respond differently to antidepressant drugs? *Neurochem Int* 2007; 51(5):246-253.

[13] Murrin LC, Sanders JD, Bylund DB. Comparison of the maturation of the adrenergic and serotonergic neurotransmitter systems in the brain: Implications for differential drug effects on juveniles and adults. *Biochem Pharmacol* 2007; 73(8):1225-1236.

[14] Herlenius E, Lagercrantz H. Development of neurotransmitter systems during critical periods. *Exp Neurol* 2004; 190:S8-S21.

[15] Tobin JR. Paradoxical effects of midazolam in the very young. *Anesthesiology* 2008; 108(1):6-7.

[16] Chugani DC, Muzik O, Juh+ísz C, Janisse JJ, Ager J, Chugani HT. Postnatal maturation of human GABAA receptors measured with positron emission tomography. *Ann Neurol* 2001; 49(5):618-626.

[17] Brooks-Kayal AR, Pritchett DB. Developmental changes in human +¦-aminobutyric acida receptor subunit composition. *Ann Neurol* 1993; 34(5):687-693.

[18] Koch SC, Fitzgerald M, Hathway GJ. Midazolam potentiates nociceptive behavior, sensitizes cutaneous reflexes, and is devoid of sedative action in neonatal rats. *Anesthesiology* 2008; 108(1):122-129.

[19] Nandi R, Fitzgerald M. Opioid analgesia in the newborn. *European Journal of Pain* 2005; 9(2):105-108.

[20] Nandi R, Beacham D, Middleton J, Koltzenburg M, Howard RF, Fitzgerald M. The functional expression of mu opioid receptors on sensory neurons is developmentally regulated; morphine analgesia is less selective in the neonate. *Pain* 2004; 111(1-2):38-50.

[21] Rahman W, Dashwood MR, Fitzgerald M, ynsley-Green A, Dickenson AH. Postnatal development of multiple opioid receptors in the spinal cord and development of spinal morphine analgesia. *Developmental Brain Research* 1998; 108(1ΓÇô2):239-254.

[22] Kretz FJ, Reimann B. Ontogeny of receptors relevant to anesthesiology. *Current Opinion in Anesthesiology* 2003; 16(3).

[23] Holladay S, Smialowicz R. Development of the murine and human immune system: differential effects of immunotixicants depend on time of exposure. *Environ Health Perspect* 2000; 108(S3):463-473.

[24] Teig N, Moses D, Gieseler S, Schauer U. Age-Related Changes in Human Blood Dendritic Cell Subpopulations. *Scand J Immunol* 2002; 55(5):453-457.

[25] Clapp DW. Developmental Regulation of the Immune System. *Semin Perinatol* 2006; 30(2):69-72.

[26] Holsapple MP, Paustenbach DJ, Charnley G, West LJ, Luster MI, Dietert RR *et al.* Symposium summary: Children's Health RiskΓÇöWhat's So Special about the Developing Immune System? *Toxicol Appl Pharmacol* 2004; 199(1):61-70.

[27] Kavelaars A, Cats B, Visser GHA, Zegers BJM, Bakker JM, Rees EP *et al.* Ontogeny of the Response of Human Peripheral Blood T Cells to Glucocorticoids. *Brain Behav Immun* 1996; 10(3):288-297.

[28] Marshall JD, Kearns GL. Developmental pharmacodynamics of cyclosporine. *Clin Pharmacol Therap* 1999; 66(1):66-75.

[29] Auslender M. New drugs in the treatment of heart failure. *Progress in Pediatric Cardiology* 2000; 12(1):119-124.

[30] Grenier MA, Fioravanti J, Truesdell SC, Mendelsohn AM, Vermilion RP, Lipshultz SE. Angiotensin-converting enzyme inhibitor therapy for ventricular dysfunction in infants, children and adolescents: a review. *Progress in Pediatric Cardiology* 2000; 12(1):91-111.

[31] Läer S, Elshoff JP, Meibohm B, Weil J, Mir TS, Zhang W *et al.* Development of a Safe and Effective Pediatric Dosing Regimen for Sotalol Based on Population Pharmacokinetics and Pharmacodynamics in Children With Supraventricular Tachycardia. *J Am Coll Cardiol* 2005; 46(7):1322-1330.

[32] Takahashi H, Ishikawa S, Nomoto S, Nishigaki Y, Ando F, Kashima T *et al.* Developmental changes in pharmacokinetics and pharmacodynamics of warfarin enantiomers in Japanese children[ast]. *Clin Pharmacol Ther* 2000; 68(5):541-555.

[33] Massicotte P, Leaker M, Marzinotto V, Adams M, Freedom R, Williams W *et al.* Enhanced thrombin regulation during warfarin therapy in children compared to adults. *Thromb Haemost* 1998; 80(4):570-574.

[34] Alcorn J, McNamara PJ. Ontogeny of hepatic and renal systemic clearance pathways in infants - Part I. *Clin Pharmacokinet* 2002; 41(12):959-998.

[35] Alcorn J, McNamara PJ. Pharmacokinetics in the newborn. *Advanced Drug Delivery Reviews* 2003; 55(5):667-686.

[36] Benedetti MS, Baltes EL. Drug metabolism and disposition in children. *Fundamental & Clinical Pharmacology* 2003; 17(3):281-299.

[37] Benedetti MS, Whomsley R, Baltes EL. Differences in absorption, distribution, metabolism and excretion of xenobiotics between the paediatric and adult populations. *Expert Opin Drug Met* 2005; 1(3):447-471.

[38] Anderson GD. Developmental Pharmacokinetics. *Semin Pediatr Neurol* 2010; 17(4):208-213.

[39] Poggesi I, Benedetti MS, Whomsley R, Lamer SL, Molimard M, Watelet JB. Pharmacokinetics in special populations. *Drug Metab Rev* 2009; 41(3):422-454.

[40] Rowland M, Tozer T. *Clinical Pharmacokinetics and Pharmacodynamics: Concepts and Applications*. 4th ed. Wolters Kluwer Health - Lippincott Williams & Wilkins. 2011.

[41] Williams JA, Hyland R, Jones BC, Smith DA, Hurst S, Goosen TC *et al.* Drug-drug interactions for UDP-glucuronosyltransferase substrates: A pharmacokinetic explanation for typically observed low exposure (AUC(i)/AUC) ratios. *Drug Metab Dispos* 2004; 32(11):1201-1208.

[42] Frye RE. Probing the world of cytochrome P450 enzymes. *Mol Interv* 2004; 4(3):157-162.

[43] Nebert DW, Russell DW. Clinical importance of the cytochromes P450. *Lancet* 2002; 360(9340):1155-1162.

[44] Nelson DR, Kamataki T, Waxman DJ, Guengerich FP, Estabrook RW, Feyereisen R *et al.* The P450 Superfamily - Update on New Sequences, Gene-Mapping, Accession Numbers, Early Trivial Names of Enzymes, and Nomenclature. *DNA Cell Biol* 1993; 12(1):1-51.

[45] Bertz RJ, Granneman GR. Use of in vitro and in vivo data to estimate the likelihood of metabolic pharmacokinetic interactions. *Clin Pharmacokinet* 1997; 32(3):210-258.

[46] Evans WE, Relling MV. Pharmacogenomics: Translating functional genomics into rational therapeutics. *Science* 1999; 286(5439):487-491.

[47] Ingelman-Sundberg M. Human drug metabolising cytochrome P450 enzymes: properties and polymorphisms. *Naunyn Schmiedebergs Arch Pharmacol* 2004; 369(1):89-104.

[48] Krishna DR, Klotz U. Extrahepatic Metabolism of Drugs in Humans. *Clin Pharmacokinet* 1994; 26(2):144-160.

[49] Shargel L, Yu A. *Applied Biopharmaceutics and Pharmacokinetics*. 4th ed. McGraw-Hill. 1999.

[50] Donato MT, Castell JV. Strategies and molecular probes to investigate the role of cytochrome P450 in drug metabolism - Focus on in vitro studies. *Clin Pharmacokinet* 2003; 42(2):153-178.

[51] Guengerich FP. Similarity of Nuclear and Microsomal Cytochromes-P-450 in the Invitro Activation of Aflatoxin-B-1. *Biochem Pharmacol* 1979; 28(19):2883-2890.

[52] Alvares A, Pratt W. Pathways of drug metabolism. In: Pratt W, Taylor P, eds. *Principles of Drug Action*. Churchill Livingstone. 1990.

[53] Clarke SE, Jones BC. Hepatic cytochromes P450 and their role in metabolism-based drug-drug interactions. In: Rodriguez A, ed. *Drug-drug Interaction*. Marcel Dekker. 2002: 55-88.

[54] de Wildt SN. Profound changes in drug metabolism enzymes and possible effects on drug therapy in neonates and children. *Expert Opin Drug Metab Toxicol* 2011; 7(8):935-948.

[55] de Wildt SN, Ito S, Koren G. Challenges for drug studies in children: CYP3A phenotyping as example. *Drug Discovery Today* 2009; 14(1-2):6-15.

[56] Hines RN. Ontogeny of human hepatic cytochromes P450. *J Biochem Mol Toxicol* 2007; 21(4):169-175.

[57] Hines RN. The ontogeny of drug metabolism enzymes and implications for adverse drug events. *Pharmacology & Therapeutics* 2008; 118(2):250-267.

[58] Adam R, Hoti E. Liver Transplantation: The current Situation. *Semin Liver Dis* 2009; 29:3-18.

[59] Arya GAJ, Balistreri WF. Pediatric liver disease in the United States: Epidemiology and impact. *J Gastroenterol Hepatol* 2002; 17(5):521-525.

[60] Samyn M, Mieli-Vergani G. Liver and biliary disease in infancy. *Medicine (Baltimore)* 2007; 35(2):61-66.

[61] Featherstone B. Causes of liver disease and dysfunction. In: North-Lewis P, ed. *Drugs and the Liver: A guide to drug handling in liver dysfunction*. London: Pharmaceutical Press. 2008: 49-72.

[62] Lacaille F. Liver transplantation and liver cell transplantation. *Clinics and Research in Hepatology and Gastroenterology* 2012;(0).

[63] Sokal E, Goldstein D, Ciocca M, Lewindon P, Ni Y, Silveira T *et al.* End-stage liver disease and liver transplant: Current situation and key issues. *J Pediatr Gastroenterol Nutr* 2008; 47(2):239-246.

[64] Villeneuve JP, Pichette V. Cytochrome P450 and liver diseases. *Curr Drug Metab* 2004; 5(3):273-282.

[65] Karakayali H, Sevmis S, Íztelik, Ozcay F, Moray G, Torgay A *et al.* Liver Transplantation for Biliary Atresia. *Transplant Proc* 2008; 40(1):231-233.

[66] Greenberger NJ, Paumgartner G. Diseases of the Gallbladder and Bile Ducts. In: Fauci AS, Kasper DL, Longo DL, Braunwald E, Hauser SL, Jameson JL *et al.*, eds. *Harrison's Principles of Internal Medicine*. McGraw-Hill Medical. 2008: 1991-2001.

[67] Fouquet V, Alves A, Branchereau S, Grabar S, Debray D, Jacquemin E *et al.* Long-term outcome of pediatric liver transplantation for biliary atresia: A 10-year follow-up in a single center. *Liver Transplantation* 2005; 11(2):152-160.

[68] Otte JB, Degoyet JD, Reding R, Hausleithner V, Sokal E, Chardot C *et al.* Sequential Treatment of Biliary Atresia with Kasai Portoenterostomy and Liver-Transplantation - A Review. *Hepatology* 1994; 20(1):S41-S48.

[69] Bals R. Alpha-1-antitrypsin deficiency. *Best Practice & Research in Clinical Gastroenterology* 2010; 24(5):629-633.

[70] Muiesan P, Vergani D, Mieli-Vergani G. Liver transplantation in children. *J Hepatol* 2007; 46(2):340-348.

[71] Kelly DA. Liver and biliary disease in childhood. *Medicine (Baltimore)* 2011; 39(10):571-575.

[72] McDiarmid SV. Management of the pediatric liver transplant patient. *Liver Transplantation* 2001; 7(11):S77-S86.

[73] El-Shabrawi MHF, Kamal NM. Medical Management of Chronic Liver Diseases in Children (Part I) Focus on Curable or Potentially Curable Diseases. *Pediatric Drugs* 2011; 13(6):357-370.

[74] Tsouka A, McLin VA. Complications of chronic liver disease. *Clinics and Research in Hepatology and Gastroenterology* 2012; 36(3):262-267.

[75] Kremer AE, Beuers U, Oude-Elferink RP, Pusl T. Pathogenesis and Treatment of Pruritus in Cholestasis. *Drugs* 2008; 68(15):2163-2182.

[76] Verbeeck RK. Pharmacokinetics and dosage adjustment in patients with hepatic dysfunction. *Eur J Clin Pharmacol* 2008; 64(12):1147-1161.

[77] Tegeder I, Lotsch J, Geisslinger G. Pharmacokinetics of opioids in liver disease. *Clin Pharmacokinet* 1999; 37(1):17-40.

[78] Macgilchrist AJ, Birnie GG, Cook A, Scobie G, Murray T, Watkinson G *et al.* Pharmacokinetics and Pharmacodynamics of Intravenous Midazolam in Patients with Severe Alcoholic Cirrhosis. *Gut* 1986; 27(2):190-195.

[79] Gines P, Arrovo V, Rodes J. Pharmacotherapy of Ascites Associated with Cirrhosis. *Drugs* 1992; 43(3):316-332.

[80] Morgan DJ, Mclean AJ. Clinical Pharmacokinetic and Pharmacodynamic Considerations in Patients with Liver-Disease - An Update. *Clin Pharmacokinet* 1995; 29(5):370-391.

[81] Verbeeck RK, Horsmans Y. Effect of hepatic insufficiency on pharmacokinetics and drug dosing. *Pharmacy World & Science* 1998; 20(5):183-192.

[82] Angermayr B, Cejna M, Karnel F, Gschwantler M, Koenig F, Pidlich J *et al.* Child-Pugh versus MELD score in predicting survival in patients undergoing transjugular intrahepatic portosystemic shunt. *Gut* 2003; 52(6):879-885.

[83] Pugh RNH, Murrayly IM, Dawson JL, Pietroni MC, Williams R. Transection of Esophagus for Bleeding Esophageal Varices. *Br J Surg* 1973; 60(8):646-649.

[84] Lucey M. Assessment for liver transplantation. In: Lucey M, Neuberger J, Shaked A, eds. *Liver Transplantation*. Georgetown: Landes Bioscience. 2003: 17-35.

[85] Malinchoc M, Kamath PS, Gordon FD, Peine CJ, Rank J, ter Borg PCJ. A model to predict poor survival in patients undergoing transjugular intrahepatic portosystemic shunts. *Hepatology* 2000; 31(4):864-871.

[86] Kamath PS, Wiesner RH, Malinchoc M, Kremers W, Therneau TM, Kosberg CL *et al.* A model to predict survival in patients with end-stage liver disease. *Hepatology* 2001; 33(2):464-470.

[87] Wiesner RH, McDiarmid SV, Kamath PS, Edwards EB, Malinchoc M, Kremers WK *et al.* MELD and PELD: Application of survival models to liver allocation. *Liver Transplantation* 2001; 7(7):567-580.

[88] Ghany M, Hoofnagle JH. Approach to the Patient with Liver Disease. In: Fauci AS, Kasper DL, Longo DL, Braunwald E, Hauser SL, Jameson JL *et al.*, eds. *Harrison's Principles of Internal Medicine*. McGraw-Hill Medical. 2008: 1918-1923.

[89] Zuckerman MJ, Menzies IS, Ho H, Gregory GG, Casner NA, Crane RS *et al.* Assessment of intestinal permeability and absorption in cirrhotic patients with ascites using combined sugar probes. *Dig Dis Sci* 2004; 49(4):621-626.

[90] Blaschke TF. Effect of liver disease on dose optimization. *International Congress Series* 2001; 1220:247-258.

[91] Johnson TN, Thomson A. Pharmacokinetics of drugs in liver disease. In: North-Lewis P, ed. *Drugs and the Liver: A guide to drug handling in liver dysfunction*. London: Pharmaceutical Press. 2008: 103-133.

[92] MacKichan J. Influence of protein binding and use of unbound (free) drug concentrations. In: Burton M, Shaw LM, Schentag JJ, Evans WE, eds. *Applied Pharmacokinetics and Pharmacodynamics. Principles of Therapeutic Drug Monitoring*. Baltimore: Lippincott Wiliams & Wilkins. 2006: 82-120.

[93] Le Couteur DG, Fraser R, Hilmer S, Rivory LP, Mclean AJ. The hepatic sinusoid in aging and cirrhosis - Effects on hepatic substrate disposition and drug clearance. *Clin Pharmacokinet* 2005; 44(2):187-200.

[94] Mclean AJ, Morgan DJ. Clinical Pharmacokinetics in Patients with Liver-Disease. *Clin Pharmacokinet* 1991; 21(1):42-69.

[95] Elbekai RH, Korashy HM, El-Kadi AOS. The effect of liver cirrhosis on the regulation and expression of drug metabolizing enzymes. *Curr Drug Metab* 2004; 5(2):157-167.

[96] Frye RF, Zgheib NK, Matzke GR, Chaves-Gnecco D, Rabinovitz M, Shaikh OS *et al.* Liver disease selectively modulates cytochrome P450-mediated metabolism. *Clin Pharmacol Ther* 2006; 80(3):235-245.

[97] Branch RA. Drugs in liver disease. *Clin Pharmacol Ther* 1998; 64(4):462-465.

[98] George J, Murray M, Byth K, Farrell GC. Differential Alterations of Cytochrome-P450 Proteins in Livers from Patients with Severe Chronic Liver-Disease. *Hepatology* 1995; 21(1):120-128.

[99] George J, Liddle C, Murray M, Byth K, Farrell GC. Pre-translational regulation of cytochrome P450 genes is responsible for disease-specific changes of individual P450 enzymes among patients with cirrhosis. *Biochem Pharmacol* 1995; 49(7):873-881.

[100] Guengerich FP, Turvy CG. Comparison of Levels of Several Human Microsomal Cytochrome-P-450 Enzymes and Epoxide Hydrolase in Normal and Disease States Using Immunochemical Analysis of Surgical Liver Samples. *J Pharmacol Exp Ther* 1991; 256(3):1189-1194.

[101] Lown K, Kolars J, Turgeon K, Merion R, Wrighton SA, Watkins PB. The Erythromycin Breath Test Selectively Measures P450Iiia in Patients with Severe Liver-Disease. *Clin Pharmacol Therap* 1992; 51(3):229-238.

[102] Johnson TN, Boussery K, Rowland-Yeo K, Tucker GT, Rostami-Hodjegan A. A Semi-Mechanistic Model to Predict the Effects of Liver Cirrhosis on Drug Clearance. *Clin Pharmacokinet* 2010; 49(3):189-206.

[103] Li YM, Lv F, Xu X, Ji H, Gao WT, Lei TL *et al.* Evaluation of liver functional reserve by combining D-sorbitol clearance rate and CT measured liver volume. *World Journal of Gastroenterology* 2003; 9(9):2092-2095.

[104] Hoyumpa AM, Schenker S. Is Glucuronidation Truly Preserved in Patients with Liver-Disease. *Hepatology* 1991; 13(4):786-795.

[105] Hasselstrom J, Eriksson S, Persson A, Rane A, Svensson JO, Sawe J. The Metabolism and Bioavailability of Morphine in Patients with Severe Liver-Cirrhosis. *Br J Clin Pharmacol* 1990; 29(3):289-297.

[106] Hildebrand M, Hellstern A, Humpel M, Hellenbrecht D, Saller R. Plasma-Levels and Urinary-Excretion of Lormetazepam in Patients with Liver-Cirrhosis and in Healthy-Volunteers. *Eur J Drug Metab Pharmacokinet* 1990; 15(1):19-26.

[107] Parker G, Bullingham R, Kamm B, Hale M. Pharmacokinetics of oral mycophenolate mofetil in volunteer subjects with varying degrees of hepatic oxidative impairment. *J Clin Pharmacol* 1996; 36(4):332-344.

[108] Taburet AM, Naveau S, Zorza G, Colin JN, Delfraissy JF, Chaput JC *et al.* Pharmacokinetics of Zidovudine in Patients with Liver-Cirrhosis. *Clin Pharmacol Therap* 1990; 47(6):731-739.

[109] Furlan V, Demirdjian S, Bourdon O, Magdalou J, Taburet AM. Glucuronidation of drugs by hepatic microsomes derived from healthy and cirrhotic human livers. *J Pharmacol Exp Ther* 1999; 289(2):1169-1175.

[110] Orlando R, Mussap M, Plebani M, Piccoli P, De Martin S, Floreani M *et al.* Diagnostic value of plasma cystatin C as a glomerular filtration marker in decompensated liver cirrhosis. *Clin Chem* 2002; 48(6):850-858.

[111] Proulx NL, Akbari A, Garg AX, Rostom A, Jaffey J, Clark HD. Measured creatinine clearance from timed urine collections substantially overestimates glomerular filtration rate in patients with liver cirrhosis: a systematic review and individual patient meta-analysis. *Nephrology Dialysis Transplantation* 2005; 20(8):1617-1622.

[112] Periáñez-Párraga L, Martinez-Lopez I, Ventayol Bosch P, Puigventos Latorre F, gado Sanchez O. Drug dosage recommendations in patients with chronic liver disease. *Rev Esp Enferm Dig* 2012; 104(4):165-184.

[113] Delco F, Tchambaz L, Schlienger R, Drewe J, Krahenbuhl S. Dose adjustment in patients with liver disease. *Drug Saf* 2005; 28(6):529-545.

[114] FDA. Guidance for Industry: Pharmacokinetics in patients with Impaired Hepatic Function: Study Design, Data Analysis, and Impact on Dosing and Labeling. 2003.

[115] EMA. Guideline on the evaluation of the pharmacokinetics of medicinal products in patients with impaired hepatic function. 2005.

[116] Andrade RJ, Isabel Lucena M. Drugs prescription in patients with chronic liver disease: rules for adjusting doses and beyond. *Rev Esp Enferm Dig* 2012; 104(4):161-164.

[117] Spray JW, Willett K, Chase D, Sindelar R, Connelly S. Dosage adjustment for hepatic dysfunction based on Child-Pugh scores. *Am J Health Syst Pharm* 2007; 64(7):690-+.

[118] WHO. WHO model formulary 2008. *http://www.who.int/selection_medicines/list/WMF2008.pdf*. 2009.

[119] Klotz U. Antiarrhythmics: Elimination and dosage considerations in hepatic impairment. *Clin Pharmacokinet* 2007; 46(12):985-996.

[120] Barrett JS, Alberighi OD, Läer S, Meibohm B. Physiologically Based Pharmacokinetic (PBPK) Modeling in Children. *Clin Pharmacol Therap* 2012; 92(1):40-49.

[121] Läer S, Barrett JS, Meibohm B. The In Silico Child: Using Simulation to Guide Pediatric Drug Development and Manage Pediatric Pharmacotherapy. *J Clin Pharmacol* 2009; 49(8):889-904.

[122] Khalil F, Läer S. Physiologically Based Pharmacokinetic Modeling: Methodology, Applications, and Limitations with a Focus on Its Role in Pediatric Drug Development. *Journal of Biomedicine and Biotechnology* 2011.

[123] Nestorov I. Whole body pharmacokinetic models. *Clin Pharmacokinet* 2003; 42(10):883-908.

[124] Jamei M, Dickinson GL, Rostami-Hodjegan A. A Framework for Assessing Inter-individual Variability in Pharmacokinetics Using Virtual Human Populations and Integrating General Knowledge of Physical Chemistry, Biology, Anatomy, Physiology and Genetics: A Tale of 'Bottom-Up' vs 'Top-Down' Recognition of Covariates. *Drug Metabolism and Pharmacokinetics* 2009; 24(1):53-75.

[125] Huang SM, Rowland M. The Role of Physiologically Based Pharmacokinetic Modeling in Regulatory Review. *Clin Pharmacol Ther* 2012; 91(3):542-549.

[126] Läer S. Pediatric Cardiovascular Drug Development and Research: Integration of Modeling and Simulation as One Future Direction. *J Cardiovasc Pharmacol* 2011; 58(3):217-227.

[127] Bellanti F, la Pasqua O. Modelling and simulation as research tools in paediatric drug development. *Eur J Clin Pharmacol* 2011; 67:S75-S86.

[128] Bouzom F, Walther B. Pharmacokinetic predictions in children by using the physiologically based pharmacokinetic modelling. *Fundamental & Clinical Pharmacology* 2008; 22(6):579-587.

[129] Rostami-Hodjegan A. Physiologically Based Pharmacokinetics Joined With In Vitro-In Vivo Extrapolation of ADME: A Marriage Under the Arch of Systems Pharmacology. *Clin Pharmacol Therap* 2012; 92(1):50-61.

[130] Edginton AN, Theil FP, Schmitt W, Willmann S. Whole body physiologically-based pharmacokinetic models: their use in clinical drug development. *Expert Opin Drug Met* 2008; 4(9):1143-1152.

[131] Dumont C, Mentré F, Gaynor C, Brendel K, Gesson C, Chenel M. Optimal Sampling Times for a Drug and its Metabolite using SIMCYP-« Simulations as Prior Information. *Clin Pharmacokinet* 2013; 52(1):43-57.

[132] Edginton AN. Knowledge-driven approaches for the guidance of first-in-children dosing. *Pediatric Anesthesia* 2011; 21(3):206-213.

[133] Johnson TN, Rostami-Hodjegan A, Tucker GT. Prediction of the clearance of eleven drugs and associated variability in neonates, infants and children. *Clin Pharmacokinet* 2006; 45(9):931-956.

[134] Edginton AN, Schmitt W, Willmann S. Development and evaluation of a generic physiologically based pharmacokinetic model for children. *Clin Pharmacokinet* 2006; 45(10):1013-1034.

[135] Johnson TN, Rostami-Hodjegan A. Resurgence in the use of physiologically based pharmacokinetic models in pediatric clinical pharmacology: parallel shift in incorporating the knowledge of biological elements and increased applicability to drug development and clinical practice. *Pediatric Anesthesia* 2011; 21(3):291-301.

[136] Bois FY. Physiologically Based Modelling and Prediction of Drug Interactions. *Basic Clin Pharmacol* 2010; 106(3):154-161.

[137] Perdaems N, Blasco H, Vinson C, Chenel M, Whalley S, Cazade F *et al.* Predictions of Metabolic Drug-Drug Interactions Using Physiologically Based Modelling. *Clin Pharmacokinet* 2010; 49(4):239-258.

[138] Edginton AN, Willmann S. Physiology-Based Simulations of a Pathological Condition Prediction of Pharmacokinetics in Patients with Liver Cirrhosis. *Clin Pharmacokinet* 2008; 47(11):743-752.

[139] Johnston C, Kirkpatrick CM, McLachlan AJ, Hilmer SN. Physiologically Based Pharmacokinetic Modeling at the Extremes of Age. *Clin Pharmacol Ther* 2013; 93(2):148.

[140] la Casa Alberighi O, Barrett JS, Läer S, Meibohm B. Response to "Physiologically Based Pharmacokinetic Modeling at the Extremes of Age". *Clin Pharmacol Ther* 2013; 93(2):149.

[141] Knibbe CAJ, Danhof M. Individualized dosing regimens in children based on population PKPD modelling: Are we ready for it? *Int J Pharm* 2011; 415(1-2):9-14.

OUTLINE

AND

AIMS OF THIS THESIS

There is a growing interest in the development and optimization of pediatric PBPK models for the prediction of the PK in children. When correctly developed and validated models are used, these predictions may serve as a decision making tool in many different steps of drug development, such as clinical trial design or safety assessment. Furthermore, more specific PBPK models could be useful to investigate drug PK in specific subpopulations such as children with hepatic dysfunction. However, it is generally acknowledged that essential information to build these models is currently not available.

In order to partially fill the knowledge gap, the general objective of this thesis was to characterize the activity of important cytochrome P450 enzymes in children with liver disease. These enzymes were selected based on their importance in drug metabolism and the known alterations in their activity in adults with hepatic impairment. In order to achieve this objective, the following aims were set:

Aim 1 was the development of a bioanalytical method for the determination of the enzyme activity in human liver microsomes. Six probe substrates were selected that are selectively metabolized by one of the assessed CYP isoforms: phenacetin, tolbutamide, S-mephenytoin, dextromethorphan, chlorzoxazone, midazolam for CYP1A2, 2C9, 2C19, 2D6, 2E1, and 3A4, respectively. Two analytical methods for the quantification of the metabolites formed during incubation, i.e. acetaminophen, 4'-OH-mephenytoin, 4-OH-tolbutamide, dextrorphan, 6-OH-chlorzoxazone, and 1-OH-midazolam were developed and validated. Firstly, **Chapter 1** describes a high performance liquid chromatography (HPLC) method coupled to a tandem mass spectrometric (MS/MS) detection using electrospray ionization (ESI). Prior to analysis, the samples were pre-treated by applying a microwave assisted derivatization with pyridine-3-sulfonyl chloride (PS), in order to increase the sensitivity and to be able to perform a single run in the positive ESI mode. Despite the increased sensitivity, another method was developed in order to increase the sample throughput. **Chapter 2** describes an ultra-high performance liquid chromatography (UPLC) MS/MS method for the quantification of the 6 metabolites, with a significantly shorter time of analysis.

Aim 2 consisted of the development of a quantification method for CYP3A4 and CYP2E1, as the abundance of the enzymes is as important as the activity. The combination of activity and abundance information may provide valuable information on the mechanisms of possible changes in activity. Moreover, abundance information is needed for adjustment of the paediatric PBPK model of SimCYP®. The concentration of CYP3A4 and 2E1 was determined using indirect enzyme-linked immunosorbent assays (ELISA). The optimization and validation of the methods for the quantification of CYP3A4 and CYP2E1 in human liver microsomes are described in **chapter 3** and **chapter 4**.

The third aim was the development of a workflow for the genotyping of the included patients. The highly polymorphic nature of many of the CYP isoforms may also influence the enzyme activity. Several acknowledged methods were combined (DNA extraction and quantification, and SNP analysis using TaqMan probes) in order to create a workflow for the genotyping of frequent and significant single nucleotide polymorphisms (SNP) of CYP2C9, 2C19 and 2D6 (**Chapter 5**).

Aim 4 was the actual determination of the *in vitro* CYP activity in children with hepatic dysfunction. During a sampling period of 5 years, 31 samples from children undergoing liver transplantation for various indications were collected at Ghent University Hospital and Cliniques Saint-Luc in Brussels. The methods described in chapters 3 to 7 were applied on the samples. **Chapter 6** describes the activity and abundance of the 6 studied isoforms in the samples. Evaluation of these data, together with patient specific information (age, weight, relevant co-medication, genetics), resulted in the identification of the determinants of the CYP activity in children with liver disease.

Due to its importance in *in vitro-in vivo* extrapolation (IVIVE), **aim 5 was to determine the microsomal protein per gram of liver (MPPGL) in our study population.** The optimal method for the determination of this factor was selected and the scaling factor was determined in patients with biliary atresia (**Chapter 7**).

The last aim, aim 6, consisted of executing a pilot study for a first evaluation of the prediction of the PK in children with liver disease (Chapter 8). Based on the information already available in SimCYP, the PK of midazolam and omeprazole (drugs often used in our study population) was predicted in our patient population. Besides being a proof-of-concept, this study also revealed the limitations of our dataset up to now, and is as such a good basis for the identification of the knowledge gaps that still need to be filled before reliable predictions can be done.

CHAPTER 1

QUANTIFICATION OF SIX PROBE METABOLITES AFTER DERIVATIZATION WITH PYRIDINE-3-SULFONYL CHLORIDE (PS) IN AN AQUEOUS ENVIRONMENT

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'Change brings opportunity'

- Nido Qubein

ABSTRACT

For the determination of the *in vitro* cytochrome P450 activity in microsomes, a quantification method for the probe metabolites, formed during incubation, is required. Due to insufficient sensitivity of a previously developed high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) method for some of the metabolites, a fast and easy derivatization method with pyridine-3-sulfonyl chloride (PS) is described. Acetaminophen (CYP1A2), dextrorphan (CYP2D6), hydroxy-chlorzoxazone (CYP2E1) and hydroxy-mephenytoin (CYP2C19) can be derivatized because of the presence of a phenolic OH, whereas hydroxy-midazolam (CYP3A4) and hydroxy-tolbutamide (CYP2C9) remain unchanged. As PS improves the ionization efficiency in the positive electrospray ionization (ESI) mode, the sensitivity of the detection is improved significantly and meets requirements for the activity determination. Native negative electrospray type molecules, moreover, become positive ESI candidates. The direct derivatization in the aqueous incubation medium, without any other sample pre-treatment steps, such as evaporation or extraction, makes this procedure easy to perform. The method using 20 s microwave irradiation was shown to equal a 10 minute reaction in a 60°C heating block, consequently simplifying and shortening the process. Collision induced fragmentation of the derivatives resulted in at least one native compound, rather than derivative, specific product ion, thereby improving the selectivity of the method in the multiple reaction monitoring mode. The HPLC-MS/MS method was validated, and was demonstrated to be sensitive, selective, precise and accurate. The absence of a relative matrix effect was established, notwithstanding that an absolute matrix effect was observed. The analysis of a sample after (human) microsomal incubation, from which some of the metabolites could not be quantified using the method without derivatization, proved the usefulness of the method.

1 INTRODUCTION

Many pre-clinical pharmacokinetic experiments determine the *in vitro* activity of the cytochrome P450 system, as these enzymes play a major role in the metabolism of many drugs. The assessment of the metabolic fate of a new drug, metabolic profiling, potential drug-drug interactions, or the activity in a specific patient population, requires the incubation of a (sub)cellular system, such as hepatocytes or microsomes, with specific probe substrates [1]. Microsomes contain 96 % of the total hepatic CYP content [2] and they are easy to use and store, which makes them a valuable tool in these experiments. During the incubation, the substrates are metabolized by a specific isoform into a specific metabolite. The appropriate substrates for the phenotyping of the six clinically most important isoforms 1A2, 2C19, 2C9, 2D6, 2E1 and 3A4 are phenacetin, S-mephenytoin, tolbutamide, dextromethorphan, chlorzoxazone and midazolam, respectively [3]. The amount of metabolite formed reflects the intrinsic (hepatic) enzymatic activity of the patient. LC-MS/MS has been used in many studies for the quantification of these metabolites. The literature based HPLC-MS/MS method for the simultaneous quantification of the six metabolites [4], implemented in our laboratory, showed insufficient sensitivity for the intended applications, i.e. the influence of distinct liver pathologies on the CYP activity. Moreover, not all metabolites could be detected in the same electrospray ionization method. Hence, the development of another analytical method was recommendable.

Even though electrospray ionization mass spectrometry (ESI-MS) is a valuable tool for the analysis of a wide range of molecules, not all compounds can be analyzed with this technique. Weakly ionisable compounds, such as neutral, nonpolar molecules, are not detected with ESI-MS. The ESI behaviour of these compounds can be altered by the attachment of a moiety carrying a formal charge or with high proton affinity, consequently improving the detectability of the analytes with ESI-MS and adding a degree of analyte selectivity. Furthermore, derivatization may allow detection of a native negative charge type analyte in the positive ion mode [5]. The potential of chemical derivatization has been proven in previously published reports, where a wide range of molecules are being derivatized using several reagents.

Most of the metabolites formed during the microsomal incubation contain a phenolic OH-function. A derivatization process commonly used for the derivatization of phenols, is 5-(dimethylamino)naphthalene-1-sulfonyl chloride (or dansyl chloride) [6-11]. Recently, Xu et al described the use of other sulfonyl chlorides for the derivatization of weakly ionisable estrogens [12,13]. The formed sulfonate can be protonated in acidic solutions,

consequently improving the ionization efficiency significantly. The use of these derivatives was suggested for other phenolic compounds [13].

A major strength of the previously published methods for the detection of (underivatized) metabolites is the lack of sample preparation: after incubation, the samples are centrifuged and the supernatant is injected as such. Therefore, a derivatization method to improve sensitivity should be as short and easy as possible. Most of the described derivatization processes consist of several evaporation, reconstitution or extraction steps. Furthermore, the reaction is performed in organic solvents. A derivatization reaction directly in the incubation medium would allow competitive comparison with non-derivatized methods. Also, as liquid chromatography is an aqueous system, the injection of an aqueous sample is preferred.

This article describes the development of a derivatization procedure of the metabolites formed during the activity assessment of the six most important CYP isoforms 1A2, 2C19, 2C9, 2D6, 2E1 and 3A4, using phenacetin, S-mephenytoin, tolbutamide, dextromethorphan, chlorzoxazone and midazolam, respectively, as probe substrates. The pyridine-3-sulfonyl derivatives were formed directly in the aqueous incubation medium during the easy and short derivatization process. The LC-MS/MS method was optimized and validated, and the complete procedure was assessed through the analysis of a human microsomal incubation sample.

2 EXPERIMENTAL

2.1 Chemicals

Phenacetin, acetaminophen (AP), tolbutamide, 4-OH-tolbutamide (HTB), S-mephenytoin, 4'-OH-mephenytoin (HME), dextromethorphan, dextrorphan (DX), chlorzoxazone, 6-OH-chlorzoxazone (HCZ), levallorphan (LA), and chlorpropamide (CP) were purchased from Sigma-Aldrich (St-Louis, MO, USA). Midazolam and 1-OH-midazolam (HMDZ) were kindly donated by Roche (Basel, Switzerland). Pyridine-3-sulfonyl chloride hydrochloride (PS) and 4-(1H-pyrazol-1-yl)benzenesulfonyl chloride (PBS) were purchased from Maybridge (Cambridge, UK). Nicotinamide adenine dinucleotide phosphate (NADPH) was obtained from Biopredic International (Rennes, France). All other chemicals were of analytical grade. Commercial rat microsomes were acquired from BD Biosciences (Wistar Han, male rat, BD Gentest, BD Biosciences, Erembodegem, Belgium). Human liver microsomes were prepared from human liver tissue (approved by the Ethics Committee of Ghent University Hospital, B67020084281).

2.2 Preparation of standards and quality controls

Primary stock standards of all metabolites and the internal standard of 1 mg/ml in methanol were prepared and stored at -20°C. Stock standards were mixed in the appropriate proportions and serially diluted in methanol. Microsomal calibration standards consisted of metabolites spiked to the microsomal incubation medium. This incubation medium was prepared by mixing 1 ml 5 mM NADPH, 1 ml 1.25 mg/ml microsomal protein, 1 ml 1.15% KCl, 1 ml 0.2M potassium phosphate buffer (pH7.4) and 0.5 ml stopreagent ($H_2O/CH_3CN/HCOOH$; 42:55:3). After vortex mixing and cool down on ice for protein denaturation, a specific amount of the stock solution mix was spiked to the incubation medium. Water was added to obtain a final volume of 5.5 ml per calibrator. Calibrators were centrifuged at 20000*xg* for 15 min, supernatant was maintained and stored at 4°C.

2.3 Chromatographic conditions

The chromatographic system consisted of a Kontron instrument (Zurich, Switzerland). The separation was carried out using a Luna C18 column (50 mm X 2.0 mm, particle size 3 µm; Phenomenex, Torrance, CA, USA) with an Alltima C18 guard column (7.5 mm X 2.1 mm, particle size 5 µm; Grace, Columbia, MD, USA). The injection volume was 10 µl. Gradient elution was performed with a flow rate of 0.2 ml/min, starting at 80% eluent A (water containing 0.1% formic acid). Eluent B (acetonitrile containing 0.1% formic acid) was linearly increased from 20% to 90% during 9 minutes. The initial conditions were regained over a 0.1 min time interval, followed by a 5 min equilibration time prior to the next injection. This resulted in an overall run time of 14 min.

2.4 MS conditions

Detection was performed using a Quattro II triple quadrupole mass spectrometer (Waters, Manchester, UK) equipped with an electrospray ionization source in the electrospray positive ion mode (ESI+). Nitrogen was used as both drying and nebulising gas. Product ions were detected using the multiple reaction monitoring (MRM) mode, using argon as collision gas. The capillary voltage and source temperature were optimized at 3.6 kV and 120°C, respectively. The collision energy and cone voltage were optimized for each compound individually. The collision energy varied from 17 eV to 30 eV and the cone voltage varied from 35 V to 50 V (see Table 1). Data were collected and processed using the MassLynx and QuanLynx software 4.0 (Waters, Manchester, UK). Table 1 illustrates the individual parameters and MRM transitions used for detection and quantification.

Table 1: Instrument parameters for the detection of the (derivatized) metabolites. PS: pyridine-3-sulfonyl chloride; HMDZ: hydroxy-midazolam; DX-PS: PS-derivatized dextrorphan; LA-PS: PS-derivatized levallorphan; HTB: hydroxy-tolbutamide; AP-PS: PS-derivatized acetaminophen; HME-PS: PS-derivatized hydroxy-mephenytoin; HCZ-PS: PS-derivatized hydroxy-chlorzoxazone; IS: internal standard.

	HMDZ	**DX-PS**	**LA-PS**	**HTB**
Targeted CYP450 isoform	CYP3A4	CYP2D6	/ (IS)	CYP2C9
Molecular mass metabolite	341.77	257.37	283.41	286.35
t_R (min)	3.75	3.9	4.34	4.46
Cone voltage (V)	40	50	45	35
Collision energy (eV)	20	30	30	18
Precursor ion	342	399	425	287
Product ion 1 (quantifier)	324	257	283	171
Product ion 2 (qualifier)	203	199	199	188
Calibration curve range (ng/ml)	2.3 - 660	1.1 - 245	/	25 - 1500
	AP-PS	**HME-PS**	**HCZ-PS**	
Targeted CYP450 isoform	CYP1A2	CYP2C19	CYP2E1	
Molecular mass metabolite	151.16	234.25	185.56	
t_R (min)	5.05	5.95	6.03	
Cone voltage (V)	40	45	35	
Collision energy (eV)	18	23	17	
Precursor ion	293	376	328	
Product ion 1 (quantifier)	151	274	264	
Product ion 2 (qualifier)	109	132	144	
Calibration curve range (ng/ml)	5.6 - 1260	6.9 - 1940	128.1 - 2560	

2.5 Derivatization

The first experiments were conducted following the method described by Xu et al. [13]. Shortly, a solution of the metabolites in 100% MeOH was evaporated to dryness under N_2. Eighty µl of 0.1M sodium bicarbonate (pH 10) and 80 µl of a 1 mg/ml PS or PBS solution in acetone were added to the vial. After mixing, the vials were placed in a heating block (Multi-Blok Heater, Lab-Line Instruments Inc, Melrose Park, Ill, USA) at 60°C for 15 minutes for PS and 30 minutes for PBS. Subsequently, the vials were cooled on ice for 10 minutes. The reaction mixture was transferred to a 250 µl insert and analyzed using an LC-MS/MS system. As described in the results and discussion section, this basic protocol was investigated for its performance characteristics, where appropriate optimized and adapted to the needs of our specific application, i.e. a biological medium containing microsomes.

After selection of the most useful approach (i.e. derivatization reagent type, solvent medium, …) and optimization of the different key process parameters (time, reaction temperature and heating method), the final protocol was: 10 µl 1.75N NaOH, 10 µl 1.25 µg/ml levallorphan (LA) and 70 µl 1 mg/ml pyridine-3-sulfonyl chloride (PS) hydrochloride in acetonitrile were added to 200 µl of incubation medium/calibrator and were mixed by vortexing. The derivatization was catalyzed by heating the vial for 20 s in an 850W microwave oven (domestic, Daewoo, KOG-376T, Korea). In order to absorb the excess microwave energy, a glass container with 60 ml water was placed next to the vials [14]. After mixing, the vial was cooled on ice for 10 minutes, and after transfer of the sample to a 250 µl insert, the sample could be readily injected.

2.6 Method validation

The analytical method was validated according to the FDA Guidance [15]. The following parameters were evaluated: selectivity, LLOQ, calibration model, accuracy, precision, and stability. Selectivity was evaluated by the analysis of blank microsomes from six different sources. The lower limit of quantification was defined as the lowest concentration for which the accuracy was 80-120% and precision (RSD %) < 20%, and was used as the lowest point of the calibration curve. Furthermore, the signal-to-noise (S/N) ratio of the LLOQ should be at least 9. Calibration models were evaluated statistically using StatGraphics 4.1 (Warrenton, Virginia, US). Accuracy of the analysis of six QC samples on three concentration levels should be within 85-115% of the nominal concentration, and within-run (n=6) and between-run (n=6) precision should not exceed 15% (RSD %).

Stability of the (derivatized) metabolites in the microsomal incubation medium was evaluated after three freeze-thaw cycles, after 10 days storage at 4°C and after 24 hours of residence in the autosampler. Matrix effect was evaluated through the calculation of the internal standard (IS)-normalized matrix factor (MF) [= peak ratio (analyte/IS) in presence of matrix ions / peak ratio (analyte/IS) in absence of matrix ions] [16] in five different matrix sources (microsomes from five different patient samples; matrix A to E) and at three concentration levels (except for HTB and HCZ-PS, 2 levels). The coefficient of variation at each concentration level should not exceed 15%. The relative intensities of the qualifier and quantifier ion were identified by the analysis of samples in absence of matrix. The maximum permitted tolerances of the ion ratios in presence of matrix were used from the EU Guidelines [17].

2.7 Application of the method

Rat microsomes were incubated with each probe substrate individually in a concentration near their apparent Km [18]. Shortly, the probe substrate was added to 1.25 mg microsomal protein/ml, 1.15% KCl and potassium phosphate buffer (pH 7.25). After pre-

incubation for 3 minutes at 37°C, the addition of NADPH initiated the reaction. After exactly 15 minutes (40 min for ME), the reaction was stopped with a reagent containing formic acid and acetonitrile ($H_2O/CH_3CN/HCOOH$; 42:55:3). Samples were frozen and stored at -20°C until analysis. After thawing, samples were derivatized following the previously described protocol, and were analyzed using the validated method.

3 RESULTS AND DISCUSSION

Due to its major role in the pharmacokinetics of many drugs, the *in vitro* activity determination of the cytochrome P450 enzyme system shows up in many different experiments during drug development. *In vitro* systems are used in the metabolic profiling of new drugs and in the evaluation of drug-drug interactions. Furthermore, changes in CYP activity in specific patient populations can be examined. In some cases, the amount of metabolite formed is (very) low (in inhibition experiments or in patients with liver disease). These experiments necessitate a sensitive quantification method for the metabolites formed.

3.1 Derivatization with PS and PBS

As our developed HPLC-MS/MS method showed insufficient sensitivity, chemical derivatization in order to improve ionization efficiency was used. Derivatization of the phenolic metabolites with commercially available pyridine-3-sulfonyl chloride (PS) and 4-(1H-pyrazol-1-yl)benzenesulfonyl chloride (PBS) was assessed. Figure 1 depicts the derivatization reaction with PS. As HTB and HMDZ lack a phenolic OH-function, they will not be derivatized by PS or PBS. However, the sensitivity of detection of these components already proved sufficient for the application and they can be detected in the positive electrospray ionization mode. Thus, derivatization is not essential for these components. In contrast, the derivatization of HME is strongly desired, as there is very poor sensitivity. Also, as HCZ needs to be detected in the negative ESI mode, derivatization may lead to detection in the positive mode. This obviates the need for within-run polarity switching (when possible) or double analysis at opposite polarities. The phenolic OH-function of DX and AP will also be derivatized.

In the first series of experiments, mixtures of standard solution (in methanol) of the metabolites were used. The metabolites were derivatized following the procedure described by Xu et al [13]. After the derivatization reaction, analysis was performed by using the multiple reaction monitoring (MRM) mode. All derivatives showed a precursor ion on $[M + 143]^+$ or $[M + 207]^+$, with M being the molecular mass of the original metabolite minus one hydrogen, and 143 and 207 representing the protonated sulfonate moiety of PS and PBS, respectively. The collision induced fragmentation of the precursor

ions of the PS derivatives resulted in specific product ions. It is important for the qualitative properties of the method that there are other diagnostic fragments rather than only the original metabolite after removal of the derivatization moiety. The product ion spectrum of the (derivatized) metabolites always showed at least one specific fragment from the original metabolite. To illustrate this, the fragment ions of AP-PS, as an example, are annotated in Figure 3. The cleavage of the S-O bond between the PS and the AP moiety leads to two fragments with *m/z* of 144 and 151. The *m/z* 109 fragment corresponds to the $(NH_3\text{-}C_6H_4\text{-}OH)^+$ ion obtained after simultaneous $CO{=}CH_2$ and PS loss. In contrast, the fragments from the PBS derivatives originated mainly from the fragmentation of the PBS moiety. For the quantification in the MRM mode, the most abundant product ion was used as quantifier. The specific fragment ion (or qualifier) was monitored in order to evaluate possible interference, as the ratio of the areas of the quantifier and qualifier should be constant. Figure 2 shows the MS/MS spectra of the (PS-derivatized) metabolites.

Prior to the MS detection, the metabolites were separated on a C18 chromatographic column. CYP enzymes are responsible for the phase I metabolism of drugs, leading to a more polar compound for renal excretion. Due to the derivatization, the polar metabolites are turned into less polar molecules, showing more retention on the reversed-phase (C18) column than prior to derivatization, and thus favourably influencing the chromatographic separation. Due to the less hydrophobic properties of PS, the PS derivatives show a lower retention time then the PBS derivatives.

Evaluation of the ionization efficiency showed a significant improvement of the detectability of both the PS and PBS derivatives compared to the non-derivatized molecules. The sensitivity of HME increased significantly. Moreover, all compounds, including HCZ, could be detected in the positive ESI mode. The decision to select PS as reagent to perform the analysis was based on several observations. Due to the more favorable fragmentation pattern, a more specific detection is possible. Also, the shorter retention times of the derivatives may decrease the total time of analysis.

3.2 Derivatization with PS in aqueous medium: optimization

Many of the previously published methods for the quantification of the metabolites after microsomal incubation only require a centrifugation step prior to injection. In order to keep the method with derivatization as simple as those methods, the evaporation to dryness under N_2 was omitted from the original procedure, and PS and sodium bicarbonate were directly added to the standard methanol solution of the metabolites. The derivatized metabolites could still be detected with sufficient sensitivity (data not shown).

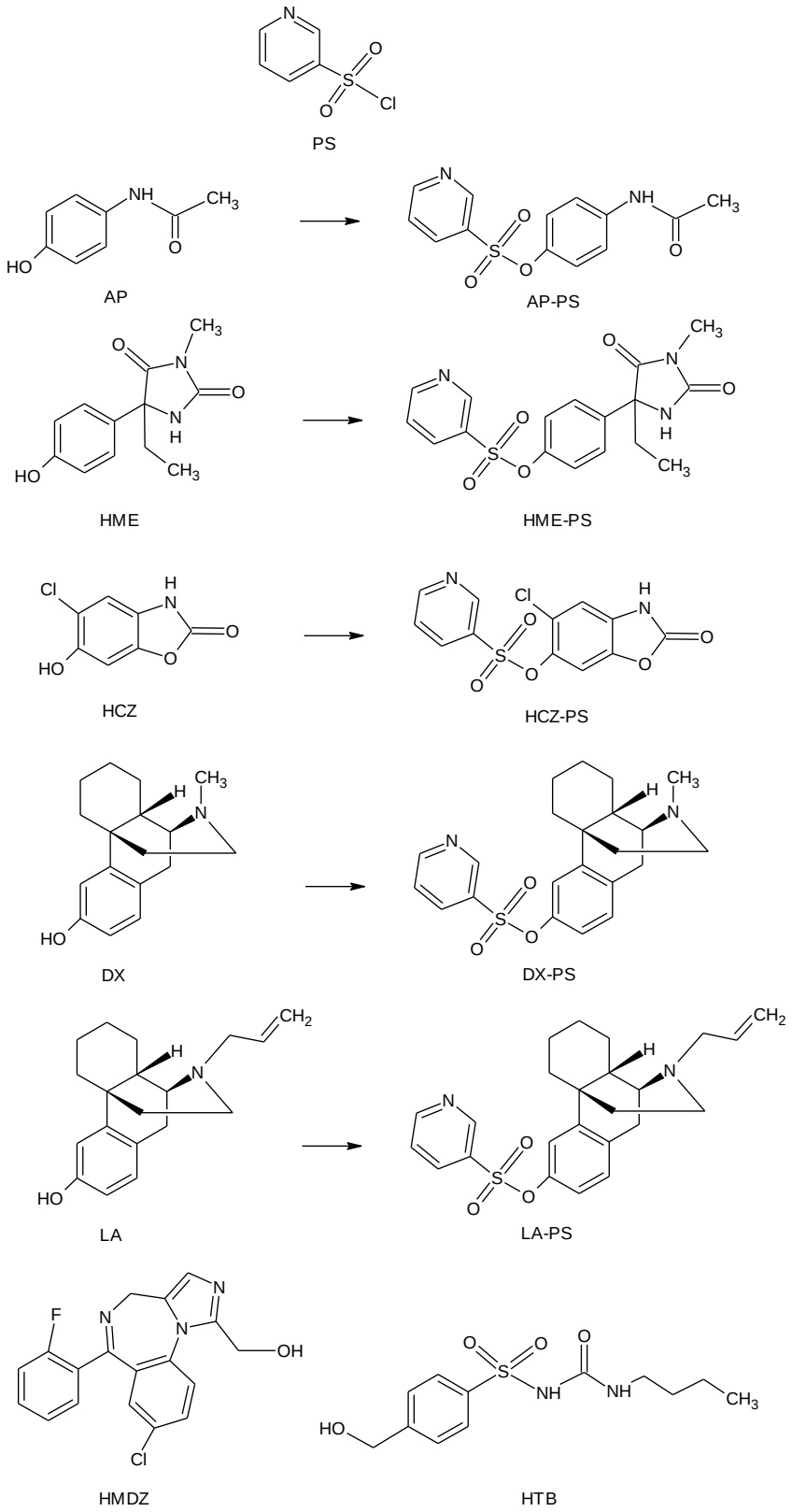

Figure 1: Molecular structures of the (derivatized) metabolites. PS: pyridine-3-sulfonyl chloride; HMDZ: hydroxy-midazolam; DX: dextrorphan; DX-PS: PS-derivatized dextrorphan; LA: levallorphan; LA-PS: PS-derivatized levallorphan; HTB: hydroxy-tolbutamide; AP: acetaminophen; AP-PS: PS-derivatized acetaminophen; HME: hydroxy-mephenytoin; HME-PS: PS-derivatized hydroxy-mephenytoin; HCZ: hydroxy-chlorzoxazone; HCZ-PS: PS-derivatized hydroxy-chlorzoxazone.

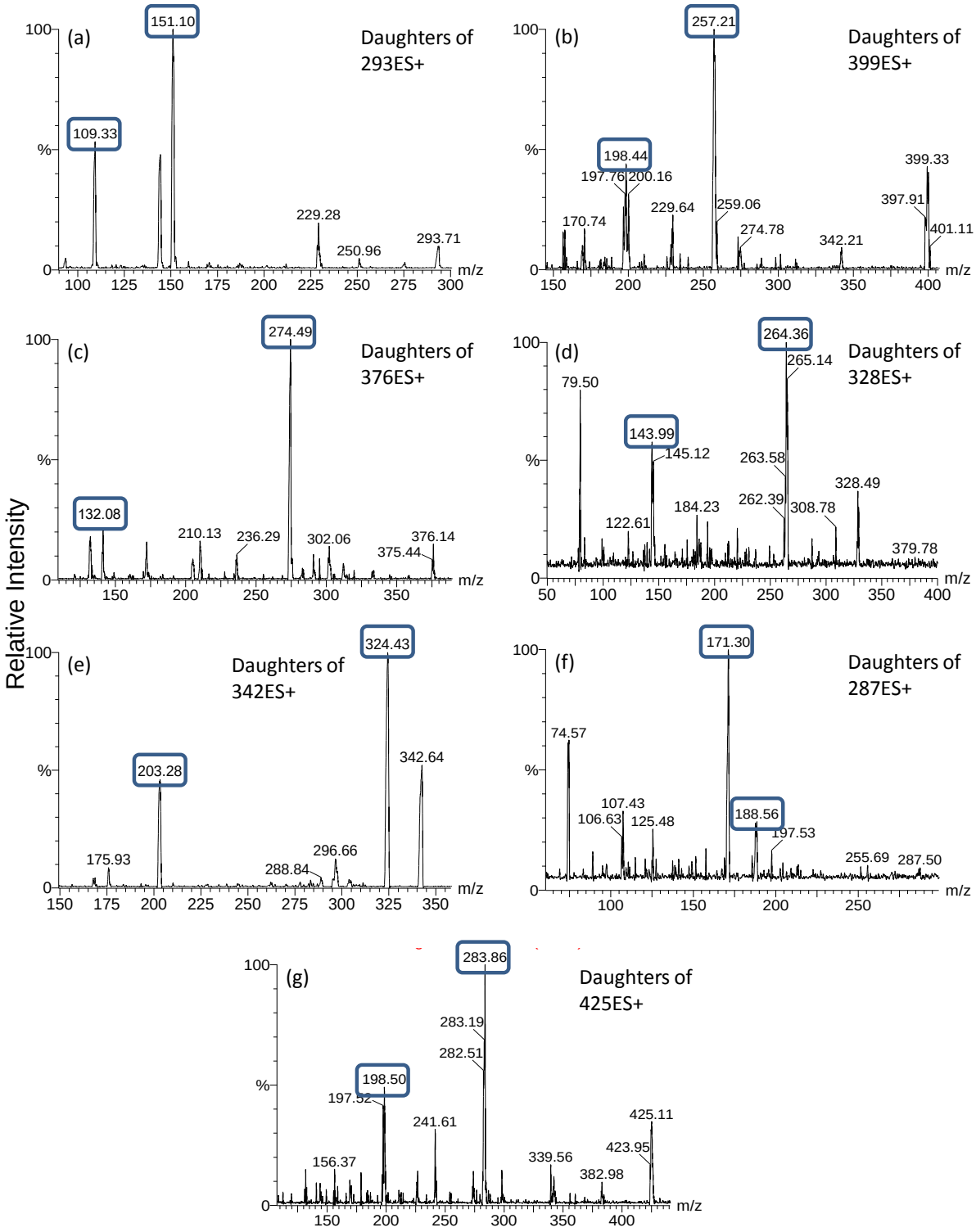

Figure 2: MS/MS spectra of (a) PS-derivatized acetaminophen (AP-PS); (b) PS-derivatized dextrorphan (DX-PS); (c) PS-derivatized hydroxy-mephenytoin (HME-PS); (d) PS-derivatized hydroxy-chlorzoxazone (HCZ-PS); (e) hydroxy-midazolam (HMDZ); (f) hydroxy-tolbutamide (HTB); and (g) PS-derivatized levallorphan (LA-PS). Highlighted ions indicate the selected product ions used in further analysis.

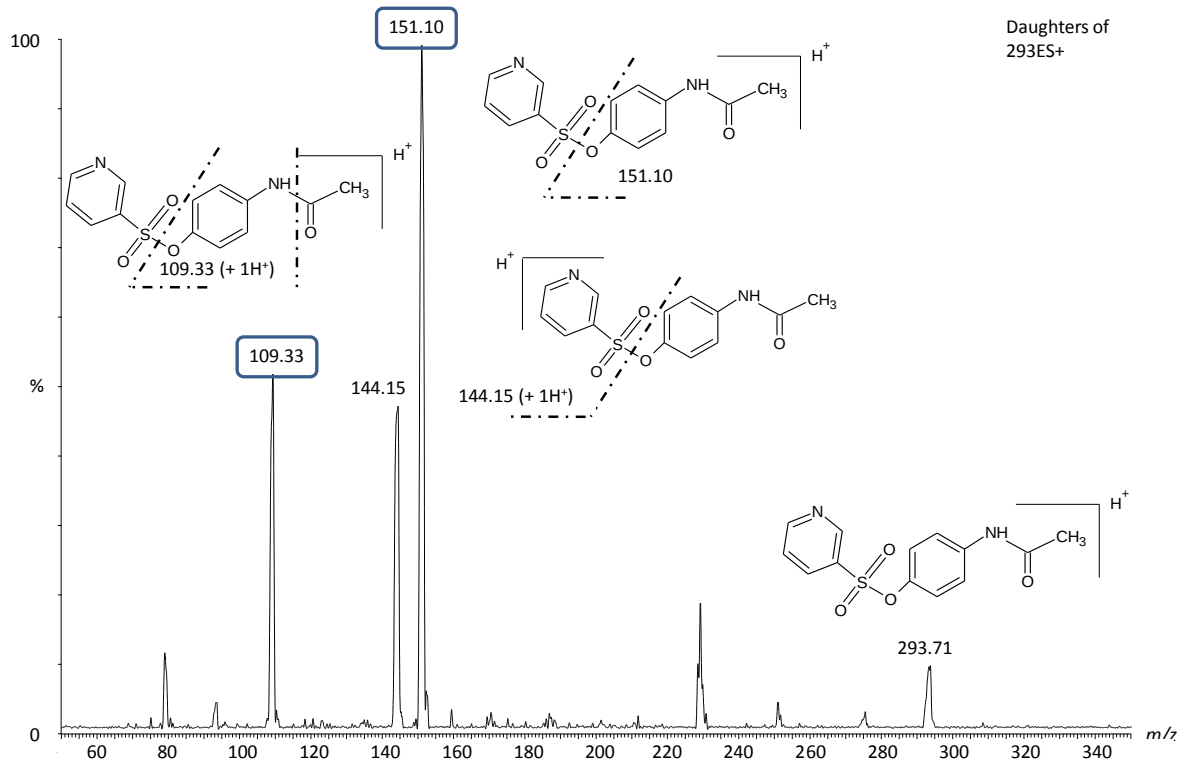

Figure 3: Fragmentation of AP-PS. The product ions m/z 144 and 151 are formed by the cleavage of the S-O bond. The specific fragment m/z 109 is obtained after a $CO{=}CH_2$ neutral loss off the AP moiety concurrently with the elimination of PS.

In order to assess the possibility of direct derivatization in the aqueous medium of the incubation samples, metabolites were spiked to a mixture of the incubation buffer (potassium phosphate, pH 7.25) and 1.15% KCl (50/50 v/v). The addition of PS (dissolved in acetone) and sodium bicarbonate to this mixture resulted in precipitation. Two parameters of the protocol were changed. Firstly, PS was dissolved in acetonitrile instead of in acetone. In accordance to the derivatization with dansyl chloride [6], an alkaline pH is important for the derivatization reaction with PS, as the nucleophilic phenolate ion reacting with the PS is formed at a pH of 9 – 10. Consequently, another buffer was considered. Beaudry et al [19] used 20 µl 100 mM NaOH in 350 µl sample and derivatization reagent in order to get a pH near pH 9. Due to the presence of a potassium phosphate buffer (pH 7.25), a greater amount of NaOH was required to obtain the correct pH. In order to avoid large dilution of the sample, a small volume (10 µl in 200 µl sample) of a higher concentrated (1.75N) NaOH solution was added. These interventions were successful, as the solution remained clear after addition of these components and the PS derivatives could be detected.

As the FDA recommends the use of calibrators in the same matrix as the actual samples, calibrators prepared with microsomal suspensions (final concentration microsomal protein

of 0.25 mg/ml) were used instead of the buffer mixture. After the confirmation that the derivatization reaction proceeded in this medium, the process was optimized with the intention to increase the yield. The optimal amount of derivatization reagent, the temperature of the heating block during reaction and the reaction time were determined. A comparison of the areas obtained when 50, 60 or 70 µl 1 mg/ml PS were added, showed that the volume was preferably 70 µl, as the yield for some of the derivatized metabolites was significantly higher in this situation. For all metabolites, temperature and reaction time were correlated: higher temperature required shorter reaction time to get the same yield. The upper limit of the reaction temperature was 80°C, as a higher temperature (90°C) led to a high variability (RSD > 15%; data not shown). Furthermore, the samples needed to be cooled down on ice for 10 minutes in order to decrease variability.

The promotion of organic reactions by microwave energy has been suggested in many studies, as reviewed by Kappe et al. [20]. Due to the direct delivery of the energy to the sample, there is a rapid rise in temperature and consequently a fast completion of the reaction [21]. As the purpose of the method under development was to be as short and easy as possible, the use of microwave-assisted heating was evaluated. A 20 second irradiation of the sample in an 850W microwave oven, showed comparable yield compared to the heating block (60°C, 10 minutes) procedure. As indicated before, a beaker with 60 ml water was placed on the microwave turning table, alongside the vials, to absorb excess energy [14,22]. The use of the microwave-assisted heating significantly decreased the total time of analysis, achieving equal overall performance and this method was finally adopted throughout.

3.3 Optimization of the LC-MS/MS analysis of PS-derivatized metabolites

The chromatographic separation was optimized through the use of gradient elution. The linear increase of eluent B (acetonitrile + 0.1% formic acid) from 20% to 90% during 9 minutes resulted in a chromatographic separation as depicted in Figure 4. As internal standard for the analysis, levallorphan (LA) was selected. Not only can this molecule be derivatized by PS, it also elutes in the middle of the analytical run.

Through multiple injections of the derivatized standard solutions, the MS parameters were tuned for each individual compound. For the source temperature, gas flows (both nitrogen and argon) and capillary voltage, a compromise was made to get optimal sensitivity for all the components (see materials and methods). The MRM transitions, cone voltage and collision energy were optimized for each (derivatized) metabolite and are depicted in Table 1.

3.4 Method validation

The method was validated according to the FDA guidelines. The optimal calibration curves were statistically calculated and the relation between ratio and concentration showed a linear correlation for all compounds. A $1/x^2$ weighting factor was used to obtain the best residuals and consequently the best accuracy. Ranges of the calibration curves were partially deduced from the article by Walsky et al [23] (mainly enzyme kinetics driven) and were decided in relation to our particular application. The LLOQ was established at the lowest concentration of the calibration curve. Precision (< 9.82% RSD) and accuracy (91.30-108.70%) at the LLOQ of all analytes were within the limits of the FDA guideline (< 20% RSD and between 80-120%, respectively).

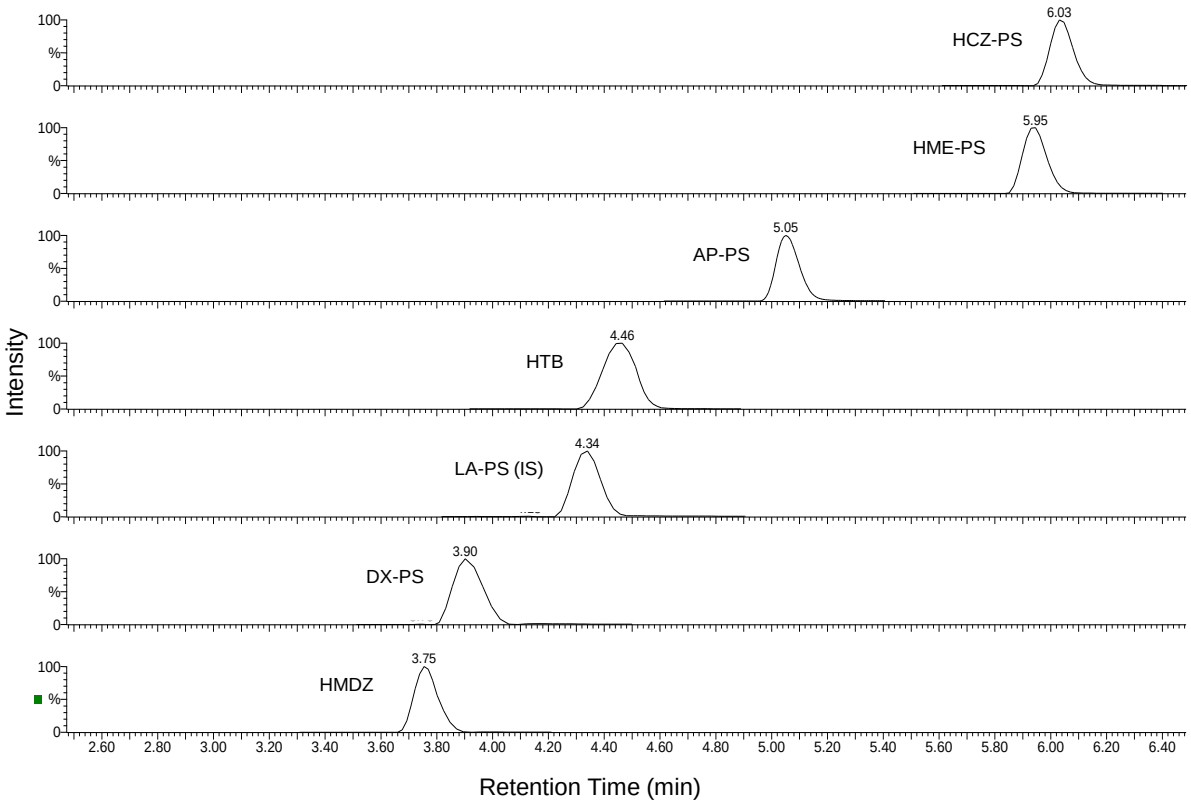

Figure 4: Chromatogram of the (PS-derivatized) metabolites and the internal standard after gradient elution. (Compound abbreviation identification as in Table 1).

The between-run and within-run precision were < 14.06% and < 9.58% (RSD), respectively, and accuracy ranged from 85.48-114.94%, for all metabolites, derivatized and underivatized ones. No interference was observed at the retention times of the analytes and the IS when analyzing blank microsomes from six independent sources. Thus, the method is considered selective.

Derivatized samples were proven to be stable for at least three freeze-thaw cycles (paired t-test, $p > 0.05$; accuracy 92.96 – 109.62%), except for DX-PS at low concentrations ($p < 0.05$). However, the mean of the calculated concentration is still within the 85-115% limit (104.09%) of the calculated concentration of the freshly prepared sample, and therefore acceptable. Except for AP-PS, stability at 4°C was acceptable (paired t-test, $p > 0.05$) for 10 days after derivatization. Nevertheless, for AP-PS the calculated concentration was still within the aforementioned limits, and therefore again acceptable. Poor autosampler stability (24 hours, room temperature around 28°C) was observed for almost all the metabolites. Cooling down (e.g. 10°C) the derivatized samples in the sampler will be mandatory.

The maximum permitted tolerances in deviation of the ion intensities are depicted in Table 2. Based on a statistically significant number of randomly chosen analyses, it can be seen that for all compounds less than 2.5% of the samples would need to be excluded based on a deviation of the ion ratio. The IS-normalized matrix factors (MF), determined by the analysis of microsomes prepared from five different patient samples (matrix A to E), are summarized in Table 3. All samples were analyzed in duplicate. For some compounds, the ionization is enhanced in the presence of the matrix (IS-normalized MF > 1). For other compounds, ionization is suppressed (IS-normalized MF < 1). The CV at the different concentration levels is < 15% for all the compounds.

Table 2: Relative ion intensities of qualifiers (% of base peak). (Compound abbreviation identification as in Table 1).

	n	Relative intensity (% of base peak)	Maximum permitted tolerance (relative)	% of samples exceeding tolerance
HMDZ	80	35.86	±25 %	2.50
DX-PS	54	6.74	±50 %	0
HTB	50	25.57	±25 %	0
HME-PS	78	18.12	±30 %	0
AP-PS	76	45.36	±25 %	1.32
HCZ-PS	71	58.54	±20 %	1.41

Table 3: Internal standard (IS)-normalized matrix factors (MF) were determined in microsomes prepared from five different patient samples (patients A to E): the maximum permitted CV (%) = 15%. Conc.: concentration; CV: coefficient of variation (Compound abbreviation identification as in Table 1).

	HMDZ			**DX-PS**			**HTB**	
(*n* = 2)	Conc. 1	Conc. 2	Conc. 3	Conc. 1	Conc. 2	Conc. 3	Conc. 2	Conc. 3
Matrix A	0.99	1.06	0.98	1.00	0.93	1.01	1.26	1.24
Matrix B	1.33	1.01	0.88	1.14	0.94	1.00	1.14	1.16
Matrix C	0.96	0.81	0.67	0.92	0.89	1.05	0.87	0.85
Matrix D	1.06	0.79	0.78	1.01	1.09	1.06	1.03	1.09
Matrix E	0.98	1.04	0.92	0.96	1.03	1.00	1.20	1.25
CV (%)	14.48	13.88	14.47	8.32	8.30	2.90	14.03	14.61
	HME-PS			**AP-PS**			**HCZ-PS**	
(*n* = 2)	Conc. 1	Conc. 2	Conc. 3	Conc. 1	Conc. 2	Conc. 3	Conc. 2	Conc. 3
Matrix A	0.81	0.98	0.58	0.95	0.89	0.73	1.02	2.89
Matrix B	1.03	0.97	0.55	1.05	0.84	0.69	1.48	2.65
Matrix C	0.82	1.18	0.70	1.02	1.00	0.79	1.16	2.75
Matrix D	1.05	1.08	0.77	1.11	1.01	0.84	1.11	2.85
Matrix E	1.03	1.14	0.63	1.11	0.99	0.69	1.27	3.15
CV (%)	12.65	8.78	13.36	6.53	7.75	9.04	14.65	6.53

3.5 Comparison with non-derivatized method

A comparison of the detection method for non-derivatized molecules and the newly developed method is summarized in Table 4. After derivatization, the detection of HCZ was made possible in positive ESI, with a lower LOQ. Furthermore, the sensitivity of the detection of AP and HME was increased significantly. The sensitivity of the detection of the metabolites that are not derivatized (HMDZ and HTB) was not affected by the procedure.

Table 4: Comparison of the LOQ's (ng/ml) of the detection of the metabolites prior and after derivatization. (Compound abbreviation identification as in Figure 1).

Prior to derivatization			**After derivatization**		
HMDZ	2.90	(ESI+)	HMDZ	2.30	(ESI+)
DX	2.10	(ESI+)	DX-PS	1.10	(ESI+)
HTB	25.00	(ESI-)	HTB	25.00	(ESI+)
AP	63.00	(ESI+)	AP-PS	5.60	(ESI+)
HME	971.40	(ESI-)	HME-PS	6.90	(ESI+)
HCZ	256.20	(ESI-)	HCZ-PS	128.10	(ESI+)

3.6 Application of the method

After incubation of rat microsomes with the probe substrates, the metabolites in the incubation medium were analyzed with the described method. Table 5 depicts the results of the analysis. These results show that derivatization was advantageous, as the amount of HME was far below the LOQ of the method without derivatization. Also, all 6 components can be quantified using the same electrospray ionization mode (ESI+).

Table 5: Results of the incubations of rat microsomes (n = 2) with the six probe substrates. Incubation conditions: 0.25 mg microsomal protein/ml; reaction time: 15 min; reaction temperature: 37°C. The amount of DX-PS exceeded the ULOQ: the samples were diluted and re-analysed. SD: standard deviation (Compound abbreviation identification as in Table 1).

	Human CYP isoform	**Metabolite concentration Mean (± SD) in ng/ml**	**Enzyme activity Mean (± SD) pmol/mg/min**
HMDZ	3A4	148.55 (± 11.49)	509.99 (± 39.45)
DX-PS	2D6	283.00 (± 12.80)	1290.18 (± 58.36)
HTB	2C9	93.10 (± 6.94)	381.48 (± 28.45)
HME-PS	2C19	10.15 (± 0.50)	50.84 (± 2.48)
AP-PS	1A2	100.00 (± 13.50)	776.22 (± 104.81)
HCZ-PS	2E1	114.75 (± 19.03)	725.59 (± 120.36)

4 CONCLUSION

A chemical derivatization method was developed for the quantification of the probe metabolites formed during *in vitro* cytochrome P450 activity determination. The derivatization with pyridine-3-sulfonyl chloride of the metabolites containing a phenolic OH-function in their structure improved the ionization efficiency in positive ES and enabled the detection of all six metabolites in the ES+ mode. The method is easy and fast, as derivatization proceeds directly in the aqueous incubation medium, without additional evaporation, extraction or reconstitution steps. The method met all requirements of sensitivity, selectivity, precision, accuracy and stability following the FDA guidelines for bioanalytical methods. Proof-of-concept samples from the intended (pharmacokinetics) application were successfully analyzed using the newly developed procedure. To our knowledge, this is the first report on using aqueous derivatization in the LC-MS analysis of CYP activity probe metabolites. Moreover, we believe that the simplicity of the approach put forward here can remove some of the disinclination towards the use of derivatization in LC-MS for hard to ionize molecules in other application fields.

REFERENCES

[1] Lahoz A, Gombau L, Donato MT, Castell JV, Gomez-Lechon MJ. In vitro ADME medium/high-throughput screening in drug preclinical development. *Mini-Reviews in Medicinal Chemistry* 2006; 6(9):1053-1062.

[2] Wilson ZE, Rostami-Hodjegan A, Burn JL, Tooley A, Boyle J, Ellis SW *et al.* Inter-individual variability in levels of human microsomal protein and hepatocellularity per gram of liver. *Br J Clin Pharmacol* 2003; 56(4):433-440.

[3] Tucker GT, Houston JB, Huang SM. Optimizing drug development: strategies to assess drug metabolism/transporter interaction potential - towards a consensus. *Br J Clin Pharmacol* 2001; 52(1):107-117.

[4] Kim MJ, Kim H, Cha IJ, Park JS, Shon JH, Liu KH *et al.* High-throughput screening of inhibitory potential of nine cytochrome P450 enzymes in vitro using liquid chromatography/tandem mass spectrometry. *Rapid Commun Mass Spectrom* 2005; 19(18):2651-2658.

[5] Quirke JME, Adams CL, Vanberkel GJ. Chemical Derivatization for Electrospray-Ionization Mass-Spectrometry .1. Alkyl-Halides, Alcohols, Phenols, Thiols, and Amines. *Anal Chem* 1994; 66(8):1302-1315.

[6] Gros C, Labouess B. Study of Dansylation Reaction of Amino Acids Peptides and Proteins. *Eur J Biochem* 1969; 7(4):463-470.

[7] Chang H, Wan Y, Naile J, Zhang XW, Wiseman S, Hecker M *et al.* Simultaneous quantification of multiple classes of phenolic compounds in blood plasma by liquid chromatography-electrospray tandem mass spectrometry. *J Chromatogr A* 2010; 1217(4):506-513.

[8] Lien GW, Chen CY, Wang GS. Comparison of electrospray ionization, atmospheric pressure chemical ionization and atmospheric pressure photoionization for determining estrogenic chemicals in water by liquid chromatography tandem mass spectrometry with chemical derivatizations. *J Chromatogr A* 2009; 1216(6):956-966.

[9] Qin F, Zhao YY, Sawyer MB, Li XF. Column-switching reversed phase-hydrophilic interaction liquid chromatography/tandem mass spectrometry method for determination of free estrogens and their conjugates in river water. *Anal Chim Acta* 2008; 627(1):91-98.

[10] Anari MR, Bakhtiar R, Zhu B, Huskey S, Franklin RB, Evans DC. Derivatization of ethinylestradiol with dansyl chloride to enhance electrospray ionization: Application in trace analysis of ethinylestradiol in rhesus monkey plasma. *Anal Chem* 2002; 74(16):4136-4144.

[11] Shou WZ, Jiang XY, Weng ND. Development and validation of a high-sensitivity liquid chromatography/tandem mass spectrometry (LC/MS/MS) method with chemical derivatization for the determination of ethinyl estradiol in human plasma. *Biomed Chromatogr* 2004; 18(7):414-421.

[12] Xu L, Spink DC. 1,2-Dimethylimidazole-4-sulfonyl chloride, a novel derivatization reagent for the analysis of phenolic compounds by liquid chromatography electrospray tandem mass spectrometry: application to 1-hydroxypyrene in human urine. *J Chromatogr B Analyt Technol Biomed Life Sci* 2007; 855(2):159-165.

[13] Xu L, Spink DC. Analysis of steroidal estrogens as pyridine-3-sulfonyl derivatives by liquid chromatography electrospray tandem mass spectrometry. *Anal Biochem* 2008; 375(1):105-114.

[14] Sun W, Gao SJ, Wang LJ, Chen Y, Wu SZ, Wang XR *et al.* Microwave-assisted protein preparation and enzymatic digestion in proteomics. *Molecular & Cellular Proteomics* 2006; 5(4):769-776.

[15] FDA. Guidance for Industry: Bioanalytical Method Validation. 2001.

[16] Viswanathan CT, Bansal S, Booth B, DeStefano AJ, Rose MJ, Sailstad J *et al.* Workshop/conference report - Quantitative bioanalytical methods validation and implementation: Best practices for chromatographic and ligand binding assays. *AAPS J* 2007; 9(1):E30-E42.

[17] Commission decision concerning the performance of analytical methods and the interpretation of results. *Official Journal of the European Communities* 2002.

[18] Yuan R, Madani S, Wei XX, Reynolds K, Huang SM. Evaluation of cytochrome P450 probe substrates commonly used by the pharmaceutical industry to study in vitro drug interactions. *Drug Metab Dispos* 2002; 30(12):1311-1319.

[19] Beaudry F, Guθnette SA, Winterborn A, Marier JF, Vachon P. Development of a rapid and sensitive LC-ESI/MS/MS assay for the quantification of propofol using a simple off-line dansyl chloride derivatization reaction to enhance signal intensity. *J Pharm Biomed Anal* 2005; 39(3-4):411-417.

[20] Kappe CO, Dallinger D. Controlled microwave heating in modern organic synthesis: highlights from the 2004-2008 literature. *Mol Divers* 2009; 13(2):71-193.

[21] Chu TY, Chang CH, Liao YC, Chen YC. Microwave-accelerated derivatization processes for the determination of phenolic acids by gas chromatography-mass spectrometry. *Talanta* 2001; 54(6):1163-1171.

[22] Bose AK, Banik BK, Lavlinskaia N, Jayaraman M, Manhas MS. MORE chemistry in a microwave. *Chemtech* 1997; 27(9):18-24.

[23] Walsky RL, Obach RS. Validated assays for human cytochrome P450 activities. *Drug Metab Dispos* 2004; 32(6):647-660.

CHAPTER 2

DEVELOPMENT AND VALIDATION OF A FAST AND SENSITIVE UPLC-MS/MS METHOD FOR THE QUANTIFICATION OF SIX PROBE METABOLITES FOR THE IN VITRO DETERMINATION OF CYTOCHROME P450 ACTIVITY

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'There is more to life

than simply increasing its speed'

- Mahatma Gandhi

ABSTRACT

A fast and sensitive UPLC-MS/MS method was developed and validated for the simultaneous quantification of six probe metabolites for the *in vitro* cytochrome P450 activity determination in hepatic microsomes from patients with hepatic impairment. The metabolites acetaminophen (CYP1A2), 4'-hydroxy-mephenytoin (2C19), 4-hydroxy-tolbutamide (CYP2C9), dextrorphan (CYP2D6), 6-hydroxy-chlorzoxazone (CYP2E1) and 1-hydroxy-midazolam (CYP3A4), together with the internal standard chlorpropamide, were separated on a Waters Acquity UPLC BEH C18 column (50 mm x 2.1 mm, 1.7 µm particle size) with VanGuard pre-column (5 mm x 2.1 mm, 1.7 µm particle size). A short gradient elution (total run time of 5.25 minutes), using water with 0.1% formic acid (eluent A) and acetonitrile with 0.1% formic acid (eluent B) at a flow rate of 400 µl/min, was used. The metabolites were detected with a triple quadrupole mass spectrometer in the multiple reaction monitoring mode. Two runs, one in the positive ionization mode and one in the negative mode, were necessary for the detection of all metabolites. The method was selective and showed good accuracy (84.59 to 109.83%) and between-day (RSD% < 5.13%) and within-day (RSD% < 9.60%) precision. The LOQ was in full accordance with the intended application, and no relative matrix effects were observed. Also, the sample incubation extracts were stable after three freeze-thaw cycles. The usability of the method was demonstrated by the incubation of pediatric microsomes with subsequent quantification of the formed metabolites and CYP activity calculation.

1 INTRODUCTION

The activity of the cytochrome P450 enzyme system may be compromised in patients with hepatic pathologies. As this enzyme system is responsible for the metabolism of the vast majority of drugs, these patients often show altered drug pharmacokinetics, which may lead to inefficient therapy or adverse reactions [1]. In order to study the differential alterations in activity of the six most important isoforms (CYP1A2, CYP2C19, CYP2C9, CYP2D6, CYP2E1, and CYP3A4), *in vitro* CYP activity can be investigated in hepatic microsomes. For the determination of the enzyme activity, these assays use the incubation of the microsomes with well established isoform-specific probe substrates, and the quantification of the resulting metabolite levels. Many different combinations of probe substrates have been used. The most commonly used and preferred in vitro probe substrates are phenacetin (PH; CYP1A2), S-mephenytoin (ME; CYP2C19), tolbutamide (TB; CYP2C9), dextromethorphan (DM; CYP2D6), chlorzoxazone (CZ, CYP2E1) and midazolam (MDZ; CYP3A4) [2].

Liquid chromatography, coupled to mass spectrometry (LC-MS) has been shown to be a valuable tool in CYP450 assays for the quantification of the metabolites, as reviewed by Youdim et al. [3] and Lahoz et al. [4]. Different LC-MS(MS) methods for simultaneous quantification of metabolites have been described [5-9]. However, to our knowledge, only two methods used a combination of probes comparable to those mentioned above [5,6]. Li et al. [6] used two different HPLC systems with isocratic elution, one coupled to an MS with a positive atmospheric pressure chemical ionization interface, and one using the negative electrospray ionization mode. In the method of Kim et al. [5], gradient elution was performed, followed by detection of most of the metabolites in the positive electrospray ionization mode, and one (HCZ) in the negative mode. Both methods showed sufficient sensitivity for the intended applications.

A marked reduction in the time of analysis of LC separations has been established by using columns with sub-2 µm particles [10]. The combination of small particles and higher solvent flows in ultra-high performance liquid chromatography (UPLC) enables a fast elution and improved resolution in the chromatographic separation. This technology therefore offers a powerful tool for a high throughput analysis of samples. Recently, a UPLC-MS/MS method for the quantification of six probe metabolites was published. However, this study lacked the inclusion of a probe for the CYP2E1 isoform activity determination [11,12]. As this isoform has been shown to be affected by liver disease [13], a probe to evaluate the activity of CYP2E1 is essential in the intended application as described above.

Matrix effect is a major issue in LC-MS method development [14]. Despite the specificity of MS/MS, co-eluting compounds may cause suppression or enhancement of the analyte response [15]. Improved chromatographic separation of the analytes or extended sample preparation has been suggested to minimize these matrix effects [16]. Therefore, base line separation of the chromatographic peaks should be the aim, rather early than late in the method development. Furthermore, base line separation also contributes to an increased selectivity of the detection method. In the previously cited studies using the same combination of probes [5,6], no complete base line separation of the metabolites was accomplished. Nevertheless, Li et al [5,6] concluded that matrix effects were absent, whereas in the method described by Kim et al [5], matrix effects were not evaluated. UPLC could be a favourable approach to yield full chromatographic separation without unacceptably prolonging sample turn over times.

This study aimed for the development of a generic fast chromatographic method that could be used for the detection of those metabolites most interesting for CYP activity evaluation (acetaminophen (AP), 4'-hydroxy-mephenytoin (HME), 4-hydroxy-tolbutamide (HTB), dextrorphan (DX), 6-hydroxy-chlorzoxazone (HCZ) and 1-hydroxy-midazolam (HMDZ)). This method is useful in either positive or negative electrospray ionization mode. Furthermore, an extensive analytical validation was performed prior to the implementation of the method. The developed and validated method was tested for its usability in incubation experiments with microsomes of pathological origin.

2 MATERIALS AND METHODS

2.1 Chemicals and biological samples

Phenacetin, acetaminophen, tolbutamide, 4-OH-tolbutamide, S-mephenytoin, 4'-OH-mephenytoin, dextromethorphan, dextrorphan, chlorzoxazone, 6-OH-chlorzoxazone and chlorpropamide (CLP) were purchased from Sigma-Aldrich (St-Louis, MO, USA). Midazolam and 1-OH-midazolam were kindly donated by Roche (Basel, Switzerland). NADPH was obtained from Biopredic International (Rennes, France). Potassium chloride, potassium dihydrogenphosphate and dipotassium hydrogenphosphate were purchased from VWR (Leuven, Belgium). All other chemicals were of analytical grade.

Microsomes were prepared from liver samples [17], collected from the diseased liver from children undergoing a liver transplantation (approved by the Ethics Committee of Ghent University Hospital, B67020084281).

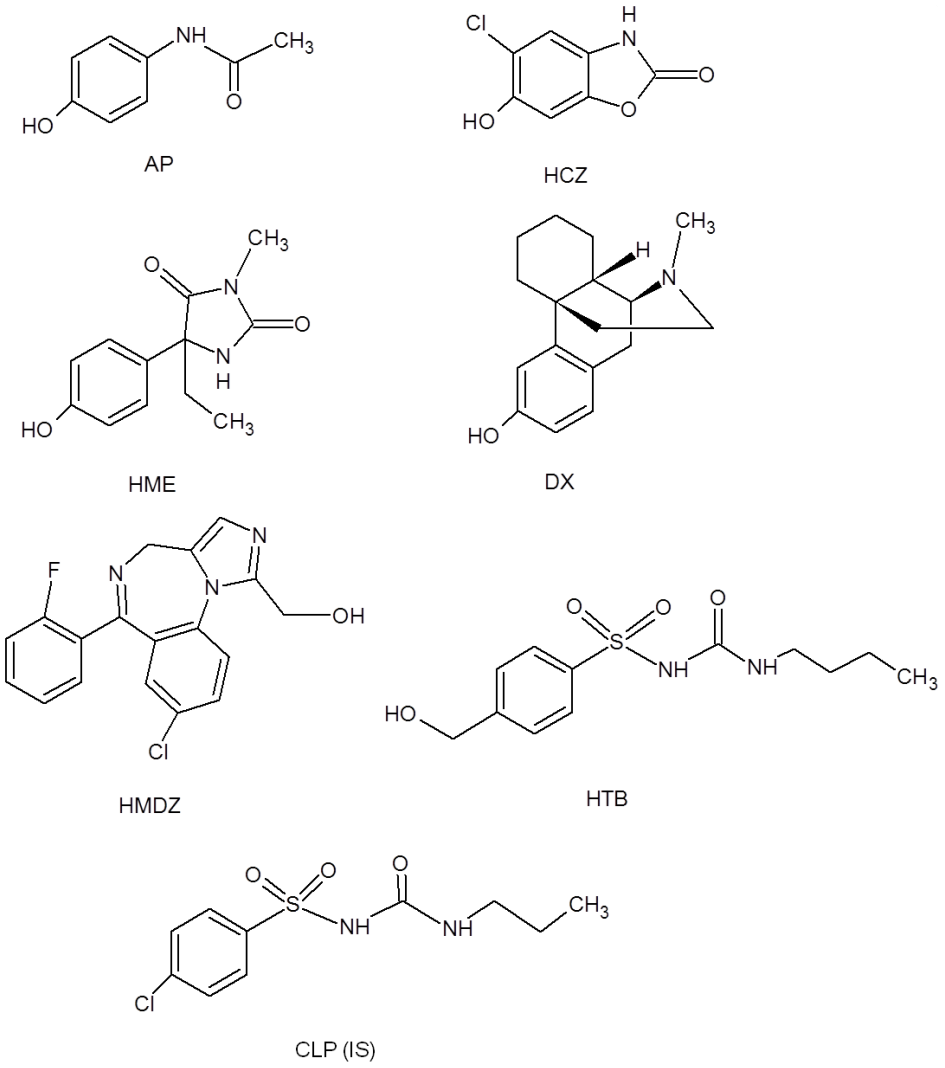

Figure 1: Molecular structures of the metabolites. (AP: acetaminophen, HCZ: 6-OH-chlorzoxazone, HME: 4'-OH-mephenytoin, DX: dextrorphan, HMDZ: 1-OH-midazolam, HTB: 4-OH-tolbutamide, CLP: chlorpropamide, IS: internal standard)

2.2 Standard solutions and calibrators

Primary stock standards of 1 mg/ml in methanol of all metabolites and the internal standard were prepared and stored at -20°C. Stock standards were mixed in the appropriate proportions and serially diluted in methanol. Microsomal calibration standards consisted of metabolites spiked to the microsomal incubation medium. This incubation medium was prepared by mixing 1 ml 5 mM NADPH, 1 ml 1.25 mg/ml microsomal protein, 1 ml 1.15% KCl, 1 ml 0.2M potassium phosphate buffer (pH 7.4) and 0.5 ml stopreagent ($H_2O/CH_3CN/HCOOH$; 42/55/3 (v/v/v)) containing 220 ng/ml internal standard (IS; chlorpropamide). After mixing and protein denaturation, a specific amount of stock solution was spiked to the incubation medium. Water was added to obtain a final volume of 5.5 ml per calibrator. Calibrators were centrifuged at 20000*xg* for 15 min at 4°C, supernatant was separated and stored at 4°C.

2.3 Chromatographic conditions

UPLC was performed on a Waters Acquity UPLC BEH C18 column (50 mm x 2.1 mm, 1.7 µm particle size) with VanGuard pre-column (5 mm x 2.1 mm, 1.7 µm particle size) using a Acquity UPLC system (Waters, Manchester, UK). The column was kept at 35 °C. An aliquot of 20 µl was injected using full loop injection. The mobile phase consisted of water containing 0.1% formic acid (eluent A) and acetonitrile containing 0.1% formic acid (eluent B). At a flow rate of 400 µl/min, the amount of eluent B was increased linearly from 5% to 80% in 4 min, kept at 80% B for 0.18 min, and then the column was left to re-equilibrate at initial conditions for 1.8 min, resulting in a total turnover time of 6 min. Due to the presence of the potassium phosphate buffer in the sample, strong wash contained 95/5 (v/v) water/methanol, and weak wash and needle wash 50/50 (v/v) water/methanol.

2.4 MS conditions

Eluting compounds were detected using a Waters Quattro Ultima triple quadrupole system (Micromass Waters, Manchester, UK). The electrospray source (orthogonal Z-spray®) used a standard 120 µm capillary. Optimal source temperature and desolvation temperature were 150 and 400 °C, respectively. Cone gas flow and desolvation gas flow (both nitrogen) were set at 175 and 575 l/min. Argon was used for the collision-induced fragmentation. Due to inadequate ionization for some compounds indebted to their particular chemical structure, both the positive and negative electrospray ionization mode were used as interface. As the instrument is not capable of effective in-run polarity switching, two runs were necessary for each cocktail sample. Data were collected and processed using the MassLynx® and QuanLynx® software (Micromass Waters).

2.5 Microsomal incubations

Microsomes were incubated with each probe substrate in a concentration near their apparent Km (see Table 1) [18]. In short, 50 µl of the probe was added to 50 µl 1.25 mg microsomal protein/ml (final concentration of 0.25 mg protein/ml), 50 µl 1.15% KCl and 50 µl 0.2M potassium phosphate buffer (pH 7.25). To initiate the reaction, 50 µl of 5 mM NADPH was added after pre-incubation of 3 minutes at 37°C (total reaction volume of 250 µl). The reaction was terminated after exactly 15 minutes (40 minutes for ME) by adding 25 µl of the stopreagent (H_2O/CH_3CN/HCOOH; 42/55/3) containing the internal standard, and cooling the mixture on ice. Subsequently, the samples were centrifuged for 10 minutes at 20000*xg* (4°C), 200 µl of the supernatant was transferred to a 250 µl insert, and samples were frozen at -20°C until analysis.

2.6 Method validation

The method was validated according the "Guidance for Industry - Bioanalytical Method Validation" recommended by the FDA [19].

2.6.1 Calibration curve

Calibration curves were constructed over a specific range for each compound (based on Walsky et al [20]). An analysis of variance with lack of fit test (StatGraphics 4.1, Warrenton, Virginia, US) was used to determine whether the selected model of the relation between analyte-to-IS ratio and concentration was adequate to describe the observed data ($p>0.10$), or whether a more complicated model was required. If necessary, a weighting factor was used to increase the accuracy. The statistical significance of the terms of the model (slope and intercept) was assessed by the comparison of calibration curves analyzed in 5 independent runs using an ANOVA for variables in the order fitted ($p>0.10$).

Quality control (QC) samples at three different concentration levels (replicate analysis; in total 6 samples) were used to either accept or reject the analytical run. At least four out of six of the QC samples should be within 15% of their nominal value, whereas two out of six QC samples (not all replicates at the same concentration level) may be outside 15% of the nominal value. Table 1 shows the concentration ranges of the different calibration curves and the concentration levels of the QC samples.

2.6.2 Precision, accuracy and LOQ

Within-day precision and accuracy were determined by analyzing six aliquots of each QC sample on the same day. Between-day precision was evaluated by analyzing the QC samples in duplicate for five days. Precision was expressed as the relative standard deviation (RSD%) of the measured QC samples and accuracy was calculated as trueness.

2.6.3 Limit of detection (LOD) and limit of quantification (LOQ)

The limit of detection (LOD) was calculated following the IUPAC definition [21] and was defined as 3 times the standard deviation of the intercept, divided by the slope or analytical sensitivity. However, as this equation only counts for linear calibration curves, the LOD of the metabolites using a quadratic calibration curve was calculated following Burkart et al [22].

The limit of quantification (LOQ) was defined as the lowest concentration, which could be measured ($n=6$) with a precision not exceeding 20% (RSD%) and with an accuracy between 80% and 120%. The lowest calibrator of the calibration curve was targeted as LOQ.

2.6.4 Selectivity and stability

Selectivity was assessed by examining peak interference (signal-to-noise ratio < 9) from six independent sources of microsomes. Stability of the sample incubation extracts was tested after storage in the autosampler (15°C) for 14 h and after three freeze-thaw cycles. The autosampler stability was tested by the analysis of 3 aliquots of a middle standard in the beginning of an analytical run, and 3 aliquots 14 h (average total run time) later. Stability was determined by statistical comparison of the calculated concentrations (t-test, 95% significance level). Freeze-thaw stability was assessed using the commonly accepted procedure: after three freeze-thaw cycles, samples were compared with regularly analyzed samples with the same amount of analyte (t-test, 95% significance level).

2.6.5 Matrix effect

Matrix effect was evaluated following Viswanathan et al. [23], through the calculation of the internal standard-normalized (IS-normalized) matrix factor (MF), using equation (1).

$$\textit{IS-normalized Matrix Factor} = \frac{\textit{peak ratio (analyte/IS) in presence of matrix ions}}{\textit{peak ratio (analyte/IS) in absence of matrix ions}} \quad (1)$$

A value different from 1, indicated an absolute matrix effect. The relative matrix effect was evaluated through the determination of the coefficient of variation (CV%) of the IS-normalized MF of five different matrix sources. This CV% should not exceed 15%.

2.6.6 Application of the method

The above described validated analytical method was used for the analysis of samples from a pharmacokinetic study. CYP450 enzyme activities were determined in three liver samples obtained from explanted livers from children undergoing liver transplantation for various reasons (biliary atresia, cystic fibrosis, progressive familial intrahepatic cholestasis). The paediatric microsomal samples were incubated with the probe substrates, and the formed metabolites were quantified.

3 RESULTS AND DISCUSSION

3.1 Method development

3.1.1 Sample preparation

Sample preparation is of major importance in LC-MS/MS analysis. In order to reduce matrix interference, complex matrix ions, such as proteins, have to be removed from the sample. Protein precipitation is the most widely used and easiest technique for this

purpose. In CYP450 assays this is typically achieved through the addition of an equal volume of organic solvent (mostly acetonitrile), or through acidification. Subsequently, the sample can be filtered [20] or centrifuged [5,24] in order to remove the proteins. The use of large volumes of organic solvent was deemed highly undesirable in this method, as this dilutes the sample at least three times, moreover providing an injection organic solvent composition incompatible with good reversed phase chromatography. In CYP450 assays, protein denaturing can also serve to end the enzymatic incubation, i.e. as a stopreagent. Taking all of this into consideration, a small volume (25 µl in 250 µl incubation volume) of a reagent containing a combination of acetonitrile and a strong acid (formic acid) was used in order to stop the enzymatic reaction and at the same time provide adequate conditions to pellet the incubation mix proteins. Addition of a small volume of 60% perchloric acid was also evaluated, but as this significantly affected peak shape, this method for sample preparation was not retained. Besides acetonitrile and formic acid, the stopreagent contained the internal standard, chlorpropamide. The terminated incubation medium was vortex mixed and placed on ice for further protein precipitation. After centrifugation for 10 minutes at 20000x*g* and 4°C, supernatant was collected and could be readily injected. As the sample preparation step is incorporated in the in vitro protocol (termination step of the incubation), sample handling is minimized and unduly dilution of the resulting extract is avoided. This in combination with a selective and sensitive MS method thus increases the quality of the result, as well as the throughput of the method [4].

3.1.2 Optimization of the mass spectrometric (MS) detection

The individual parameters for the detection with the triple quadrupole MS were optimized by the infusion of a standard solution of the metabolites (1 µg/ml (100 ng/ml for DX) in methanol/water 50/50 (v/v) + 0.1% formic acid). Detection of all metabolites (depicted in Figure 1) was evaluated in the positive electrospray ionization (ESI +) mode. No or poor MS responses were observed for HCZ and HTB in the positive mode. In contrast, intense MS signals were observed for AP, DX and HMDZ. Infusion of HCZ and HTB in the negative ESI (ESI -) mode showed strong responses. Therefore, detection of AP, DX and HMDZ was performed using the ESI + mode, whereas HCZ and HTB need the ESI- mode. As HME showed poor MS response in both ESI + and ESI - mode during infusion, this compound was initially detected in both modes. The MS parameters were optimized in order to achieve the highest MS response. The optimal cone voltages were determined, and an acceptable signal for the detection of HME in ESI + was obtained by an increased source (150°C) and desolvation temperature (400°C). Consequently, the preferred detection mode of HME was finally ESI +. The collision energy (using argon gas) was optimized for each metabolite, based on the product ion mass spectra. Collision energy

was varied until the intensity of the precursor ion $[M + H]^+$ was 10 to 20 % of the intensity of the quantifier ion (i.e. the product ion with the highest response).

For the actual detection using multiple reaction monitoring (MRM), the two most abundant product ions were selected for each analyte; except for HTB, where only one product ion was formed during fragmentation. The most abundant product ion served as quantifier (for the quantification), the other as qualifier (confirmation of the identification).

Based on Kim et al. [5], chlorpropamide was selected as internal standard, as this molecule can be detected in both ESI + and ESI - mode. The internal standard was added to the sample at the end of the incubation reaction (in the stopreagent) and was used to correct for variability of the analytical system. Table 1 depicts the MS parameters of all the metabolites and the internal standard.

3.1.3 Optimization of the chromatographic separation

In order to obtain a short analysis time, a sub 2-µm particle size UPLC column was selected. Considering the chemical diversity in structures of the metabolites, gradient elution was applied, using water containing 0.1% formic acid as eluent A. Eluent B (acetonitrile + 0.1% formic acid) was increased gradually from 5% to 80% during 4 minutes using a 0.4 ml/min flow. A chromatogram in both ionization modes is shown in Figure 2. All peaks were base line separated.

An increase in flow rate (> 0.4 ml/min) also allowed good separation of the peaks. However, quantification with the MS in MRM was no longer adequate, as the MS system could not switch masses as fast as needed to obtain sufficient scans per peak (i.e. a minimum of 10 points across each peak). This led to a decreased sensitivity, and thus, a flow rate of 0.4 ml/min was defined to be optimal. The final gradient already enabled an analysis in only 5.25 minutes, including the equilibration time required for the next run.

3.2 Method validation

3.2.1 Calibration curves

Lack of fit analysis of the model describing the relation between the analyte-to-IS ratio and concentration demonstrated a linear correlation for AP, DX and HMDZ, and a quadratic correlation for HTB, HME and HCZ ($p<0.10$). Due to the large concentration range, a weighting factor of $1/x^2$ and $1/x$ was necessary for the linear and the quadratic models, respectively, to obtain the best residuals, and consequently the best accuracy. When five independent calibration curves were compared statistically, they were proven to have an equal slope and intercept ($p > 0.10$).

Table 1: Incubation conditions and analytical parameters for the individual metabolites and internal standard.

	CYP1A2	**CYP2C9**	**CYP2C19**	**CYP2D6**
Substrate	Phenacetin	Tolbutamide	S-mephenytoin	Dextromethorphan
Km (µM)	50	100	100	5
Metabolite	Acetaminophen (AP)	4-OH-tolbutamide (HTB)	4'-OH-mephenytoin (HME)	Dextrorphan (DX)
t_R (min)	0.99	2.02	1.64	1.48
Ionization mode	ESI +	ESI -	ESI +	ESI +
Capillary voltage (kV)	+ 3.25	- 2.80	+ 3.25	+ 3.25
Cone voltage (V)	12	24	24	22
Collision energy (eV)	28	12	14	28
Precursor ion (m/z)	152.10	285.09	235.41	258.00
Quantifier (m/z)	110.00	185.60	150.00	156.70
Qualifier (m/z)	93.00		133.00	132.80
Range (nM)	18.52 - 8333	2.79 - 5238.34	18.43 - 8293.56	2.13 - 959.29
QC low (nM)	52.09	21.83	51.83	6.00
QC medium (nM)	1111.20	698.45	1105.81	127.91
QC high (nM)	4166.98	2619.17	4146.78	479.65
	CYP2E1	**CYP3A4**	**Internal standard**	
Substrate	Chlorzoxazone	Midazolam	Chlorpropamide (CLP)	
Km (µM)	50	5		
Metabolite	6-OH-chlorzoxazone (HCZ)	1-OH-midazolam (HMDZ)		
t_R (min)	1.44	2.08	2.75	
Ionization mode	ESI -	ESI +	ESI +	ESI -
Capillary voltage (kV)	- 2.80	+ 3.25	+ 3.25	- 2.80
Cone voltage (V)	25	22	25	20
Collision energy (eV)	14	19	11	11
Precursor ion (m/z)	183.83	342.04	276.86	274.89
Quantifier (m/z)	119.80	323.70	174.69	189.57
Qualifier (m/z)	147.70	202.80	191.65	125.85
Range (nM)	49.09 - 5522.79	4.29 - 1929.60		
QC low (nM)	86.29	12.06		
QC medium (nM)	1840.93	257.28		
QC high (nM)	3451.77	964.80		

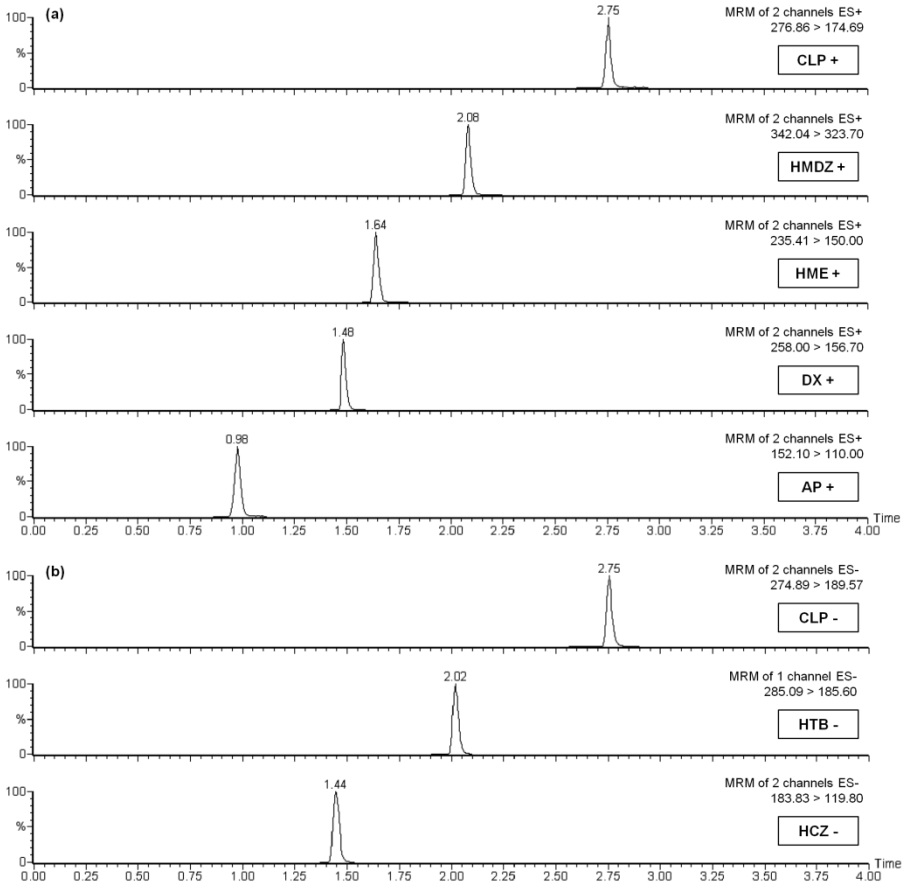

Figure 2: Representative chromatograms of the metabolites in positive (a) and negative (b) electrospray ionization mode, obtained after the injection of QC medium (concentrations: see Table 1). Concentration of the internal standard: 20 ng/ml. (compound abbreviation identification as in Figure 1).

3.2.2 Limit of detection (LOD) and limit of quantification (LOQ)

Limits of detection (LOD) and quantification (LOQ) of all metabolites are depicted in Table 2. Precision and accuracy of the LOQs met the requirements of the FDA (RSD <20%, and between 80% and 120%, respectively). The obtained LOQs are evaluated as adequate for the intended pharmacokinetic application(s), as very low CYP enzyme activities can still be detected, important in diseased liver situations.

3.2.3 Precision and accuracy

The between-day and within-day precision were better than 5.13% and 9.60% (RSD%), respectively, and the accuracy ranged from 84.59 to 109.83% (see Table 3). Thus, the method proved to be precise and accurate.

Table 2: Limit of detection (LOD) and limit of quantification (LOQ) of all metabolites. The precision and accuracy of the LOQs met requirements (< 20%, and between 80% and 120%, respectively) (compound abbreviation identification as in Figure 1).

		AP	DX	HME	HMDZ	HCZ	HTB
LOD (ng/ml)		0.134	0.088	0.912	0.095	1.871	0.173
LOQ (ng/ml)		2.8	0.55	4.32	1.47	9.11	0.8
Precision (RSD%)	n = 6	5.13	2.64	4.5	5.33	4.13	3.27
Accuracy (%)	n = 6	107.52	101.51	110.93	92.63	89.62	98.02

Table 3: Validation data: within-day precision, between-day precision and accuracy (compound abbreviation identification as in Figure 1).

		AP	DX	HME	HMDZ	HCZ	HTB
Within-day precision (RSD%) n = 6	QC low	4.48	1.37	1.65	1.97	1.72	2.61
	QC medium	2.16	2.41	2.04	2.66	2.03	2.33
	QC high	5.13	2.37	1.14	4.61	1.1	1.47
Between-day precision (RSD%) n = 6	QC low	6.29	3.75	5.88	4.63	9.6	3.34
	QC medium	3.01	4.36	5.27	3.84	6.16	3.64
	QC high	2.86	2.69	3.27	4.82	4.29	3.84
Accuracy (%) n = 6	QC low	87.75	86.26	91.1	93.52	85.32	95.04
	QC medium	104.61	107.61	105.07	102.61	84.59	99.59
	QC high	107.39	107.46	107.93	97.04	106.83	109.83

3.2.4 Selectivity and stability

No interference was observed at the retention times of the analytes and the IS when analyzing blank microsomes from six independent sources (signal-to-noise ratio > 9; data not shown).

Statistical analysis of the autosampler stability only showed significant differences of the HME and DX concentration after 14h ($p < 0.05$). However, the mean of the calculated concentration is still within the 85-115% interval of the nominal value (DX: 88.48%, HME: 92.11%) and therefore acceptable. Consequently, sample incubation extracts may be placed in the autosampler for up to 14h.

The evaluation of the freeze-thaw stability showed similar results: HME QC high, HMDZ QC low and high and HCZ QC mid showed statistically significant differences ($p < 0.05$), but the mean of the calculated concentration is within the 85-115% interval of the

nominal value. This means that (large) batches of incubations can be stored temporarily upon final analysis.

3.2.5 Matrix effect

Co-eluting compounds may cause enhancement or suppression of the ionization of the analyte. Due to the scarce sample preparation of the biological matrix (only protein precipitation), matrix effects were expected. In order to try to reduce these matrix effects base line separation of the metabolites was pursued. As a quantitative measure of matrix effect, the IS-normalized matrix factor (MF) was determined at three different concentration levels for all six metabolites, following Viswanathan et al [23]. The absence of an absolute matrix effect is not indispensable for a valid bioanalytical method. Variable matrix effects in individual subjects, however, would cause a problem of reproducibility of the method. As shown in Table 4, an absolute matrix effect (IS-normalized MF ≠ 1) was observed for some of the metabolites at some of the concentrations (HMDZ and HCZ). Nevertheless, no relative matrix effects were seen, as the coefficients of variation (CV %) at each concentration level were < 15% for all compounds. The observed absolute matrix effects are in contrast with the study described by Li et al., where ion enhancement or suppression from the matrix was found negligible [6]. This is probably due to the more selective nature of the sample preparation, consisting of a liquid-liquid extraction, followed by evaporation and reconstitution in mobile phase. Despite the lack of matrix effects in their method, the sample preparation is much more extended than the fast method used in the study described in this article (protein precipitation followed by centrifugation).

These results indicate that the selection of an appropriate internal standard is essential for the analytical method to be valid. Also, as the FDA prescribes, calibrators and QC samples need to be prepared in the same matrix as the samples.

3.2.6 Application of the method

The incubations of the microsomes originating from diseased livers from children undergoing liver transplantation resulted in the formation of metabolite concentrations as depicted in Table 5. With these metabolite concentrations, enzyme activities were calculated. Some of the isoforms showed very low activities, but nevertheless, these activities could be determined. These results show that the quantification method can be used in the *in vitro* determination of the enzyme activity of the six most important CYP isoforms. Interpretation of the results, however, is not included in this manuscript in view of the ongoing nature of the study, as well as the scope of this publication.

Table 4: Internal standard-normalized matrix factors of the six metabolites at three concentration levels. Despite the absolute matrix effect observed for some of the metabolites (MF ≠ 1), no relative matrix effect was seen (CV% < 15%) (compound abbreviation identification as in Figure 1; conc: concentration).

		Mean (*n* = 5)	CV (%)			Mean (*n* = 5)	CV (%)
AP	Conc 1	0.9	6.89	HME	Conc 1	0.95	10.77
	Conc 2	0.94	4.49		Conc 2	1	3.94
	Conc 3	0.99	4.06		Conc 3	1.08	3.99
DX	Conc 1	0.92	6.84	HCZ	Conc 1	1.25	4.46
	Conc 2	1.01	3.33		Conc 2	0.96	5.14
	Conc 3	0.99	3.75		Conc 3	0.98	6.62
HMDZ	Conc 1	1.23	4.30	HTB	Conc 1	0.92	9.82
	Conc 2	1.25	5.11		Conc 2	0.91	5.34
	Conc 3	1.22	3.92		Conc 3	0.91	3.00

Table 5: Enzyme activities calculated after incubation of paediatric microsomes, with the following conditions: 0.25 mg microsomal protein/ml incubation mix, probe substrates near Km, reaction time: 15 minutes, reaction temperature 37°C (n = 3). SD: standard deviation (compound abbreviation identification as in Figure 1).

	CYP	**Metabolite concentration** Mean (±SD) in ng/ml			**Enzyme activity** Mean (±SD) in pmol/mg/min		
		Sample 1	Sample 2	Sample 3	Sample 1	Sample 2	Sample 3
AP	1A2	3.51 (±0.28)	3.84 (±0.22)	6.07 (±0.57)	27.23 (±2.15)	29.84 (±1.72)	47.09 (±4.41)
DX	2D6	13.46 (±0.82)	32.76 (±4.29)	70.52 (±3.74)	61.38 (±3.73)	149.3 (±19.54)	321.51 (±17.07)
HME	2C19	< LLOQ	31.13 (±1.93)	17.13 (±1.68)	< LLOQ	155.9 (±9.64)	85.81 (±8.40)
HMDZ	3A4	22.27 (±2.04)	65.50 (±5.21)	6.43 (±0.55)	76.47 (±7.02)	224.9 (±17.89)	22.07 (±1.87)
HCZ	2E1	57.35 (±4.89)	30.95 (±2.39)	214.7 (±15.90)	362.6 (±30.92)	195.7 (±15.11)	1358 (±100.5)
HTB	2C9	12.31 (±1.17)	38.62 (±3.12)	62.49 (±7.18)	50.4 (±4.81)	158.2 (±12.78)	256.1 (±29.43)

4 CONCLUDING REMARKS

This paper presents the development and validation of a fast and sensitive UPLC-MS/MS method for the determination of the *in vitro* CYP450 enzyme activity. Especially in populations with liver dysfunction, a sensitive quantification method is required, as these patients often show a reduced activity. The UPLC-MS/MS approach in addition allows short sample analysis turn over times, which is interesting for high sample loads. The presented method was validated for selectivity, precision and accuracy. Despite the observed absolute matrix effects, a relative matrix effect could be ruled out, thus corroborating the validity of the obtained quantitative measurements. The sensitivity of the method was shown to be adequate for the intended pharmacokinetic applications, i.e. the incubation experiments with microsomes originating from liver samples from children with severe hepatic dysfunction. The low activities of some of the CYP isoforms could still be calculated. This method will be used in pre-clinical pharmacokinetic experiments.

REFERENCES

[1] Verbeeck RK. Pharmacokinetics and dosage adjustment in patients with hepatic dysfunction. *Eur J Clin Pharmacol* 2008; 64(12):1147-1161.

[2] Tucker GT, Houston JB, Huang SM. Optimizing drug development: strategies to assess drug metabolism/transporter interaction potential - towards a consensus. *Br J Clin Pharmacol* 2001; 52(1):107-117.

[3] Youdim KA, Saunders KC. A review of LC-MS techniques and high-throughput approaches used to investigate drug metabolism by cytochrome P450s. *J Chromatogr , B: Biomed Sci Appl* 2010; 878(17-18):1326-1336.

[4] Lahoz A, Donato MT, Castell JV, Gomez-Lechon MJ. Strategies to in vitro assessment of major human CYP enzyme activities by using liquid chromatography tandem mass spectrometry. *Curr Drug Metab* 2008; 9(1):12-19.

[5] Kim MJ, Kim H, Cha IJ, Park JS, Shon JH, Liu KH *et al.* High-throughput screening of inhibitory potential of nine cytochrome P450 enzymes in vitro using liquid chromatography/tandem mass spectrometry. *Rapid Commun Mass Spectrom* 2005; 19(18):2651-2658.

[6] Li XY, Chen XY, Li Q, Wang LL, Zhong DF. Validated method for rapid inhibition screening of six cytochrome P450 enzymes by liquid chromatography-tandem mass spectrometry. *J Chromatogr , B: Biomed Sci Appl* 2007; 852(1-2):128-137.

[7] Testino SA, Patonay G. High-throughput inhibition screening of major human cytochrome P450 enzymes using an in vitro cocktail and liquid chromatography-tandem mass spectrometry. *J Pharm Biomed Anal* 2003; 30(5):1459-1467.

[8] Yao M, Zhu MS, Sinz MW, Zhang HJ, Humphreys WG, Rodrigues AD *et al.* Development and full validation of six inhibition assays for five major cytochrome P450 enzymes in human liver microsomes using an automated 96-well microplate incubation format and LC-MS/MS analysis. *J Pharm Biomed Anal* 2007; 44(1):211-223.

[9] Lahoz A, Donato MT, Picazo L, Gomez-Lechon MJ, Castell JV. Determination of major human cytochrome P450s activities in 96-well plates using liquid chromatography tandem mass spectrometry. *Toxicology in Vitro* 2007; 21(7):1247-1252.

[10] Xu RNX, Fan LM, Rieser MJ, El-Shourbagy TA. Recent advances in high-throughput quantitative bioanalysis by LC-MS/MS. *J Pharm Biomed Anal* 2007; 44(2):342-355.

[11] Alden PG, Plumb RS, Jones MD, Rainville PD, Shave D. A rapid ultra-performance liquid chromatography/tandem mass spectrometric methodology for the in vitro analysis of Pooled and Cocktail cytochrome P450 assays. *Rapid Commun Mass Spectrom* 2010; 24(1):147-154.

[12] Peng SX, Barbone AG, Ritchie DM. High-throughput cytochrome P450 inhibition assays by ultrafast gradient liquid chromatography with tandem mass spectrometry using monolithic columns. *Rapid Commun Mass Spectrom* 2003; 17(6):509-518.

[13] George J, Murray M, Byth K, Farrell GC. Differential Alterations of Cytochrome-P450 Proteins in Livers from Patients with Severe Chronic Liver-Disease. *Hepatology* 1995; 21(1):120-128.

[14] Yu ZQ, Peng PA, Sheng GY, Fu J. Determination of hexabromocyclododecane diastereoisomers in air and soil by liquid chromatography-electrospray tandem mass spectrometry. *J Chromatogr A* 2008; 1190(1-2):74-79.

[15] Palma P, Famiglini G, Trufelli H, Pierini E, Termopoli V, Cappiello A. Electron ionization in LC-MS: recent developments and applications of the direct-EI LC-MS interface. *Analytical and Bioanalytical Chemistry* 2011; 399(8):2683-2693.

[16] Niessen WMA, Manini P, Andreoli R. Matrix effects in quantitative pesticide analysis using liquid chromatography-mass spectrometry. *Mass Spectrom Rev* 2006; 25(6):881-899.

[17] Wilson ZE, Rostami-Hodjegan A, Burn JL, Tooley A, Boyle J, Ellis SW *et al.* Inter-individual variability in levels of human microsomal protein and hepatocellularity per gram of liver. *Br J Clin Pharmacol* 2003; 56(4):433-440.

[18] Yuan R, Madani S, Wei XX, Reynolds K, Huang SM. Evaluation of cytochrome P450 probe substrates commonly used by the pharmaceutical industry to study in vitro drug interactions. *Drug Metab Dispos* 2002; 30(12):1311-1319.

[19] FDA. Guidance for Industry: Bioanalytical Method Validation. 2001.

[20] Walsky RL, Obach RS. Validated assays for human cytochrome P450 activities. *Drug Metab Dispos* 2004; 32(6):647-660.

[21] Long GL, Winefordner JD. Limit of Detection. *Anal Chem* 1983; 55(7):A712-&.

[22] Burkart JA. General procedures for limit of detection calculations in the industrial hygiene chemistry laboratory. *Appl Ind Hyg* 1986; 1(3):153-155.

[23] Viswanathan CT, Bansal S, Booth B, DeStefano AJ, Rose MJ, Sailstad J *et al.* Workshop/conference report - Quantitative bioanalytical methods validation and implementation: Best practices for chromatographic and ligand binding assays. *AAPS J* 2007; 9(1):E30-E42.

[24] Lin T, Pan K, Mordenti J, Pan L. In vitro assessment of cytochrome P450 inhibition: Strategies for increasing LC/MS-Based assay throughput using a one-point IC50 method and multiplexing high-performance liquid chromatography. *J Pharm Sci* 2007; 96(9):2485-2493.

CHAPTER 3

DEVELOPMENT AND VALIDATION OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY FOR THE QUANTIFICATION OF CYTOCHROME 3A4 IN HUMAN LIVER MICROSOMES

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"Elisa, Elisa

Elisa les autres on s'en fout,

Elisa, Elisa

Elisa rien que toi, moi, nous"

- Serge Gainsbourg

ABSTRACT

Little is known about the influence of hepatic pathologies on cytochrome P450 (CYP) mediated drug metabolism in children. The determination of the abundance of the different isoforms in paediatric microsomes may provide valuable information on the mechanisms of possible changes in activity. Until now, western blotting was mostly used for abundance measurements, but this technique only provides semi-quantitative data. Therefore, this study aimed to develop and validate an indirect ELISA for the quantification of the most important CYP isoform, CYP3A4, in human liver microsomes, using commercially available reagents. Samples, calibrators and validation samples were diluted to a final concentration of 10 µg microsomal protein/ml. A polyclonal antibody raised against the full length human protein was used as primary antibody, horseradish peroxidase conjugated secondary antibodies for detection. The assay was validated for sensitivity, working range and calibration, accuracy and precision. Amounts of CYP3A4 between 2 and 300 pmol/mg microsomal protein could be quantified with a 5-parameter logistics function with 1/x weighting factor. Coefficients of variation of intra and inter assay variability were between 9.54 and 13.98 % (16.34% at LLOQ), and between 10.51 and 14.55% (19.44% at LLOQ), respectively. The relative error (%RE) varied between -5.96 and 6.68% (11.53% at LLOQ), and the total error between 11.93 and 21.23% (30.97% at LLOQ). The cross-reactivity of the method with human CYP2E1 showed to have no significant effect on the accuracy of the results. Successful analysis of five samples from an ongoing study demonstrated the usefulness of the method.

1 INTRODUCTION

Cytochrome P450 (CYP) enzymes are a superfamily of enzymes involved in the metabolism of endogenous substrates, many drugs and other xenobiotics. The main CYP isoforms involved in drug metabolism are CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4. The presence of these isoforms has initially been determined by Shimada et al [1]. The most abundant isoform is CYP3A4 (~30% of total CYP). This protein, composed of 503 amino acids [2], is responsible for the metabolism of over 50% of the drugs currently on the market [1]. CYP3A4 is one of the four members of the CYP3A subfamily. Apart from CYP3A4, CYP3A5 is the only member of the CYP3A family that was detected in significant concentrations in the liver [1], albeit in much lower concentrations than CYP3A4 [3]. Only very rare polymorphic sites of CYP3A4 were identified. This isoform is probably well preserved because of its role in the metabolism of many endogenous and environmental factors [3].

CYP-mediated drug metabolism has been shown to be impaired in adult patients with hepatic dysfunction [4]. Furthermore, the degree of impairment in adults correlates with the severeness of the hepatic dysfunction [5], as well as with the aetiology of the disease [6]. In children, however, no similar investigation was performed up till now. The effect of pathology on drug metabolism can be investigated through phenotyping reactions in regard to CYP activity. Furthermore, the abundance of the relevant isoforms can be determined. CYPs are mainly located in the liver and 96% of the CYPs can be found in the subcellular fraction called microsomes [7]. As microsomes are easy to prepare and have excellent long term stability, they are a good choice to perform the characterizations mentioned before (phenotyping and abundance). Previous studies in adults showed a positive correlation between activity and abundance [1,8]. This relationship between enzyme activity and abundance may offer valuable information on the causes of possible changes in enzyme activity. A reduced abundance has been described in patients with liver cirrhosis, as clearly reviewed by Villeneuve et al [5]. Post-translational mechanisms, on the other hand, may result in low activities but regular abundances of quantifiable CYP protein.

To determine CYP abundance in e.g. microsomes, many studies have used the technique of western blotting. These methods, however, are time consuming and electrophoresis is relatively prone to technical complications. Also, quite large amounts of microsomal protein are necessary. Therefore, Snawder et al developed an indirect enzyme linked immunosorbent assay (ELISA), using primary antibodies against CYPs in rats [8]. These methods for immunoquantification have a substantially higher throughput and are easier to perform than western blots. This is a clear advantage in studies with a larger number

of samples. Barter et al. developed an indirect competitive ELISA for the quantification of human CYP3A4 and CYP3A5 [9], using primary antibodies against the C-terminus of the 3A4 enzyme [10]. Immunologically targeting only part of the protein might introduce bias in the correlation between activity and abundance as protein dysfunctions (e.g. truncation) in the non-targeted but active part of the protein would go undetected.

Therefore, the study described here aims to develop a sensitive indirect ELISA using commercially available primary antibodies raised against the full length human protein, in order to obtain supportive information for a pharmacokinetic study on the impact of disease on hepatic biotransformation in children. The method was thoroughly validated according to the recommendations of DeSilva et al [11]. Furthermore, as a proof of concept, samples from the ongoing pharmacokinetic study were analyzed.

2 MATERIALS AND METHODS

2.1 Chemicals

Sodium carbonate and bicarbonate, sodium chloride, and Tween 20® were purchased from VWR (Leuven, Belgium), tris(hydroxy-methyl)aminomethane from Sigma-Aldrich (Buchs, Switserland), and hydrochloric acid from Acros Organics (Geel, Belgium). All chemicals were at least reagent grade.

2.2 Calibrators: recombinant CYP3A4

For the preparation of the calibrators and for certain validation aspects, microsomes prepared from insect cells infected with a virus engineered to express human CYP3A4 were used (2000 pmol CYP3A4/ml; 2.1 mg microsomal protein/ml BD Supersomes™, BD Gentest, Franklin Lakes, USA). As negative controls (blanks) for the analysis, Control Supersomes™ (BD Gentest) were used. These control supersomes were prepared from the same type of insect cells, but no human CYPs were expressed. After optimization of the protocol, the final composition and concentrations of the calibrators were 300, 150, 60, 30, 15, 7.5, 4 and 2 pmol rCYP3A4/mg protein in a pH 9.4 carbonate-bicarbonate plating buffer. Additional Control Supersomes™ were added to each calibrator, in order to obtain a final concentration of total microsomal protein of 10 µg/ml. The validation samples (VS), with final concentrations of 2, 5, 100, 200 and 300 pmol rCYP3A4/mg protein, were prepared similarly. For the cross-reactivity experiment, Supersomes™ of insect cells which expressed human CYP2E1 (BD Gentest) were used.

2.3 Patient samples: microsomes

Liver samples were collected from the diseased liver from children undergoing a liver transplantation (approved by the Ethics Committee of Ghent University Hospital,

B67020084281). Microsomes were prepared following the method of Wilson et al [7]. Protein content of the microsomal samples was determined by the method of Bradford [12].

2.4 Primary and secondary antibodies

For the indirect ELISA, two types of antibodies were necessary. The primary antibody to bind the antigen was a polyclonal antibody raised in rabbit against the full-length human CYP3A4 protein (MaxPab® antibody, Abnova, Taiwan). As the secondary antibody, a goat anti-rabbit IgG conjugated with horse radish peroxidase (HRP) was purchased from Thermo Scientific (Rockford, USA).

2.5 Development of indirect ELISA for CYP3A4 in human microsomes

Microsomal samples were diluted to a concentration of 10 µg of microsomal protein/ml using a carbonate-bicarbonate buffer pH 9.4. One hundred microliters of the pH 9.4 carbonate-bicarbonate plating buffer dilution of the calibrators or unknown samples were loaded on a black 96-well MaxiSorp® micro-titre plate (Nunc, Roskilde, Denmark). After an overnight incubation at 4°C, the plating solution was removed. Subsequently, plates were washed 3 times by the addition of 300 µl wash buffer (Tris-buffered saline (TBS) with 0.05% (v/v) Tween 20®, pH 7.2), soaking during 2.5 minutes and aspiration of the buffer. Nonspecific binding sites were blocked with 300 µl/well of StartingBlock™ blocking buffer in TBS with Tween 20® (Thermo Scientific, Rockford, USA) and incubation for 1h at room temperature (±23°C). After aspiration of the blocking buffer, plates were washed 3 times as described previously. The primary antibody was diluted in blocking buffer (1:3200), and 100 µl was added to each well, followed by incubation of the plates for 1h at 37°C. Primary antibody was aspirated and plates were washed as described previously. For the detection of the primary antibodies, 100 µl of secondary antibody dilution in blocking buffer (1:20000) was added and plates were then incubated at room temperature for 1 h. The unbound secondary antibody was removed and plates were washed, and 100 µl of premixed HRP substrate (QuantaBlu™ Fluorogenic peroxidase substrate and peroxide, Thermo Scientific, Rockford, USA) was added to each well. After 30 minutes at 37°C, the reaction was stopped with the stop solution from the QuantaBlu™ kit, and fluorescence was determined at an excitation wavelength of 320 nm, and emission at 405 nm (Ascent Fluoroscan, Thermo Scientific, Rockford, USA). Calibration curves were fitted and data were analyzed using the Masterplex® Readerfit 2010 software (Hitachi, San Francisco, CA, USA) and Microsoft® Excel (v 2007). The amounts of CYP3A4 were expressed in pmol/mg microsomal protein.

The final protocol was obtained after the optimization of several parameters. Optimal concentrations of primary and secondary antibodies were determined by a checkerboard

titration experiment. In this experiment, samples with CYP3A4 at 3 concentration levels (0, 5, and 100 pmol/mg protein) were analyzed using the combination of 10 concentrations of primary antibody (2500, 1250, 625, 417, 312.5, 208, 156, 75, 40, and 20 ng/ml), with 4 concentrations of secondary antibody (25, 50, 75, and 100 ng/ml, according to the manufacturers guidelines). Furthermore, the optimal amount of protein to load in each well (0.5, 1 or 1.5 µg protein), the blocking buffer, incubation times and temperature were determined.

2.6 Validation of indirect ELISA for CYP3A4 in human microsomes

The method was validated for assay sensitivity, linearity of dilution, spiking recovery, calibration model and working range, intra and inter assay variability and precision, and cross-reactivity.

The calibration model was assessed as described by DeSilva et al [11]. The curve was fitted for six independent assay runs. The appropriateness of the model was evaluated by analysis of the relative error (%RE) of the back-calculated calibration points (%RE = 100 x (calculated concentration - nominal concentration)/nominal concentration). The %RE should be ≤20% (≤25% at LLOQ) for ≥75% of the calibrators within a curve. Furthermore, the mean %RE and %CV calculated from all runs (n=6) should both be ≤15% for each calibrator, except at the LLOQ where both should be ≤20%.

Sensitivity of the method was determined by calculating the mean response of 10 blank samples plus 3 standard deviations. The concentration calculated with this response was defined as the lowest concentration that could be distinguished from a blank sample, and was used as the lower limit of the working range. The upper limit of the working range was defined based on literature information (based on naturally occurring CYP3A4 abundance in microsomes) and compliance with the validation criteria was tested. Linearity of dilution was determined by diluting a sample with a known concentration above the upper limit of the working range 1:2, 1:3, 1:4 and 1:6. The recovery (%) of the observed concentration to the expected concentration should be within the 85-115% interval. In order to evaluate matrix effects, a spiking recovery experiment was set up. Samples were prepared at five concentration levels in both dilution buffer (carbonate-bicarbonate, pH 9.4) and in blank matrix (blank supersomes, final protein concentration of 10 µg/ml). The recovery (%) of the observed concentration to the nominal concentration was calculated and evaluated against the 85-115% interval in both matrices.

Assessment of the intra assay and inter assay precision and accuracy was also performed according to the recommendations of DeSilva et al [11]. Method precision and accuracy were estimated by the analysis of validation samples prepared in the sample matrix at

five concentration levels: anticipated LLOQ, less than 3 times LLOQ, medium, high and anticipated upper limit of the working range. For each validation sample, at least 2 independent determinations were done each run, for a minimum of 6 runs. The statistical methods used for the evaluation of the repeated measurements of each validation sample were described by DeSilva et al [11]. The coefficient of variation used to express the intra assay precision was calculated by dividing the pooled intra assay standard deviations of the calculated assay run means with the sample nominal concentration. The standard deviation needed for the calculation of the %CV for the inter assay precision was calculated by the method of analysis of variance (ANOVA). Method accuracy (%RE) was determined by the percent deviation of the weighted sample mean from the sample nominal concentration. The a priori set target limits were an intra assay and inter assay precision (%CV) and absolute value of the mean bias (%RE) ≤ 20% (25% at LLOQ). In addition, the total error of the method (= sum of %CV and absolute value of the %RE) should be ≤ 30% (40% at LLOQ).

The cross-reactivity was assessed following MacFarlane et al [13], according to the equation:

[(observed concentration - control concentration)/ supplemented concentration] * 100,

with observed concentration = calculated CYP3A4 concentration, control concentration = calculated CYP3A4 concentration in a sample without supplementation of rCYP2E1, and supplemented concentration = concentration of rCYP2E1 supplemented. Also, the calculated mean concentrations from the samples with supplementation of recombinantly expressed CYP2E1 at 4 concentration levels (zero - low – expected physiologically – high) were compared with one-way ANOVA (using Microsoft Excel® 2007).

In-study validation was performed during each patient sample analysis run. Two determinations of validation samples at 3 concentration levels (less than 3 times LLOQ, medium and high) were measured. These results were evaluated with the 4:6:30 rule [11], i.e. 4 out of the 6 VS should have a total error below 30%, and the 2 samples not meeting this requirement should not be at the same concentration level. Furthermore, ≥75% of the standard points should have a %RE of ≤ ±15%.

2.7 Application of the method

Five patient samples from an ongoing pharmacokinetic study were analyzed with the validated method. After determination of the total protein content, the microsomal suspensions were diluted in the plating buffer to a final protein concentration of 10 µg/ml. For each of the samples, two wells were loaded with hundred µl of the dilution.

The actual concentration of the samples was determined by the average concentration of the two wells.

3 RESULTS

3.1 Validation

Based on six independent assay runs, a five-parameter logistics (5-PL) curve with a 1/x weighting factor was validated (Figure 1). Eight calibrators within the range of 2 to 300 pmol CYP3A4/mg protein were used to fit the model. The %RE of the individual calibration points, and the intercurve mean %RE and %CV of each calibrator are shown in Table 1, and were all within the specified limits. The signal bigger than the mean signal of blank microsomes + 3 SD corresponded with a concentration of 2 pmol/mg protein.

Table 1: Validation of the standard curve: Relative error (%) of the back-calculated standard concentrations, mean relative error and coefficient of variation (%CV).

	Nominal concentration (pmol CYP3A4/mg protein)							
Batch run	**2**	**4**	**7.5**	**15**	**30**	**60**	**150**	**300**
1	6.00	-8.00	4.00	-6.67	7.93	-1.65	-2.50	1.67
2	5.50	-9.50	8.00	-6.60	4.17	-0.25	-1.90	1.77
3	-0.50	2.50	-0.53	-1.33	1.23	0.22	-0.93	1.16
4	6.00	-7.25	0.40	-0.53	2.27	0.70	-2.31	1.42
5	1.00	-3.75	3.47	-3.87	1.80	-1.75	0.23	0.13
6	-6.00	12.00	0.00	-6.53	3.03	2.80	-3.59	1.72
Mean %RE	4.25	-5.56	2.97	-3.78	3.90	-0.25	-1.91	1.51
%CV	3.05	5.78	3.77	3.44	2.84	1.02	0.71	0.27

The results from the spiking recovery experiment for the evaluation of the matrix effects are shown in Table 2. Dilution of the stock solution of rCYP3A4 with plating buffer revealed bad recovery, whereas samples diluted in blank matrix and with a final protein concentration of 10 µg/ml, showed a recovery within the 85-115% limits.

Dilution of a sample with a known concentration of 450 pmol CYP3A4/mg protein, which is above the upper limit of the working range of 300 pmol CYP3A4/mg protein, resulted in concentrations with a recovery within the 85-115% limits (Table 3).

Table 2: Spiking recovery experiment: Percent recovery of the observed concentration compared to the nominal concentration. Samples diluted in plating buffer showed very poor recovery at the lower concentrations.

Nominal concentration (pmol CYP3A4/mg protein)	**Observed concentration** (pmol CYP3A4/mg protein)		**Recovery %**	
	Diluted in blank matrix	Diluted in plating buffer	Diluted in blank matrix	Diluted in plating buffer
2	1.84	0.58	92	28.9
5	5.18	3.32	104	66.4
100	107	84.3	107	84.3
200	187	180	93.4	90.3
300	279	275	93.1	91.7

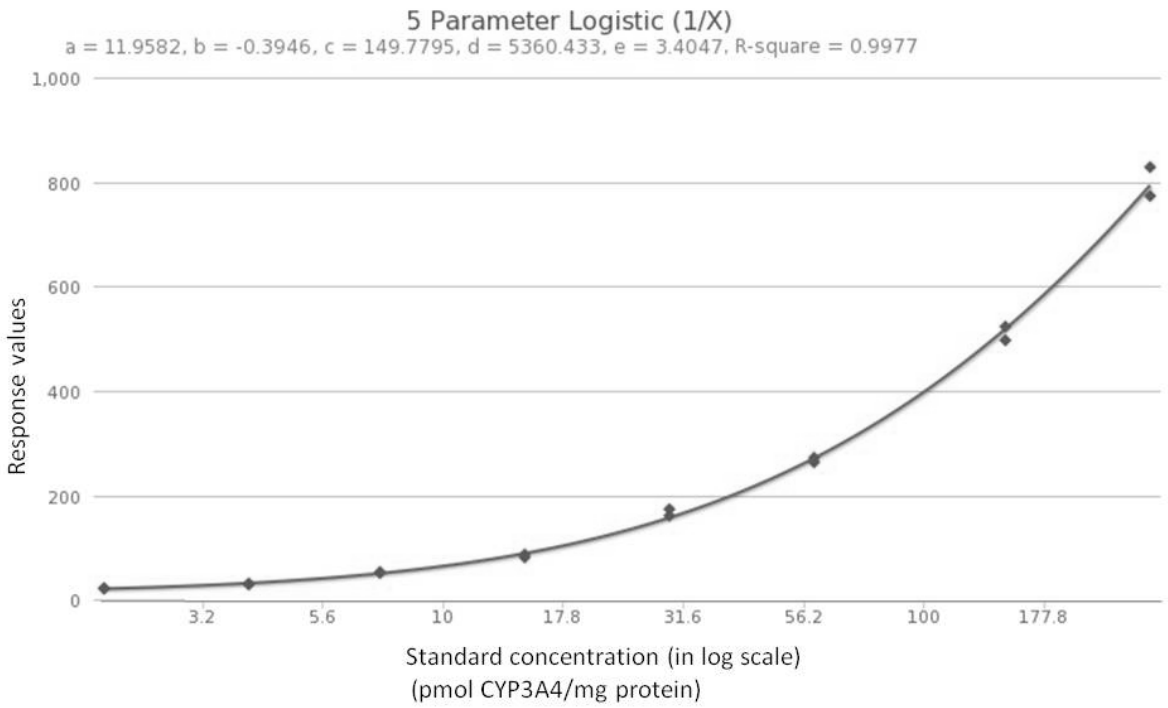

Figure 1: Representative calibration curve (range 2-300 pmol CYP3A4/mg protein) from the indirect ELISA for human CYP3A4, calculated with a five-parameter logistics curve, with 1/x weighting factor.

Table 3: Recovery of the diluted samples (in blank matrix, with a final protein concentration of 10 µg/ml), compared to the expected concentration of 450 pmol CYP3A4/mg protein.

Dilution Factor (DF)	**Observed concentration** (pmol CYP3A4/mg protein) x DF	**Recovery %**
2	426.2	94.7
3	436.5	97.0
4	431.6	95.9
6	433.3	96.3

The results of the statistical analysis of the precision and accuracy at the 5 concentration levels of the validation samples are presented in Table 4. Intra assay and inter assay precision were between 9.54 and 16.34%, and 10.51 and 19.44%, respectively. The mean bias (%RE) was always ≤ ±11.53%. Furthermore, the total error of the method at the 5 concentration levels was always substantially below the required 30% (≤40% at LLOQ).

Table 4: Precision and accuracy assessment.

		Nominal concentration (pmol CYP3A4/mg protein)				
Characteristic	**Statistic**	**2**	**5**	**100**	**200**	**300**
# Results	N	18	18	18	18	18
Accuracy	Mean bias (%RE)	11.53	6.68	0.69	-1.41	-5.96
Precision	Intra assay(%CV)	16.34	13.98	10.27	11.62	9.54
	Inter assay (%CV)	19.44	14.55	11.24	11.62	10.51
Total error (Accuracy + Precision)	|Mean bias| + Inter assay %CV	30.97	21.23	11.93	13.03	16.47

The cross-reactivity with human CYP2E1 was assessed through the calculation of the percentage of cross-reactivity (Table 5). The % deviation from the nominal concentration value was always within the method (inter assay) precision interval. One can conclude that the deviation from nominal seen is due to normal variability and cross reactivity can be considered insignificant. This was confirmed by the ANOVA, which showed a p-value > 0.05.

Table 5: Cross-reactivity experiment with recombinant CYP2E1.

Supplemented concentration CYP2E1 (pmol/mg protein)	**Mean observed concentration CYP3A4** (pmol/mg protein) (± SD)	**% cross reactivity**
0	125.7 ± 12.4	
20	124.5 ± 7.5	-5.9
140	140.5 ± 6.3	10.5
280	132.5 ± 8.5	2.4

3.2 Application of the method

Table 6 depicts the results of the analysis of microsomes prepared from explanted liver tissue from five children with biliary atresia.

Table 6: CYP3A4 content of microsomes prepared from explanted liver tissue from five children with biliary atresia.

Sex	**Age at transplantation** (months)	**CYP3A4 abundance** (pmol/mg protein)
M	4.7	54.32
M	7.3	4.14
F	8.9	133.36
F	9.4	6.55
F	9.5	18.76

4 DISCUSSION

An indirect ELISA was developed for the quantification of CYP3A4 in human liver microsomes. A fit-for-purpose optimization was performed in order to optimize all parameters in relation to the intended applications [14].

Commercially available polyclonal antibodies against full length human protein were used for the primary detection of the antigen. This type of antibody was considered essential for this method, as the antigen, i.e. the microsomal vesicle membrane bound CYP3A4 enzyme, is being adsorbed to the wall of the microplate. As it is unpredictable which part or side of the enzyme would be exposed for antibody binding, it would be advantageous to add polyclonal antibodies to maximize the chance of antigen-antibody interaction. The down side of this approach could possibly be the fact that truncated and non-functional protein, e.g. due to pathological conditions, would most probably also be measured. In contrast, monoclonal antibodies targeted at the active part of the protein would not include truncated and non-functional protein in the abundance measure. We, however, think the use of monoclonal antibodies would introduce bias in the activity/abundance

picture because abundance measurement then already includes an activity estimation. The optimal concentrations of primary and secondary antibody were determined by a checkerboard titration experiment (see methods). Based on outcome parameters such as a background signal as low as possible, combined with sufficient sensitivity for the low concentrations and no saturation for the higher concentration, a combination of 312.5 ng/ml of primary antibody, and 50 ng/ml of secondary antibody was further used. Incubation times and temperatures of the antibodies were also optimized, aiming for optimal sensitivity. Also, the amount of protein to be plated was tested. 0.5 µg protein per well showed insufficient sensitivity, as the response from the spiked sample containing 2 pmol/mg protein could not be distinguished from the blank samples. 1 µg was shown to be the optimal plating concentration, as a higher amount (1.5 µg protein per well) resulted in a significantly higher background signal. For reducing the non-specific binding, two types of commercially available blocking buffers were compared. The Starting Block buffer finally chosen provided an assay with highest precision.

The selection of the calibration model was achieved based on the %RE of the back-calculated concentrations. A visual inspection of the relationship between the response and the concentration already suggested the use of a 5-parameter logistics (5-PL) function, due to clear asymmetry (compared to a 4-parameter logistics function) of the curve (see Figure 1). A comparison of the mean %RE in back-calculated concentrations from an unweighted and a weighted 5-PL model is depicted in Figure 2. A weighting factor was shown to be necessary in order to obtain sufficient accuracy at the lower concentration level. The 5-PL function with 1/x weighting factor met all requirements of %RE and %CV to validate the calibration model (see Table 1).

The working range was selected based on previously determined mean concentrations of CYP3A4 in adults [8]. Nevertheless, as no information is present on this parameter in children (our research focus), nor on the effect of liver disease on the abundance in children, the possibility of lower or higher concentrations should be taken into account. The aspired lower limit of the working range was based on the mean abundance of CYP3A4 in adult liver (i.e. ± 100 pmol/mg protein). If liver disease would reduce the abundance with 90%, the remaining 10 pmol/mg protein should still be detectable. However, Snawder et al. reported a minimum of 19.7 pmol CYP3A4/mg protein in adult microsomes [8]. If the method should be able to detect a 90% reduction, an LOQ of 1.97 pmol/mg protein would then be required. Our validated lower limit of 2 pmol/mg protein meets this requirement. Achieving this LLOQ meant optimizing for sensitivity. For example, a clear increase in sensitivity, or decrease in background signal, was obtained by diluting the antibodies (both primary and secondary) in the commercially prepared blocking buffer, instead of in a 2% BSA solution in the wash buffer (TBS with 0.05%

Tween 20®, pH 7.2). The results from the spiking recovery experiment (Table 2) show a clear influence of the matrix proteins on the response. If the microsomes used for the calibrator samples would be diluted in a buffer without additional proteins, a low concentration in a sample would be severely overestimated. This is probably due to non-specific binding of the antibodies. Therefore, all calibrators and validation samples should be diluted in blank matrix, in order to have an equal final microsomal protein content in all samples (10 µg/ml).

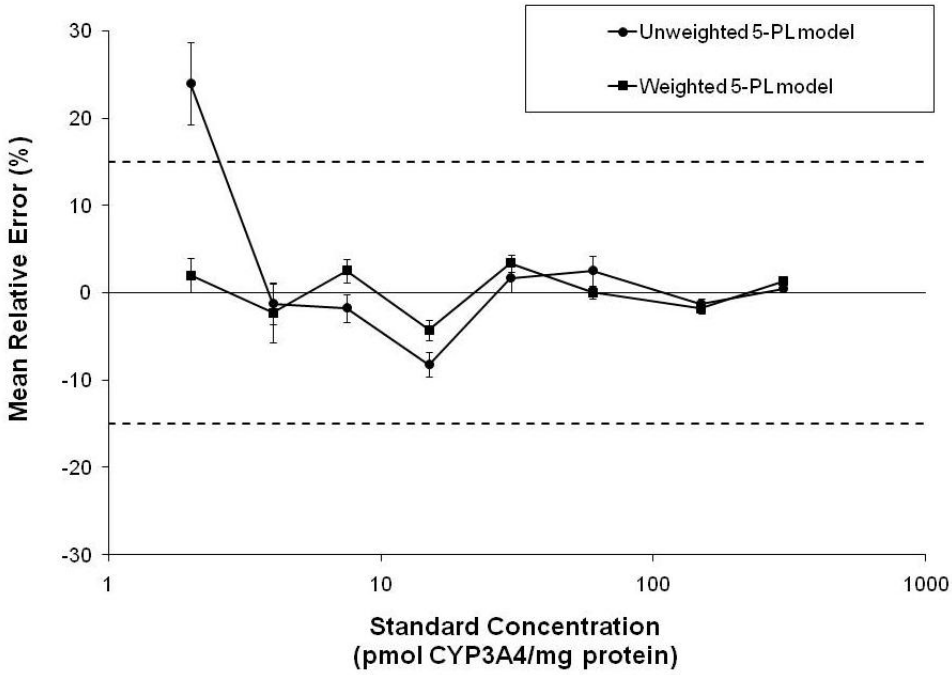

Figure 2. Comparison of the mean (± standard error) percent relative errors in back-calculated concentrations from an unweighted and weighted 5-PL function. The weighted model showed a better fit than the unweighted model, which showed an unacceptable accuracy at the lower range of the curve.

A dilution experiment was set up in order to define the approach for the determination of samples with a concentration above the upper limit of the working range (300 pmol/mg protein). A sample was spiked at a high CYP concentration, and was subsequently diluted 2, 3, 4 and 6 fold. Again, it was important to adjust final protein content using blank microsomes. Table 3 shows that the concentrations, calculated after multiplying the observed concentration with the dilution factor, all were within the 85-115% interval of the nominal concentration, indicating dilution of high dosed samples will not deteriorate the quantitative measurement results.

The variability of the results for the same sample analyzed under repeatability conditions and the intermediate precision are determined by the intra and inter assay precision, respectively. The coefficient of variation calculated to express these variabilities should not exceed 20% (25% at the LLOQ). The precision of this assay was evaluated at 5

concentration levels by performing 6 independent assay runs (with n = 3 within each run). Furthermore, these 6 assay runs were used for the assessment of the assay's accuracy (expressed as %RE). The assay's total error, i.e. the sum of the absolute mean value of the %RE and the inter assay %CV, should be below 30% (40% at LLOQ). The results from these analyses are summarized in Table 4. Both precision (intra and inter assay) and accuracy, as well as the total error, showed results below these limits at all 5 concentrations.

The CYPs are a group of hemoproteins classified according to their homology in amino acid sequence. Consequently, isoforms from the same family have ≥40 % homology, whereas members from the same subfamily show at least 55% similarity [15]. This homology may lead to cross-reactivity of the primary antibody. The ability of this assay to differentiate between CYP3A4 and other human CYPs was tested through the addition of CYP2E1 to a sample with a known concentration of CYP3A4. Even at a physiologically high abundance of CYP2E1, the method showed sufficient selectivity towards the detection of CYP3A4 (Table 5).

The method was tested through the analysis of samples from an ongoing PK study. The CYP3A4 abundance could be determined in all five samples, which proved the applicability of the method.

5 CONCLUSION

An indirect ELISA was developed for the quantification of CYP3A4 in human liver microsomes. After optimization of several parameters, the method was successfully validated. Concentrations of CYP3A4 between 2 and 300 pmol/mg microsomal protein could be selectively determined with adequate accuracy and precision. All samples, including validation samples and dilutions of samples with a concentration above the working range, should have a concentration of total protein of 10 µg/ml, as proven by the spiking recovery experiment. This method will be applied to samples from a pharmacokinetic study investigating the impact of disease on the metabolic capacity of the liver in children. The analysis of five samples from the study showed that the method has adequate sensitivity for the intended application.

REFERENCES

[1] Shimada T, Yamazaki H, Mimura M, Inui Y, Guengerich FP. Interindividual Variations in Human Liver Cytochrome-P-450 Enzymes Involved in the Oxidation of Drugs, Carcinogens and Toxic-Chemicals - Studies with Liver-Microsomes of 30 Japanese and 30 Caucasians. *J Pharmacol Exp Ther* 1994; 270(1):414-423.

[2] Molowa DT, Schuetz EG, Wrighton SA, Watkins PB, Kremers P, Mendezpicon G *et al.* Complete Cdna Sequence of A Cytochrome-P-450 Inducible by Glucocorticoids in Human-Liver. *Proc Natl Acad Sci U S A* 1986; 83(14):5311-5315.

[3] Ingelman-Sundberg M. Human drug metabolising cytochrome P450 enzymes: properties and polymorphisms. *Naunyn Schmiedebergs Arch Pharmacol* 2004; 369(1):89-104.

[4] Verbeeck RK. Pharmacokinetics and dosage adjustment in patients with hepatic dysfunction. *Eur J Clin Pharmacol* 2008; 64(12):1147-1161.

[5] Villeneuve JP, Pichette V. Cytochrome P450 and liver diseases. *Curr Drug Metab* 2004; 5(3):273-282.

[6] Bastien MC, Leblond F, Pichette V, Villeneuve JP. Differential alteration of cytochrome P450 isoenzymes in two experimental models of cirrhosis. *Can J Physiol Pharmacol* 2000; 78(11):912-919.

[7] Wilson ZE, Rostami-Hodjegan A, Burn JL, Tooley A, Boyle J, Ellis SW *et al.* Inter-individual variability in levels of human microsomal protein and hepatocellularity per gram of liver. *Br J Clin Pharmacol* 2003; 56(4):433-440.

[8] Snawder JE, Lipscomb JC. Interindividual variance of cytochrome P450 forms in human hepatic microsomes: Correlation of individual forms with xenobiotic metabolism and implications in risk assessment. *Regul Toxicol Pharmacol* 2000; 32(2):200-209.

[9] Barter Z, Perrett H, Yeo K, Allorge D, Lennard M, Rostami-Hodjegan A. Determination of a Quantitative Relationship between Hepatic CYP3A5*1/*3 and CYP3A4 Expression for Use in the Prediction of Metabolic Clearance in Virtual Populations. *Biopharmaceutics & Drug Disposition* 2010; 31(8-9):516-532.

[10] Edwards RJ, Adams DA, Watts PS, Davies DS, Boobis AR. Development of a comprehensive panel of antibodies against the major xenobiotic metabolising forms of cytochrome P450 in humans. *Biochem Pharmacol* 1998; 56(3):377-387.

[11] DeSilva B, Smith W, Weiner R, Kelley M, Smolec JM, Lee B *et al.* Recommendations for the bioanalytical method validation of ligand-binding assays to support pharmacokinetic assessments of macromolecules. *Pharm Res* 2003; 20(11):1885-1900.

[12] Bradford MM. Rapid and Sensitive Method for Quantitation of Microgram Quantities of Protein Utilizing Principle of Protein-Dye Binding. *Anal Biochem* 1976; 72(1-2):248-254.

[13] MacFarlane GD, Scheller DG, Ersfeld DL, Shaw LM, Venkatarmanan R, Sarkozi L *et al.* Analytical validation of the PRO-Trac II ELISA for the determination of tacrolimus (FK506) in whole blood. *Clin Chem* 1999; 45(9):1449-1458.

[14] Lee JW, Devanarayan V, Barrett YC, Weiner R, Allinson J, Fountain S *et al.* Fit-for-purpose method development and validation for successful biomarker measurement. *Pharm Res* 2006; 23(2):312-328.

[15] Nelson DR, Kamataki T, Waxman DJ, Guengerich FP, Estabrook RW, Feyereisen R *et al.* The P450 Superfamily - Update on New Sequences, Gene-Mapping, Accession Numbers, Early Trivial Names of Enzymes, and Nomenclature. *DNA Cell Biol* 1993; 12(1):1-51.

CHAPTER 4

QUANTIFICATION OF CYTOCHROME 2E1 IN HUMAN LIVER MICROSOMES USING A VALIDATED INDIRECT ELISA

This section is a modified version of the publication:

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"Things should be made as simple as possible,

but not any simpler"

- Albert Einstein

ABSTRACT

CYP2E1 is an important cytochrome P450 isoform in many endogenous processes and in the metabolism of organic solvents, a number of drugs and pre-carcinogens. Information on the abundance of the enzyme may be valuable in various types of research in the field of toxicology and pharmacology. An indirect ELISA for the quantification of CYP2E1 in human liver microsomes was developed and successfully validated. All samples, including validation samples and calibrators, were diluted to a final concentration of microsomal protein of 10 µg/ml. Detection of the antigen was obtained through binding of a polyclonal antibody raised against the full length protein, followed by the addition of horseradish peroxidase conjugated secondary antibodies and enzymatic detection. A five-parameter logistics function with 1/x weighting was used for quantification within the concentration range of 4-256 pmol CYP2E1/mg microsomal protein. The method showed acceptable intra- and inter-assay precision, with calculated coefficients of variation of 6.3-15.2% and 11.3-21.0%, respectively. The relative error varied between -2.3 and 8.9%, and the total error between 16.0 and 27.2%. No significant cross reactivity with other abundant CYP isoforms was observed. The method was evaluated through the analysis of samples from a pharmacokinetic study, and the comparison with the CYP2E1 activity in those samples.

1 INTRODUCTION

Cytochrome P450 (CYP) enzymes are key enzymes in the metabolism of many endogenous compounds and numerous xenobiotics. One member of the CYP superfamily is the isoform CYP2E1, which has broad substrate specificity. Besides being the main enzyme in the biotransformation of organic solvents, it also metabolizes certain drugs and pre-carcinogens. CYP2E1 is reasonably conserved between mammalian species, probably due to its important endogenous role [1]. The enzyme is highly inducible by alcohol, and many of its substrates often are also inducers. Due to its important function in the biotransformation of xenobiotics, information on CYP2E1 may be needed in various types of studies in the fields of, amongst others, toxicology and pharmacokinetics. Changes in the enzyme in specific situations may occur on the level of mRNA expression, protein expression or enzyme activity. For example, Liddle et al investigated the effects of growth hormone on the mRNA expression, the protein level and the activity of CYP3A4 [2]. They observed a similar change in all three measures, and thus suggested that the changes occurred at a pretranslational level. The same factors were evaluated by George et al in patients with cirrhosis [3,4]. For some isoforms (CYP1A2, 3A, and 2C), a good correlation was observed between the three measures, indicating pretranslational alterations in liver diseases. For CYP2E1 however, there was no strong correlation between the mRNA and protein levels, suggesting both pre- and posttranslational effects of the disease. These examples show the importance of abundance measurements in the characterization of enzymes in a specific situation.

The CYP enzymes are mainly located in the liver and 96% of these enzymes are present in the subcellular fraction called microsomes [5]. Microsomes are easy to prepare and have an excellent long term stability [6,7]. Therefore, they are a good choice to perform abundance measurements. About 10% of the total CYP content of the liver consists of CYP2E1 [1,8]. In adults, an average abundance of 50 pmol CYP2E1/mg microsomal protein was detected. Several techniques can be used to determine CYP abundance. Western blotting is the most widely used technique, but it has some disadvantages, such as the time consuming process and the susceptibility to technical difficulties. Moreover, it provides only semi-quantitative data. In order to overcome these disadvantages, Snawder et al developed an indirect ELISA for the quantification of several CYPs in rat liver microsomes [8]. This method has a higher throughput and is easier to perform than a western blot. However, the described method is applied to rat samples, not to human samples, and a primary antibody raised against only a part of the protein is used. Consequently, some proteins may be missed during analysis, as it is unpredictable which part of the protein is available for antigen-antibody interaction after adsorption of the microsomally embedded antigen to the microplate.

In this article, we report the development and validation of an indirect enzyme-linked immunosorbent assay for the quantification of CYP2E1 in human liver microsomes, using a polyclonal primary antibody raised against the full length protein.

2 MATERIALS AND METHODS

2.1 Chemicals

Tween 20®, sodium chloride, potassium chloride, and sodium carbonate and bicarbonate were purchased from VWR (Leuven, Belgium), hydrochloric acid from Acros Organics (Geel, Belgium), and tris(hydroxy-methyl)aminomethane, and chlorzoxazone from Sigma-Aldrich (Buchs, Switserland). NADPH was obtained from Biopredic International (Rennes, France). All other chemicals were at least reagent grade.

2.2 Recombinant CYP enzymes

Microsomes prepared from insect cells infected with a virus engineered to express human CYP2E1 were used (2000 pmol rCYP2E1/ml, 8.4 mg microsomal protein/ml, BD Supersomes™, BD Gentest, Franklin Lakes, USA) to prepare calibrators and validation samples. Corresponding Control Supersomes™ (BD Gentest), i.e. microsomes prepared from the same type of insect cells but without expression of human CYP2E1, were used as negative controls (blanks) for the analysis. The final composition and concentrations of the calibrators were 256, 128, 64, 32, 16, 8, 4 and 2 pmol rCYP2E1/mg protein in a pH 9.4 carbonate-bicarbonate plating buffer. A final concentration of total microsomal protein of 10 µg/ml was obtained by adding Control Supersomes™, if necessary. The validation samples (VS), with final concentrations of 4, 10, 50, 130 and 256 pmol rCYP2E1/mg protein, were prepared similarly. For the cross-reactivity experiments, the Supermix Supersomes™ of BD Gentest were used. These were prepared from the same type of insect cells as described above, but which expressed human CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4.

2.3 Determination of CYP2E1 in human microsomes

Prior to plating one µg of microsomal protein/well in a black 96-well MaxiSorp® micro-titer plate (Nunc, Roskilde, Denmark), the microsomal samples were diluted to a concentration of 10 µg of microsomal protein/ml using a carbonate-bicarbonate buffer pH 9.4. Plates were incubated overnight at 4°C, after which the plating solution was removed. Subsequently, plates were washed 3 times by adding 300 µl wash buffer (Tris-buffered saline (TBS) with 0.05% (v/v) Tween 20®, pH 7.2), soaking during 2.5 minutes and aspiration of the buffer. Three hundred µl of StartingBlock™ blocking buffer in TBS with Tween 20® (Thermo Scientific, Rockford, USA) was added as a blocking agent, and plates were incubated for 1h at room temperature (±23°C). After aspiration of the

blocking buffer, plates were washed 3 times as described previously. The primary antibody, i.e. a polyclonal antibody raised in rabbit against the full-length human CYP2E1 protein (MaxPab® antibody, Abnova, Taiwan), was diluted in blocking buffer (1:1600). Hundred µl was added to each well, followed by incubation of the plates for 1h at 37°C. Primary antibody was removed and plates were washed as described above. Subsequently, 100 µl of secondary antibody dilution in blocking buffer (1:10000) goat anti-rabbit IgG conjugated with horse radish peroxidase (HRP) from Thermo Scientific (Rockford, USA) was added and plates were incubated at for 1 h at 37°C. Unbound secondary antibody was removed, plates were washed, and 100 µl of premixed HRP substrate (QuantaBlu™ Fluorogenic peroxidase substrate and peroxide, Thermo Scientific, Rockford, USA) was added to each well. The reaction was stopped after 60 minutes at 37°C with the stop solution from the QuantaBlu™ kit, and fluorescence was determined at an excitation wavelength of 320 nm, and emission at 405 nm (Ascent Fluoroscan, Thermo Scientific, Rockford, USA). Curve fitting and data analysis was performed using the Masterplex® Readerfit 2010 software (Hitachi, San Francisco, CA, USA) and Microsoft® Excel (v 2007). The calculated amounts of CYP2E1 were expressed in pmol/mg microsomal protein.

The antibodies were stored and handled following manufacturer's recommendations in order to guarantee optimal stability. The primary antibody was stored in small aliquots at -80°C in order to avoid repeated freeze-thawing. The reconstitution solution of the secondary antibody contained glycerol in order to prolong long term stability at -20°C. During the experiments, all solutions were stored on ice.

2.4 Method validation

Assay sensitivity, calibration model, linearity of dilution, spiking recovery, working range, intra and inter assay variability and precision, and cross-reactivity were evaluated prior to analysis of study samples.

For the evaluation of the calibration model, a curve was fitted for each of six independent assay runs. The suitability of the model was evaluated by analysis of the relative error (%RE) of the back-calculated calibration points within each run (%RE = 100 x (calculated concentration - nominal concentration)/nominal concentration). The %RE should be ≤20% (≤25% at LLOQ) for ≥75% of the calibrators within a curve. Furthermore, the mean %RE and mean %CV calculated from all runs (n=6) should both be ≤15% for each calibrator, except at the LLOQ where both should be ≤20% [9].

The mean response of 10 blank samples plus 3 standard deviations was calculated to determine the assay sensitivity. The concentration corresponding with this response was defined as the lowest concentration that could be distinguished from a blank sample, and

was used as the lower limit of the working range. The upper limit of the working range was defined based on literature information (based on naturally occurring CYP2E1 abundance in microsomes) and tested to comply with validation criteria. Linearity of dilution was evaluated by diluting a sample with a known concentration (500 pmol CYP2E1/mg protein) above the upper limit of the working range 1:3, 1:4 and 1:6. The recovery (%) of the observed concentration to the expected concentration should be within the 85-115% interval. A spiking recovery experiment was used to assess matrix effects. Hereto, samples were prepared at five concentration levels in both dilution buffer (carbonate-bicarbonate, pH 9.4) and in blank matrix (blank supersomes, final protein concentration of 10 µg/ml). The recovery (%) of the observed concentration to the nominal concentration was calculated and evaluated against the 85-115% interval in both matrices.

Intra assay and inter assay precision and accuracy were also evaluated according to the recommendations of and the statistical methods described by DeSilva et al [9]. The formulas used to perform this evaluation can be found at the end of the publication by De Silva et al. Validation samples were prepared in the sample matrix at five concentration levels: anticipated LLOQ, less than 3 times LLOQ, medium, high and anticipated upper limit of the working range. Three independent determinations were done each run, for a minimum of 6 runs. The intra assay precision was estimated by the coefficient of variation, obtained after dividing the pooled intrabatch standard deviation of measured concentration values from the calculated run means with the sample nominal concentration. The standard deviation needed for the calculation of the %CV for the inter assay precision was calculated by the method of analysis of variance (ANOVA). Method accuracy (%RE) was determined by the percent deviation of the weighted sample mean from the sample nominal concentration. The target limits were an intra assay and inter assay precision (%CV) and absolute value of the mean bias (%RE) ≤ 20% (25% at LLOQ). In addition, the total error of the method (= sum of %CV and absolute value of the %RE) should be ≤ 30% (40% at LLOQ).

Possible cross-reactivity with the most abundant CYP isoforms in liver microsomes was evaluated in 2 of the validation runs. In the first run, a mix of recombinantly expressed CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 (“Supermix”) was added at 4 concentration levels (zero - low – intermediate – high) to a sample with a fixed concentration of CYP2E1. In order to cover the complete range of known important and abundant CYPs, cross-reactivity with recombinant CYP2A6 and CYP2B6 (Corning® Supersomes™, Corning BV Life Sciences EMEA, Amsterdam, The Netherlands) was assessed in the second run. CYP2A6 was added at 4 concentration levels (zero - low – intermediate (and physiologically expected) – high), whereas CYP2B6 was only added in

a low and intermediate concentration, due to the high protein content of the Supersomes and the consequent impossibility to prepare a sample with a protein concentration of 10 µg/ml. The cross-reactivity was evaluated following MacFarlane et al [10], according to the equation:
[(observed concentration - control concentration)/ supplemented concentration] * 100, with observed concentration = calculated CYP2E1 concentration of the supplemented sample, control concentration = calculated CYP2E1 concentration of the sample with no other CYP isoform(s) added, and supplemented concentration = concentration of the supplemented CYP(s). Additionally, the calculated mean concentrations were compared with one-way ANOVA (using Microsoft Excel® 2007).

During sample analysis, in-study validation was performed within each run through the measurement of two validation samples, each of 3 concentration levels (less than 3 times LLOQ, medium and high). In order to accept the run, these results should meet the 4:6:30 rule [9], i.e. 4 out of the 6 VS should have a total error below 30%, and the 2 samples not meeting this requirement should not be at the same concentration level. Furthermore, ≥75% of the calibration points should have a %RE of ≤ ±15%.

2.5 Proof of concept: application of the method and CYP activity determination

Liver tissue samples were taken from the explanted livers from children undergoing liver transplantation, after obtaining written informed consent from the parent(s), as approved by the Ethics Committee of Ghent University Hospital (B67020084281). Microsomes were prepared following the method of Wilson et al [5] and subsequently analyzed with the validated method. Total protein content was estimated with the method of Bradford et al [11], and the microsomal suspensions were diluted to a final protein concentration of 10 µg/ml in the plating buffer (carbonate-bicarbonate, pH 9.4). For each of the samples, two wells were loaded with 100 µl of the dilution, and the average concentration in those two wells was considered the actual concentration of the samples.

Furthermore, CYP2E1 activity was determined through incubation of the microsomes with chlorzoxazone (CZ). The reaction was initiated by the addition of NADPH to the reaction mixture containing the microsomes, KCl, phosphate buffer and CZ. After 15 minutes, reactions were stopped using a mixture of formic acid, acetonitrile, and water (3:55:42 *(v:v:v)*), and the amount of hydroxy-chlorzoxazone (HCZ) was quantified using UPLC-MS/MS [12].

The relationship between the activity and abundance was evaluated graphically using locally weighted scatter plot smoothing (LOWESS). Moreover, the Pearson's correlation coefficient was calculated. All data analysis was performed using R® v.2.13 (R foundation for statistical computing, Vienna, Austria).

3 RESULTS AND DISCUSSION

Quantification of a specific CYP isoform in microsomes requires recombinant CYP enzymes (rCYP) for the preparation of calibrators. The rCYP2E1 enzymes used in this protocol were expressed in a baculovirus/insect cell system. This expression system has a high yield of functional CYP enzymes [13]. Moreover, it is capable to perform post-translational modifications, such as phosphorylation or O-linked glycosylation [14]. The obtained microsomes containing the enzyme are preferred over the purified enzyme, as purified rCYPs have a different conformation compared to the enzyme embedded in the endoplasmatic membrane [15].

The final protocol for the quantification of CYP2E1 in microsomes using indirect ELISA was obtained after the optimization of several parameters, such as primary and secondary antibody concentration, optimal amount of protein to load in each well, the choice of blocking buffer, incubation times and temperatures.

3.1 Choice of primary and secondary antibody

Cytochrome P450 enzymes are embedded in the membrane of the smooth endoplasmatic reticulum, and remain as such during the preparation of the microsomes. Upon adsorption of the antigen, i.e. the CYP enzyme, to the wall of the microplate, some parts will be available for interaction with the antibody. However, it is unpredictable which parts are exposed and thus available. Therefore, the use of a polyclonal antibody raised against the full length human protein would be favorable in order to increase the probability of antigen-antibody interaction.

3.2 Optimization of the protocol

Several parameters were optimized in order to achieve a background signal as low as possible, in combination with sufficient sensitivity. Previously published studies describe average concentrations of 50 pmol CYP2E1/mg protein, and a minimum amount of 11 pmol CYP2E1/mg protein [8]. Based on these observations, a sensitivity of about 5 pmol CYP2E1/mg protein was aimed for. As such, decreases of up to 50% in CYP2E1 abundance could still be detected.

The method for the evaluation of the assay sensitivity, i.e. the analysis of 10 blank samples, followed by the calculation of the concentration corresponding to the mean response plus 3 standard deviations, was used in order to determine the optimal conditions. Those conditions leading to the highest sensitivity were eventually chosen for validation. An assay sensitivity of 4 pmol CYP2E1/mg protein was obtained, thus allowing the detection of large decreases in CYP2E1 abundance compared to healthy adult individuals. Comparison of the sensitivity with other published methods for the

quantification of CYP2E1 is difficult, as often the method is described, but without the validation parameters. One study, published by Kornilayev et al [16], describes the method characteristics of the quantification of CYP2E1 tryptic peptides using both ELISA and western blot. The ELISA method shows comparable sensitivity to our method, whereas the sensitivity of the western blot method was 10 pmol CYP2E1/mg protein. The same issue arises when trying to compare the sensitivity of our method to previously published western blot methods: the validation parameters are seldom described. Moreover, due to the semi-quantitative nature of western blot methods, where often the amount of investigated protein is relatively expressed compared to a control sample or other sample, an absolute comparison of the sensitivity is difficult to perform.

In order to determine the optimal assay conditions, the most favorable concentrations of primary and secondary antibody were determined by a checkerboard titration experiment. Samples containing 3 concentrations of CYP2E1 (0, 5 and 100 pmol/mg protein) were analyzed using different combinations of primary antibody concentration (156, 312, 468, 625, 938, 1250 ng/ml) and secondary antibody concentration (50, 75, and 100 ng/ml, according to the manufacturers guidelines). The combination of 625 ng/ml of primary antibody, and 100 ng/ml of secondary antibody was further used. Furthermore, in order to increase sensitivity by decreasing the background signal, the antibodies (both primary and secondary) were diluted in Starting Block buffer, a commercially prepared buffer, instead of in a 2% BSA solution in the wash buffer (TBS with 0.05% Tween 20®, pH 7.2). The dilution in the blocking buffer most likely decreased non-specific binding. The optimal amount of protein to load in each well was evaluated by comparing the signals after plating 0.5 µg, 1 µg and 1.5 µg protein per well. One µg protein was chosen above 0.5 µg and 1.5 µg, which resulted in insufficient sensitivity and higher background signal, respectively. The incubation times and temperatures of the antibodies were also optimized, aiming for optimal sensitivity. Two types of commercially available blocking buffers were compared in order to aim for minimal non-specific binding. The highest precision was obtained by using the Starting Block buffer.

3.3 Validation

3.3.1 Calibration model

After the determination of the assay sensitivity as described above, the working range of the assay was selected based on previously determined (mean) concentrations of CYP2E1 in adults [8]. The lowest calibrator was determined to be 4 pmol CYP2E1/mg protein, the upper limit was chosen at 256 pmol CYP2E1/mg protein. Consequently, very low

abundances, corresponding with a 50% reduction of the lowest reported concentration, as well as rather high abundances can be measured.

The calibration model was selected based on the %RE of the back-calculated concentrations. Due to the clear asymmetry (compared to a 4-parameter logistics function) of the curve, a 5-parameter logistics (5-PL) was suggested (see Figure 1). Evaluation of the mean %RE in back-calculated concentrations showed the necessity of a 1/x weighting factor, in order to obtain sufficient accuracy at the lower concentrations. This model, a 5-PL function with 1/x weighting factor, was validated based on six independent assay runs. All requirements of %RE and %CV were met (see Table 1).

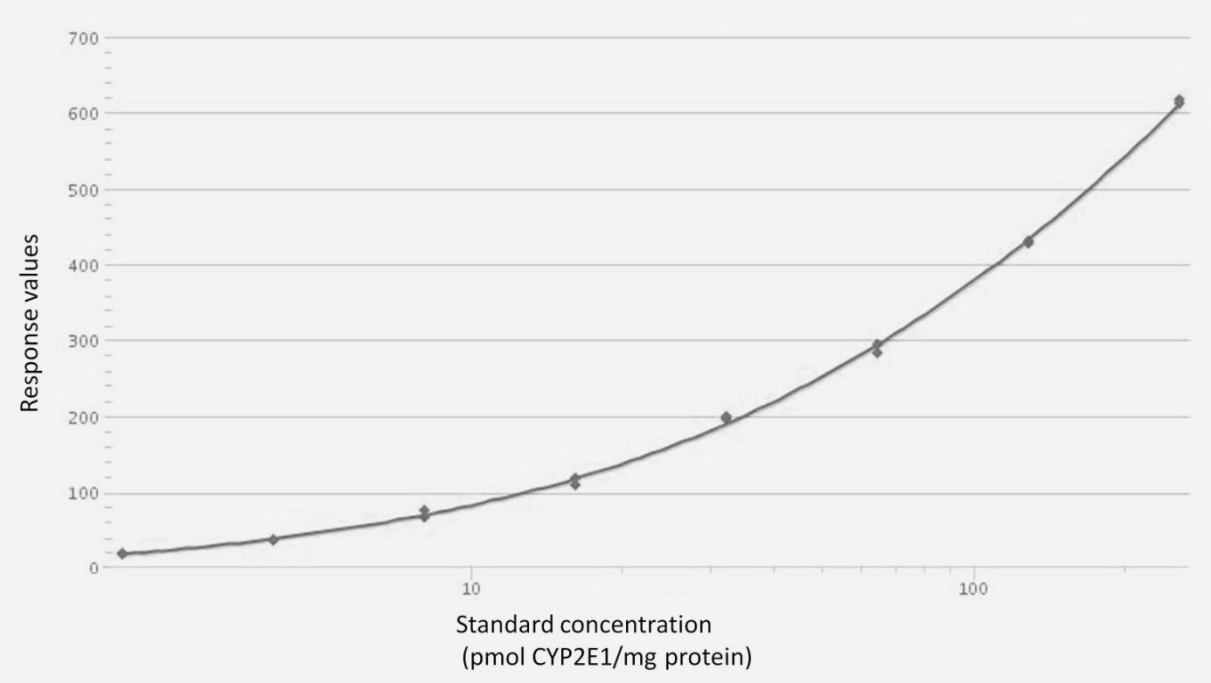

Figure 1: Representative 5-PL calibration curve with 1/x weighting factor.

3.3.2 Spike and recovery

A clear influence of the matrix proteins on the response was demonstrated by the spiking recovery experiment. As depicted in Table 2, the samples of rCYP2E1 diluted in plating buffer showed a low recovery, outside the 85-115% limits. In contrast, the samples diluted in blank matrix, thus having a final protein content of 10 µg/ml, showed a recovery within those limits. These results show that all calibrators and validation samples should be diluted in blank matrix, in order to have an equal final microsomal protein content in all samples (10 µg/ml). If this would not be done, and the calibrator samples would be diluted in a buffer without additional proteins, a low concentration in a sample would be severely overestimated, probably due to non-specific binding of the antibodies.

Table 1: Validation of the calibration model: %RE of the back-calculated concentrations was ≤20% (≤25% at LLOQ) for ≥75% of the calibrators within each batch run. The mean %RE and %CV calculated from all runs (n=6) were both be ≤15% for each calibrator (≤20% at LLOQ).

	Nominal concentration (pmol CYP2E1/mg protein)						
Batch run	**4**	**8**	**16**	**32**	**64**	**128**	**256**
1	-7.00	2.00	1.90	-3.00	1.70	-0.40	0.50
2	-8.80	4.50	-5.30	4.60	-0.20	-1.20	0.40
3	-10.0	8.30	-7.90	5.40	-2.90	1.00	0.80
4	-5.80	-2.40	2.80	1.00	-0.90	0.50	-0.40
5	-5.50	-2.80	-7.60	12.10	-3.80	-0.70	0.40
6	0.80	10.4	2.60	-1.90	-3.20	2.00	-0.70
Mean %RE	-6.00	3.30	-2.20	3.00	-1.50	0.20	0.20
%CV	4.00	5.20	5.30	5.40	2.10	1.20	0.50

Table 2: Spike-and-recovery experiment: samples diluted in plating buffer show poor recovery, especially around the LLOQ, all samples should have a total protein content of 10 µg/ml (dilution in blank matrix) in order to obtain a good recovery.

Nominal concentration (pmol CYP2E1/mg protein)	**Observed concentration** (pmol CYP2E1/mg protein)		**Recovery %**	
	Diluted in blank matrix	Diluted in plating buffer	Diluted in blank matrix	Diluted in plating buffer
4	3.68	2.93	91.9	73.3
10	11.6	10.1	116	101
50	52.9	47.9	106	95.7
100	101	90.0	101	89.9
130	131	122	101	94.1

3.3.3 Dilution experiment

As CYP2E1 is a highly inducible enzyme, high amounts of CYP2E1 can be expected. Increasing the upper limit of the working range was not possible due to the total protein content of the calibrators, which could not exceed 10 µg/ml. Therefore, a dilution experiment was set up in order to define the approach for the determination of samples with a concentration above the upper limit of the working range (256 pmol CYP2E1/mg protein). The recoveries of the 3-fold, 4-fold, and 6-fold dilutions in blank matrix of a sample with a high CYP2E1 concentration are shown in Table 3. After multiplication of the observed concentration with the dilution factor, all samples were within the 85-115% interval of the nominal concentration. Thus, dilution of samples with a concentration

above the upper limit of quantification will provide reliable quantitative measurement results.

Table 3: Dilution of a sample with a concentration of CYP2E1 above the upper limit of quantification of 256 pmol CYP2E1/mg protein.

Dilution Factor (DF)	**Observed concentration** (pmol CYP2E1/mg protein) x DF	**Recovery %**
3	454	90.8
4	447	89.3
6	480	96.0

3.3.4 Accuracy and precision

The results of the accuracy and precision evaluation are depicted in Table 4. The intra and inter assay precisions determine the variability of the results for the same sample analyzed under repeatability conditions and the intermediate precision, respectively. The assay precision was evaluated at 5 concentration levels in 6 independent assay runs, with n=3 within each run. The coefficient of variation did not exceed 20% (25% at the LLOQ) for the intra assay precision (6.3 – 15.2%RSD), as well as for the intermediate precision (11.3 – 21.0%RSD), except for the 50 pmol CYP2E1 sample, where a minor deviation of the limit was seen. The assay accuracy (expressed as the %RE) was determined using the same 6 assay runs. The absolute value of the mean bias did not exceed the limit of 20% (25% at the LLOQ), indicating a good accuracy. Moreover, the assay's total error was below 27.2 % at all concentration levels.

Table 4: Accuracy and precision evaluation

		Nominal concentration (pmol CYP2E1/mg protein)				
Characteristic	**Statistic**	**4**	**10**	**50**	**130**	**256**
# Results	N	18	18	18	18	16
Accuracy	Mean bias (%RE)	0.9	8.9	6.0	4.7	-16.4
Precision	Intra assay(%CV)	15.2	6.3	7.0	6.4	6.8
	Inter assay (%CV)	19.7	16.5	21.0	11.3	9.8
Total error (Accuracy + Precision)	|Mean bias| + Inter assay %CV	20.5	25.4	27.2	16.0	26.2

3.3.5 Cross reactivity

The homology in amino acid sequence between CYP isoforms from the same family (>40%) or subfamily (>55%) may lead to cross-reactivity of the primary antibody. The selectivity of the primary antibody was tested through the addition of CYP isoforms (CYP2A6, CYP2B6, CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4), selected based on their high abundance or high importance in drug metabolism [1]. Table 5 summarizes the results from the cross-reactivity experiments. The percentage of cross reactivity appears to be quite high at low supplemented concentrations of CYP2B6 (25%). At a higher and physiologically more relevant concentration, a much lower % cross-reactivity was calculated (13.5%). As the same trend in results was observed for the cross-reactivity with the Supermix, this probably rather reflects the uncertainty of the measurement at the different concentration levels, than cross-reactivity. This is corroborated by the mean observed CYP2E1 concentration which is, irrespective of the supplemented concentration of cross reactant, always basically equal. Also, the recovery was within the 85-115% interval of the concentration of the non-supplemented CYP2E1 sample for all the samples. Consequently, the method showed sufficient selectivity towards the detection of CYP2E1 in the presence of other abundant CYP isoforms, as shown in Table 5.

3.4 Application of the method

The validated method for the quantitative determination of CYP2E1 was applied for the analysis of samples from an ongoing PK study. Additionally, the CYP2E1 activity was determined through the evaluation of the chlorzoxazone hydroxylase activity. Figure 2 shows a weak correlation (Pearson's correlation = 0.371) between the two variables after logarithmic transformation. Protein alterations in the studied pharmacokinetic situation can consequently not solely be attributed to pre-translational changes.

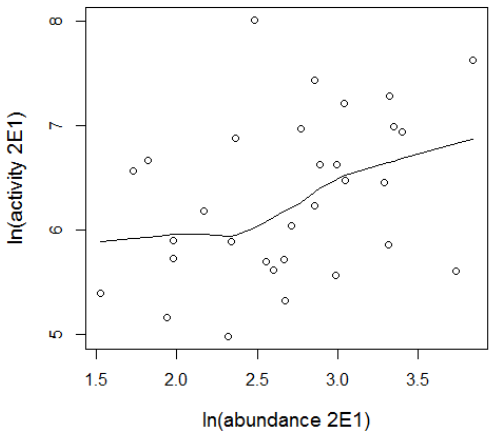

Figure 2: Correlation of CYP2E1 abundance as determined by ELISA with the chlorzoxazone hydroxylase activity: protein levels were weakly correlated with CZ-OH activity.

Table 5: Cross-reactivity with CYP2A6, CYP2B6 and the BD Supermix®, containing human CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4. Cross-reactivity was calculated as: [(observed concentration - control concentration)/ supplemented concentration] * 100, with observed concentration = calculated CYP2E1 concentration of the supplemented sample, control concentration = calculated CYP2E1 concentration of the sample with no other CYP isoform(s) added, and supplemented concentration = concentration of the supplemented CYP(s).

	Supplemented concentration (pmol CYP/mg protein)	Mean observed concentration CYP2E1 (pmol/mg protein) (± SD)	% cross reactivity	% recovery
Run 1				
CYP2E1		52.7 ± 2.2		
Supermix	20	47.9 ± 2.4	-23.9	90.9
	50	47.6 ± 2.7	-10.1	90.4
	350	46.0 ± 3.1	-1.91	87.3
Run 2				
CYP2E1		53.1 ± 1.6		
CYP2A6	10	53.7 ± 2.5	5.39	101
	50	57.1 ± 4.8	7.96	107
	250	57.9 ± 2.0	1.91	109
CYP2B6	10	55.6 ± 2.3	25	105
	50	59.9 ± 4.2	13.5	113

4 CONCLUSION

This article describes the development and full validation of an indirect ELISA for the quantification of CYP2E1 in human liver microsomes in a concentration range of 4 and 256 pmol CYP2E1/mg microsomal protein. The method was proven to be accurate and precise. The spiking recovery experiment showed the importance of an equal concentration of total protein in all samples, validation samples and calibrators. The polyclonal antibody against the full length protein showed acceptable cross-reactivity with the other abundant CYP isoforms. Analysis of samples from a pharmacokinetic study showed the suitability of this ELISA in the quantification of CYP2E1. We conclude that a valuable alternative to Western blot analysis has been presented, appropriate for use in various fields of research, e.g. toxicology and pharmacokinetics.

5 REFERENCES

[1] Ingelman-Sundberg M. Human drug metabolising cytochrome P450 enzymes: properties and polymorphisms. *Naunyn Schmiedebergs Arch Pharmacol* 2004; 369(1):89-104.

[2] Liddle C, Goodwin BJ, George J, Tapner M, Farrell GC. Separate and Interactive Regulation of Cytochrome P450 3A4 by Triiodothyronine, Dexamethasone, and Growth Hormone in Cultured Hepatocytes. *Journal of Clinical Endocrinology & Metabolism* 1998; 83(7):2411-2416.

[3] George J, Murray M, Byth K, Farrell GC. Differential Alterations of Cytochrome-P450 Proteins in Livers from Patients with Severe Chronic Liver-Disease. *Hepatology* 1995; 21(1):120-128.

[4] George J, Liddle C, Murray M, Byth K, Farrell GC. Pre-translational regulation of cytochrome P450 genes is responsible for disease-specific changes of individual P450 enzymes among patients with cirrhosis. *Biochem Pharmacol* 1995; 49(7):873-881.

[5] Wilson ZE, Rostami-Hodjegan A, Burn JL, Tooley A, Boyle J, Ellis SW *et al.* Inter-individual variability in levels of human microsomal protein and hepatocellularity per gram of liver. *Br J Clin Pharmacol* 2003; 56(4):433-440.

[6] Yamazaki H, Inoue K, Turvy CG, Guengerich FP, Shimada T. Effects of freezing, thawing, and storage of human liver samples on the microsomal contents and activities of cytochrome p450 enzymes. *Drug Metab Dispos* 1997; 25(2):168-174.

[7] Pearce RE, McIntyre CJ, Madan A, Sanzgiri U, Draper AJ, Bullock PL *et al.* Effects of freezing, thawing, and storing human liver microsomes on cytochrome P450 activity. *Arch Biochem Biophys* 1996; 331(2):145-169.

[8] Snawder JE, Lipscomb JC. Interindividual variance of cytochrome P450 forms in human hepatic microsomes: Correlation of individual forms with xenobiotic metabolism and implications in risk assessment. *Regul Toxicol Pharmacol* 2000; 32(2):200-209.

[9] DeSilva B, Smith W, Weiner R, Kelley M, Smolec JM, Lee B *et al.* Recommendations for the bioanalytical method validation of ligand-binding assays to support pharmacokinetic assessments of macromolecules. *Pharm Res* 2003; 20(11):1885-1900.

[10] MacFarlane GD, Scheller DG, Ersfeld DL, Shaw LM, Venkatarmanan R, Sarkozi L *et al.* Analytical validation of the PRO-Trac II ELISA for the determination of tacrolimus (FK506) in whole blood. *Clin Chem* 1999; 45(9):1449-1458.

[11] Bradford MM. Rapid and Sensitive Method for Quantitation of Microgram Quantities of Protein Utilizing Principle of Protein-Dye Binding. *Anal Biochem* 1976; 72(1-2):248-254.

[12] De Bock L, Boussery K, Colin P, De Smet J, T'jollyn H, Van Bocxlaer JFP. Development and validation of a fast and sensitive UPLC-MS/MS method for the quantification of six probe metabolites for the in vitro determination of cytochrome P450 activity. *Talanta* 2012; 89:209-216.

[13] Asseffa A, Smith SJ, Nagata K, Gillette J, Gelboin HV, Gonzalez FJ. Novel Exogenous Heme-Dependent Expression of Mammalian Cytochrome-P450 Using Baculovirus. *Arch Biochem Biophys* 1989; 274(2):481-490.

[14] Yun CH, Yim SK, Kim DH, Ahn T. Functional expression of human cytochrome P450 enzymes in Escherichia coli. *Curr Drug Metab* 2006; 7(4):411-429.

[15] Waterman MR. Heterologous Expression of Cytochrome-P-450 in Escherichia-Coli. *Biochem Soc Trans* 1993; 21(4):1081-1085.

[16] Kornilayev BA, Alterman MA. Utility of polyclonal antibodies targeted toward unique tryptic peptides in the proteomic analysis of cytochrome P450 isozymes. *Toxicology in Vitro* 2008; 22(3):779-787.

CHAPTER 5

GENOTYPING THE IMPORTANT SINGLE NUCLEOTIDE POLYMORPHISMS (SNPS) OF CYP2C9, 2C19 AND 2D6

"A four-letter alphabet called DNA."

- Matt Ridley

ABSTRACT

The polymorphic nature of many of the CYP isoforms leads to large interindividual differences in drug clearance and respons. Single nucleotide polymorphisms (SNPs) are the most common genetic mutations and may lead to altered activity, either abolished or reduced, or increased. CYP2C9, 2C19, and 2D6 belong to the Class II CYP isoforms, which are highly polymorphic. Based on the frequency in the Caucasian population and on the significant effect on the enzyme activity, the following SNPs were studied: CYP2C9*2, 2C9*3, 2C19*2, 2C19*17, 2D6*4, 2D6*10, 2D6*41. A protocol for the analysis of these SNPs was set up using well established methods. After the extraction of the DNA from liver tissue samples with a Qiagen QIAamp DNA Mini kit, it was quantified using the Picogreen® assay. TaqMan® genotyping assays were performed in order to detect the presence of the SNPs, and the patients were consequently characterized as homozygous wild type, heterozygous or homozygous SNP. The analysis of the patient samples was shown to be reliable, as the observed minor allele frequencies were similar to previously reported frequencies based on the analysis of large populations.

1 INTRODUCTION

Polymorphisms of drug-metabolizing genes have by far the largest role in interindividual differences in drug response and drug clearance, and are highly relevant in the consequent adverse drug reactions [1,2]. Genetic polymorphism is defined as a stable variation in a given locus of the genetic sequence, which is detected in 1% or more of a specific population [3]. The cytochrome P450 (CYP) enzymes, key drug-metabolizing enzymes, have a polymorphic nature. The most common genetic mutations seen in human CYP genes are single nucleotide polymorphisms (SNP). The functionally important SNPs are the non synonymous SNPs, as they occur in a coding region and cause amino-acid changes in the corresponding enzyme [3]. The mutations can cause enzymes with abolished, reduced, altered or increased activity [2]. A population can be divided based on four phenotypes: poor metabolizers (PM), intermediate metabolizers (IM), extensive metabolizers (EM), and ultra rapid metabolizers (UM). The distribution of these phenotypes in a population is ethnicity dependent [3].

The main CYP enzymes can be divided into two classes [4]:

- *Class I*, composed of CYP1A1, CYP1A2, CYP2E1 and CYP3A4: these isoforms are well conserved, do not have important functional polymorphisms, and are active in the metabolism of pre-carcinogens and drugs.
- *Class II*, composed of CYP2B6, CYP2C9, CYP2C19 and CYP2D6, which are highly polymorphic and active in the metabolism of clinically important drugs, but not of pre-carcinogens.

In order to uniformly name the alleles throughout the scientific community, a website was established, where information and recommended nomenclature of various allelic forms of P450s are continuously updated. The site also contains a link to relevant literature references and the dbSNP database (http://www.cypalleles.ki.se/).

Different methods for genotyping are available, amongst which restriction fragment length polymorphism (RFLP) analysis, denaturing high-performance liquid chromatography (dHPLC), mass spectrometry, microarrays, allele-specific amplification using real-time PCR, and whole-exome sequencing [5,6]. RFLP analysis is one of the hallmark methods to identify known mutations. It has some major drawbacks, however, as it is labor-intensive, costly and not suitable for large-scale clinical applications. dHPLC is a rapid, cost-effective and accurate method for the identification of known and unknown sequence variations in PCR products. Mass spectrometry, more specific matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), also provides a sensitive method for the detection of polymorphisms. These latter two

methods, however, require specialized instruments. Other methods generating a large amount of data on multiple mutations from several genes within one experiment, are the microarray or whole-exome sequencing. Despite this major advantage, the expenses of the special equipment required for this technology are often limiting factors for the implementation of the methods. The rapid evolution in these approaches, however, could lead to a reduction on the cost per sample in the near future.

Also, most of the previous methods all require a post-PCR analysis, whereas this is not necessary in allele-specific amplification methods, such as TaqMan™ DNA probes. In this method, the DNA region containing the SNP is amplified during PCR. Two probes that differ only at the polymorphic site, and are thus specific for one or the other allele, are also incorporated in the reaction. At the 5' end of the probe a specific reporter dye is attached, and a quencher dye is covalently attached to the 3' end. Thus, the fluorescence of the reporter dye is quenched. The probes anneal specifically to the complementary sequence between the forward and reverse primer sites. The DNA polymerase extends the primers bound to the genomic DNA template, and cleaves the probes that are hybridized to the target. Cleavage of the probes separates the reporter dye from the quencher dye, which results in an increased fluorescence of the reporter. As the reporter dyes attached to the two primers have different fluorescence signals, the signals generated after PCR amplification indicate which alleles are present in the sample. As such, the genotype of the patient sample can be determined. The advantages of this method are the ease to perform the experiment, as (1) no post-PCR processing is necessary, (2) ready-to-use, validated assays are available, and (3) this technology can easily be automated to increase throughput.

In the light of a pharmacokinetic study, we aimed to establish a workflow for the genotyping of the patient samples, in order to add an extra variable in the evaluation of the CYP activity.

2 MATERIALS AND METHODS

2.1 Samples

Liver tissue samples were taken from the explanted livers of 31 children undergoing a liver transplantation at Ghent University Hospital (Ethical Committee B67020084281) and Saint-Luc Clinics Brussels.

2.2 DNA extraction from liver tissue

DNA was extracted from the liver tissue using the QIAamp DNA Mini kit from Qiagen (Santa Clarita, CA, USA). The composition of the buffers in this kit is proprietary

information. About 25 mg of liver tissue was excised from the sample and was cut up into small pieces. The minced tissue was put into 180 µl of Buffer ATL and 20 µl of Proteinase K (>600 mAU/ml). After vortex mixing, the sample was incubated overnight at 56°C in a shaking heating block. Four µl of RNase A (100 mg/ml) was added, the sample was vortex mixed and incubated for 2 minutes at room temperature (RT). Buffer AL was added, followed by incubation at 70°C for 10 min. Two hundred µl of ethanol (96-100%) was added, and the entire mixture was applied onto a QIAmp Mini spin column. After centrifugation at 6000*xg* for 1 min, the filtrate was discarded. Subsequently, 500 µl Buffer AW1 was added onto the column, the sample was centrifuged (6000*xg* for 1 min), and the filtrate was again discarded. The same procedure was then repeated using Buffer AW2. Elution buffer, Buffer AE, was applied on the column (200 µl) and incubated for 5 min at RT. The genomic DNA was recovered after centrifugation for 1 min at 6000*xg*. In order to increase the DNA yield, the elution step was repeated. Both eluates were pooled, and the DNA was stored at -20°C.

2.3 Quantification of DNA

The concentration of DNA was determined with a PicoGreen® dsDNA (double-stranded DNA) assay kit (Invitrogen, Life Technologies, Carlsbad, CA, USA). In short, calibrators containing 0 to 30 ng dsDNA were prepared, and all patient DNA samples were diluted 1:5, 1:10 and 1:50 in 1X Tris-EDTA (TE) buffer. Forty-nine µl of 1X TE was added into the wells of a black 384-well plate (Greiner Bio-One, Frickenhausen, Germany). Subsequently, 1 µl of sample (undiluted or diluted) or calibrator was added to the TE in the appropriate well. After the addition of 50 µl of PicoGreen® reagent to each well, the fluorescence was determined at an excitation wavelength of 480 nm, and emission at 520 nm (Magellan™ v6 and Safire^2, Tecan, Männedorf, Switserland).

2.4 SNP analysis using TaqMan probes

The samples were genotyped using TaqMan® Drug Metabolism Genotyping Assays from Applied Biosystems (Life Technologies, Carlsbad, CA, USA). DNA samples were diluted to a concentration of 10 ng/2.25 µl, and for each assay, 2.25 µl of the dilution was added to a well of a black Corning® Thermowell GOLD 384-well PCR plate (Corning, Tewksbury, MA, USA). Moreover, three no-template controls (NTC) were plated for each assay. A reaction mix was prepared for each of the assays, containing TaqMan® Universal PCR Master Mix (2X) and the SNP working solution. The Master Mix consists of AmpliTaq Gold® DNA Polymerase, a blend of dNTPs with dTTP/dUTP and Uracil-DNA Glycosylase (UDG) to minimize carry-over PCR contamination, and a passive internal reference based on the ROX™ dye. The working solution contains: (a) 2 unlabelled PCR primers, forward and reverse (primers at 900 nM final concentration); (b) 1 VIC® dye – MGB (minor

groove binder) labeled probe detects the Allele 1 sequence (probes at 200 nM final concentration); (c) 1 FAM™ dye – MGB labeled probe detects the Allele 2 sequence (probes at 200 nM final concentration). The evaluated SNPs are depicted in Table 1. A volume of 2.75 µl of the reaction mix was added to the appropriate wells. After centrifugation of the plate, PCR (GeneAmp® PCR System 9700 Thermal Cycler, Applied Biosystems) was performed. Cycling conditions were an initial denaturation at 95°C for 10 min, followed by 50 cycles of denaturation at 92°C for 15 sec, and annealing and extension at 60°C for 90 sec. Upon termination of the PCR process, the fluorescence of the microplate is measured (Tecan Safire², with Magelan v6.3 software) at 3 pairs of excitation and emission wavelengths: (1) 538 – 554 nm for VIC®; (2) 494 – 522 nm for FAM™; and (3) 587 – 607 nm for ROX™. The IS corrected fluorescence signals, VIC®/ROX™ and FAM™/ROX™, were used for further data analysis.

Table 1: Evaluated SNPs

Allele	Nucleotide change (gene)	NCBI dbSNP identification numbers
CYP2C9*2	3608C>T	rs1799853
CYP2C9*3	42614A>C	rs1057910
CYP2C19*2	19154G>A	rs4244285
CYP2C19*17	-806C>T	rs12248560
CYP2D6*4	1846G>A	rs3892097
CYP2D6*10	100C>T	rs1065852
CYP2D6*41	2988G>A	rs28371725

Data analysis was performed with Microsoft® Excel (v 2007). The normalized intensity of the reporter dyes of each individual sample were plotted on an allelic discrimination plot. The clusters (homozygote VIC®, homozygote FAM™, or heterozygote) were determined graphically, and each sample was assigned to a particular cluster.

3 RESULTS AND DISCUSSION

3.1 DNA extraction from liver tissue

DNA can be extracted from blood or from tissue samples. In order to minimize the burden for the patients, no blood samples were taken for the study on the PK of drugs in children with liver disease. The liver samples, considered surgical waste, were therefore the only available source of DNA. Commercially available kits, from which the composition of the buffers is unfortunately proprietary information, offer a straightforward procedure to extract DNA from any tissue sample. Following the manufacturer's guidelines, about 25 mg of liver tissue from each patient (mean 26.27

±2.27 mg) was used. The amount of DNA was quantified using Picogreen, a fluorochrome that selectively binds double stranded DNA (dsDNA). Upon binding to the dsDNA, the Picogreen causes a high fluorescence enhancement. As the unbound dye has virtually no fluoresce, little background occurs [7]. A typical calibration curve is shown in Figure 1. The average yield of DNA after extraction of the patient liver samples was 1.12 ± 0.47 µg DNA/mg tissue.

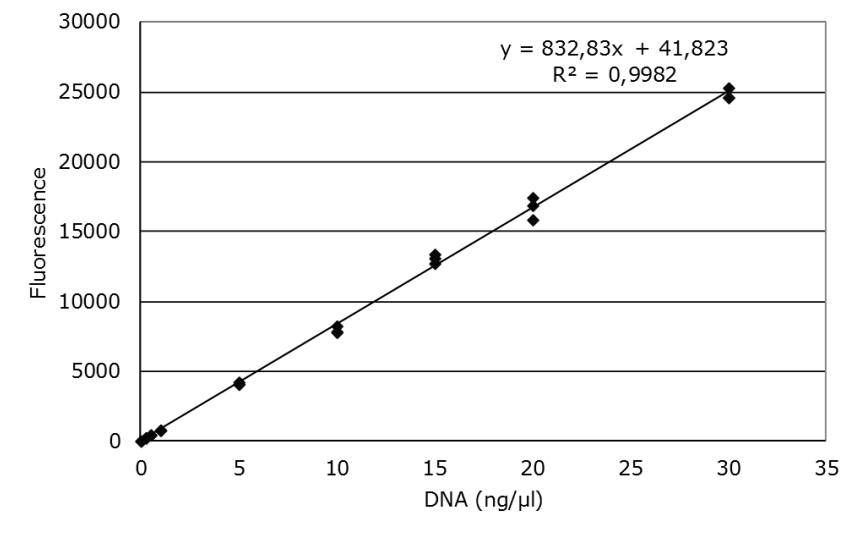

Figure 1: Typical calibration curve (range 0 – 30 ng/µl) for the quantification of DNA using the Picogreen dsDNA kit.

3.2 Selection of relevant SNPs

Our study contained 3 *Class II* CYPs (CYP2C9, 2C19, and 2D6) for which SNPs needed to be assessed. The available literature on CYP polymorphisms was consulted in order to determine the relevant SNPs for our patient population, i.e. Caucasians. Those SNPs were selected that have a significant prevalence in the Caucasian population, as well as a significant influence on the enzyme activity. Evidently, many other SNPs may occur within the genotype of the patients, but those often have a very low frequency of occurrence. Moreover, many SNPs, some even with high frequency, have been shown to leave CYP activity practically unaltered. A summary of the SNPs, their frequency and influence on the activity is given in Table 2.

3.2.1 CYP2C9

To date, 44 haplotypes of CYP2C9 have been identified (http://www.cypalleles.ki.se/, last update 13-nov-2013). In addition, there are other SNPs for which the haplotype has not yet been determined. The most common CYP2C9 allelic variants in Caucasians are CYP2C9*2 and *3. Other mutations or haplotypes were described as being rare [8].

Table 2: Allelic frequency in the Caucasian population and effect of the SNP on the enzyme activity.

Isoform	Allele	Polymorphism	Allele frequency (%)	Enzyme activity
CYP2C9	*2	3608C>T	8 - 19	decreased
	*3	42614A>C	3.3 - 16.2	decreased
CYP2C19	*2	19154G>A	13 - 15	null
	*17	-806C>T	18	increased
CYP2D6	*4	1846G>A	12 - 25	null
	*10	100C>T	2 - 6	decreased
	*41	2988G>A	8.4	decreased

The nonsynonymous 3608C>T mutation in the CYP2C9*2 allele causes an Arg144Cys exchange, resulting in a decrease in enzyme activity of 20-30% towards CYP2C9 substrates [3]. About 1% of the Caucasians are homozygous carriers, 22% are heterozygous carriers [9]. Xie et al. performed a quantitative meta-analysis in order to define the population frequencies of CYP2C9*2 variant alleles. The CYP2C9*2 variant was shown to be absent in East Asian populations, whereas Ethiopian and African-Americans carry CYP2C9*2 with an overall allele frequency of 3.2%. The allele frequencies reported in Caucasians show a high heterogeneity of 8% to 19%, as confirmed by the reported allele frequencies by Sullivan-Klose et al. and Yasar et al. of 8% and 10.7%, respectively [9,10].

The CYP2C9*3 allele is characterized by the 42416A>C SNP, causing an Ile359Leu exchange. The reduction in activity caused by the CYP2C9*3 allele can be up to 70%. This allele is less frequent than the *2 allele, but still 0.4% and 15% of Caucasians are homozygous or heterozygous carriers, respectively. Similar to the allele frequency of *2, a large heterogeneity was seen in the allele frequency of *3 in Caucasians (3.3 – 16.2 %). Lower frequencies were described in East Asian populations (1.1-3.3%) and Blacks (1.3%) [8].

For both alleles (*2 and *3), the loss in activity is possibly due to enzyme conformational changes that reduce the enzyme's ability to bind substrates [3]. Haining et al. suggested it is more likely that the consequence of the mutation in CYP2C9*3 is a change in catalytical activity, rather than a gross alteration in substrate orientation, as the mutation is situated in the putative, distal substrate binding domain [11]. The decrease in *in vitro* activity of CYP2C9 due to these alleles has been described by several studies [11-13]. Moreover, many studies were conducted to assess the effect of common CYP2C9 SNPs on the clinical use of CYP2C9 substrates, such as the anticoagulant warfarin [13,14], the anticonvulsant phenytoin [15], and the angiotensin II receptor

antagonist losartan [16]. These results, and the influence of CYP genotype on the PK and PD of other frequently used drugs, such as NSAIDs, torasemide (diuretic), and fluvastatin, have been reviewed comprehensively [3,17].

3.2.2 CYP2C19

Up till now, 48 allelic variants of CYP2C19 have been described (http://www.cypalleles.ki.se/, last update 27-may-2013). CYP2C19*2 and *3 are the two genetic defects responsible for the majority of PMs for CYP2C19 [18], but the *3 variant is extremely rare in Caucasians (allele frequency of 0.04%) [19]. The *2 variant accounts for 75-85% of the CYP2C19 PMs in Caucasians. Allele frequencies of 13-15% were described for *2 [18,20]. The other variants responsible for the PMs are *4, *5, *6, *7, and *8 [18]. About 1-8% of the Caucasian population is CYP2C19 PM. The substitution of 19154G>A (gene, 681G>A cDNA) in exon 5 in the *2A variant causes a splicing defect, leading to a premature stop codon [21]. The subvariants *2B and *2C also carry additional SNPs [22]. CYP2C19*2A, *2B and *2C are null alleles resulting in a total loss of activity, due to the formation of a truncated protein [21,23].

The large interindividual variation in CYP2C19 activity is not only due to the existence of the previously described alleles causing loss of protein activity. Sim et al. described the presence of a CYP2C19 gene variant causing ultra rapid drug metabolism [24]. The CYP2C19*17 allele is characterised by a Ile331Val exchange, due to the presence of the -806C>T SNP in the 5'-flanking region of the gene. This SNP results in an increased transcription of the CYP2C19 gene, and a consequent rapid metabolism of CYP2C19 substrates. An allele frequency of 18% was described in Caucasians [24].

Approximately 10% of the commonly used drugs are metabolized by CYP2C19 [3,17,25]. The clinical implications of the CYP2C19 genotype are elaborately reviewed previously [3,17,25]. Proton pump inhibitors are a widely used group of drugs mainly metabolized by CYP2C19. The influence of both PM [26] and UM [27] metabolism on omeprazole kinetics has been extensively described. Other commonly used drugs studied in this context are citalopram [28] and the antiplatelet therapy with clopidogrel (UM [29], and PM [30]).

3.2.3 CYP2D6

A considerable variation in CYP2D6 activity occurs within a population. The interindividual variation in enzyme expression and activity is largely the responsibility of genetic variation, as, unlike other CYPs, CYP2D6 is not inducible [3]. The website of the human cytochrome P450 allele nomenclature committee (http://www.cypalleles.ki.se/, last update 27-may-2013) reports a large list of variants and subvariants (from *1A to

*105). Due to these mutations, four distinct metabolizer types of CYP2D6 (PM, IM, EM, and UM) can be distinguished in each population due to a complete absence of enzyme activity, reduced activity, normal activity, or increased activity. Besides a large number of low-frequency alleles, the most important variants of CYP2D6 associated with the PM phenotype are *2, *3, *4, *5, *10, *17, and *41.

Seven to ten percent of the Caucasian population is a poor metabolizer for CYP2D6 [31,32]. CYP2D6*2 and *4 account for 80% of all allelic variants [33]. The *2 variant results in a functional protein with an activity comparable to that of the wild type enzyme, and was consequently not analyzed in our study. The 1846G>A substitution in the *4 allele results in an incorrectly spliced primary transcript, leading to a truncated protein with complete loss of activity [34,35]. CYP2D6*4 is by far the most frequent null allele in Caucasians, with allelic frequencies of 12 to 25% in Caucasians [31,36]. About 70-90% of the PMs can be explained by this variant [33,37,38].

The 100C>T nucleotide change associated with the *10 variant leads to a Pro34Ser exchange. This results in a very unstable enzyme with abnormal folding and reduced affinity for the substrates [39]. The metabolic capacity of the enzyme is situated between the EM and PM type, and individuals carrying this mutation were therefore classified as IM. This variant is very common in Asian populations (33-50%), but rarer in Caucasians. Despite its low frequency (~2-6% [36]), this SNP was selected for investigation, as it accounts for 10-20% of individuals with IM phenotype.

Another variant leading to the IM phenotype is the CYP2D6*41 allele, which can be specifically identified by genotyping for 2988G>A [40]. This mutation is involved in a low expression of CYP2D6 in affected individuals, due to a complex effect on splicing of the gene [41,42]. The analysis of this SNP with an allele frequency of about 8.4% in Caucasians, an additional 60% of the IM in Caucasians can be explained [40].

Due to the existence of many polymorphisms and different metabolizing types, significant differences in the pharmacokinetics and – dynamics of drugs metabolized by CYP2D6 have been described [3,17]. The drug classes that could be affected by these polymorphisms are, amongst others, antidepressants (tricyclic, SSRIs, and others), a variety of antipsychotics, and anti-arrhythmic drugs.

3.3 Allelic determination

The individual samples were classified into one of the three groups (homozygous for allele 1, homozygous for allele 2, or heterozygous) for each of the studied CYPs based on the allelic discrimination plots. The presence of an individual data point in one of the three clusters enabled the classification of the sample.

The validity of the analysis was examined through the calculation of the allele frequencies within our patient population, and by comparing the result with the frequencies reported in the manufacturer's specification sheet. As shown in Table 3, the calculated minor allele frequencies in our patient samples were comparable to the expected frequencies.

Table 3: Minor allele frequencies of the studied SNPs in our patient population

Isoform	Allele	Minor allele frequency	Allele frequency in patient samples
CYP2C9	*2	0.17	0.065
	*3	0.10	0.081
CYP2C19	*2	0.14	0.194
	*17	0.17	0.194
CYP2D6	*4	0.19	0.242
	*10	0.21	0.242
	*41	0.11	0.097

The 31 patient samples were analyzed for the presence or absence of the 7 previously discussed SNPs. In Figures 2(a) to 2(g), the allelic discrimination plots are depicted. Interpretation of the graphs resulted in the classification of the patients in one of the clusters as summarized in Table 4.

Table 4: Classification of the 31 patients for the studied SNPs

Isoform				
CYP2C9		wt/wt	wt/*2	*2/*2
	n	27	4	0
		wt/wt	wt/*3	*3/*3
	n	26	5	0
CYP2C19		wt/wt	wt/*2	*2/*2
	n	20	10	1
		wt/wt	wt/*17	*17/*17
	n	20	10	1
CYP2D6		wt/wt	wt/*4	*4/*4
	n	19	9	3
		wt/wt	wt/*10	*10/*10
	n	19	9	3
		wt/wt	wt/*41	*41/*41
	n	25	6	0

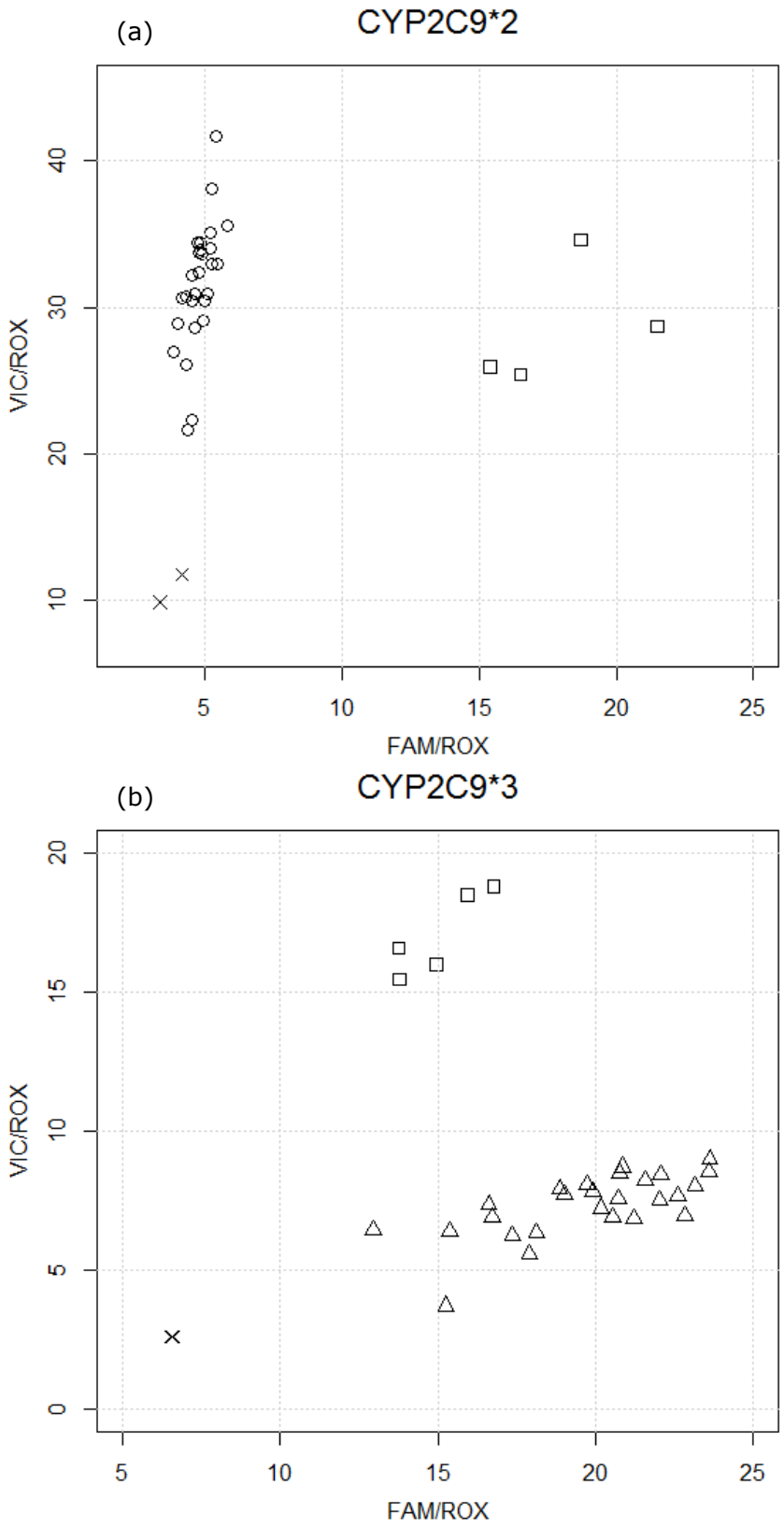

Figure 2(a) to (g): Allelic discrimination plots of the analysis of the 31 patients samples. Four (or less) clusters could be distinguished in the plots: o: homozygous VIC® cluster, □: heterozygous cluster, △ homozygous FAM™ cluster, ×: no-template controls (NTC).

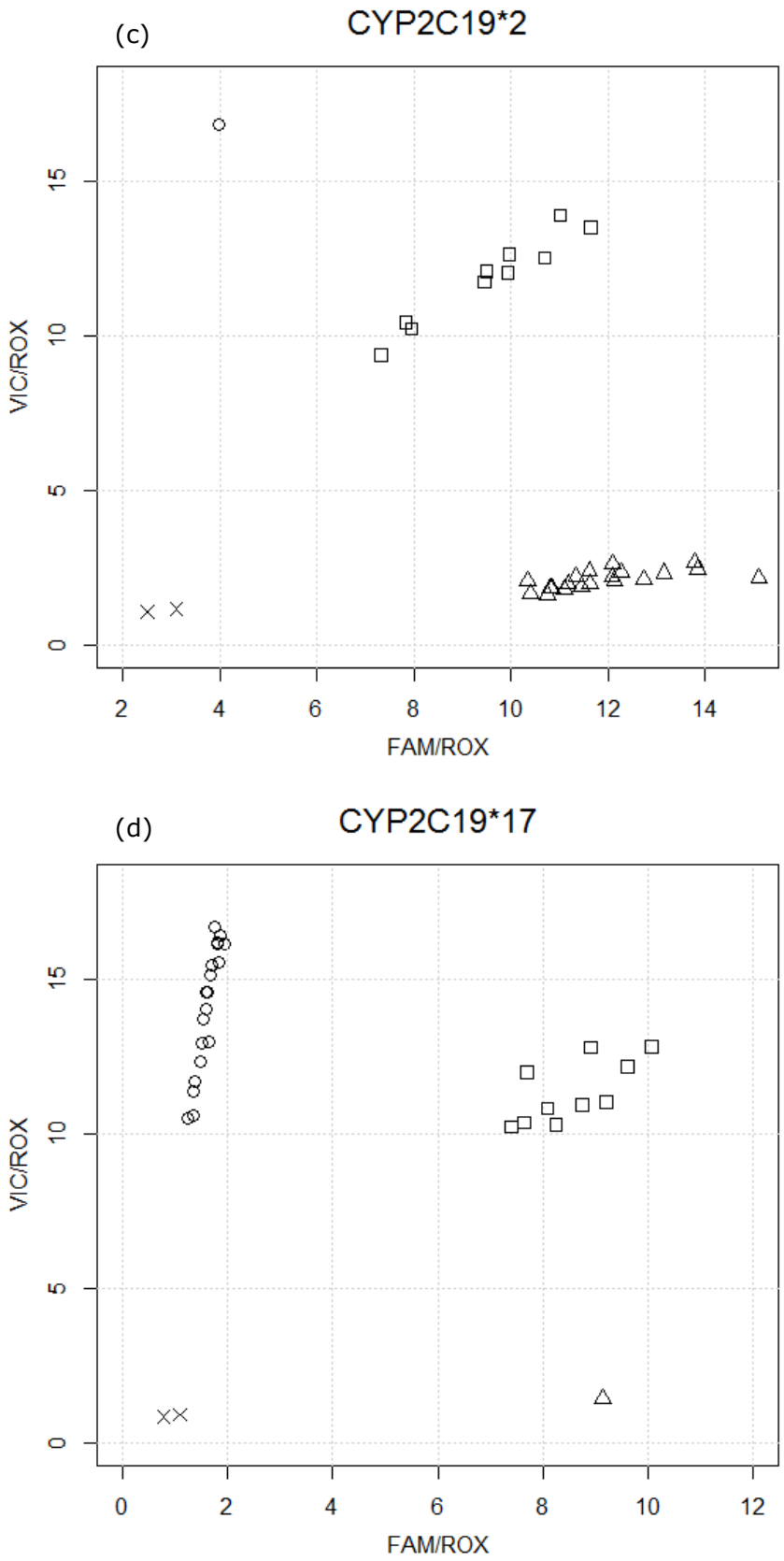

Figure 2 (continued): Allelic discrimination plots of the analysis of the 31 patients samples. Four (or less) clusters could be distinguished in the plots: o: homozygous VIC® cluster, □: heterozygous cluster, △ homozygous FAM™ cluster, ×: no-template controls (NTC).

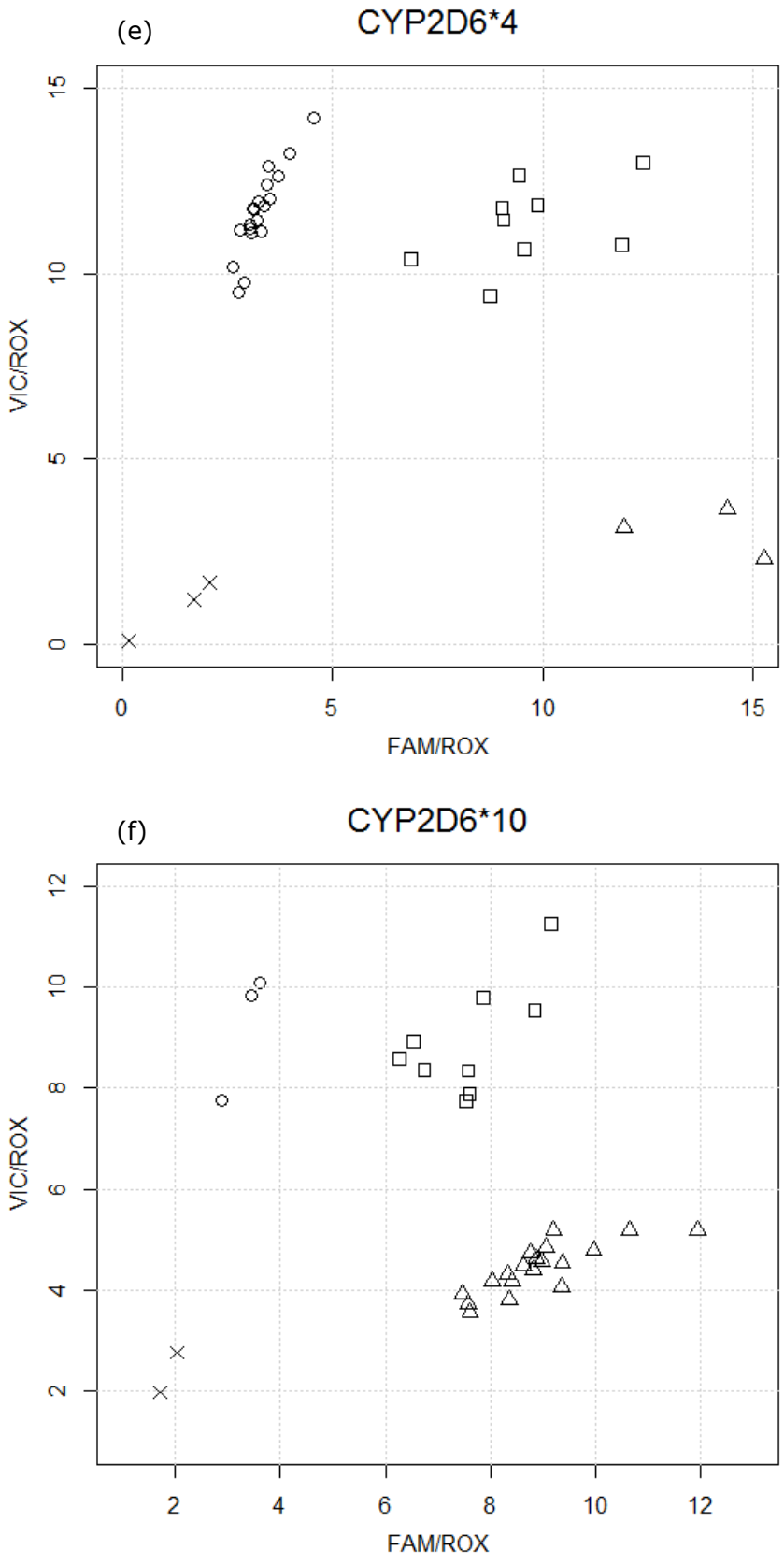

Figure 2 (continued): Allelic discrimination plots of the analysis of the 31 patients samples. Four (or less) clusters could be distinguished in the plots: o: homozygous VIC® cluster, □: heterozygous cluster, △ homozygous FAM™ cluster, ×: no-template controls (NTC).

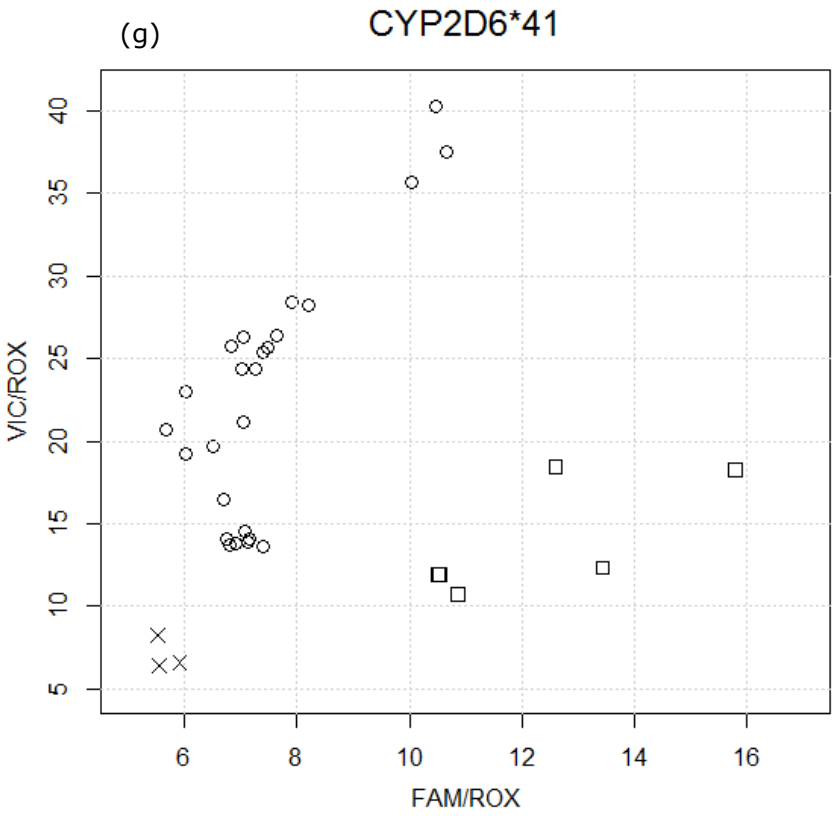

Figure 2 (continued): Allelic discrimination plots of the analysis of the 31 patients samples. Four (or less) clusters could be distinguished in the plots: o: homozygous VIC® cluster, □: heterozygous cluster, △ homozygous FAM™ cluster, ×: no-template controls (NTC).

4 CONCLUSION

A workflow was successfully created for the genotyping of SNPs of CYP2C9, 2C19 and 2D6, relevant in pharmacokinetic studies. These SNPs were carefully selected based on their frequency of occurrence in the Caucasian population, as well as on their significant effect on enzyme activity. Established methods for the extraction and quantification of DNA, using a Qiagen QIAamp DNA Mini kit and the Picogreen® assay, respectively, were combined. Straightforward TaqMan analysis was performed for the analysis of several SNPs. Analysis of a set of samples showed that the method is reliable, as the observed minor allele frequencies were similar to previously reported frequencies based on the analysis of large populations.

REFERENCES

[1] Ingelman-Sundberg M, Sim SC, Gomez A, Rodriguez-Antona C. Influence of cytochrome P450 polymorphisms on drug therapies: Pharmacogenetic, pharmacoepigenetic and clinical aspects. *Pharmacology & Therapeutics* 2007; 116(3):496-526.

[2] Ingelman-Sundberg M. Human drug metabolising cytochrome P450 enzymes: properties and polymorphisms. *Naunyn Schmiedebergs Arch Pharmacol* 2004; 369(1):89-104.

[3] Zhou SF, Liu JP, Chowbay B. Polymorphism of human cytochrome P450 enzymes and its clinical impact. *Drug Metab Rev* 2009; 41(2):89-295.

[4] Rodriguez-Antona C, Ingelman-Sundberg M. Cytochrome P450 pharmacogenetics and cancer. *Oncogene* 2006; 25(11):1679-1691.

[5] Jannetto PJ, Laleli-Sahin E, Wong SH. Pharmacogenomic genotyping methodologies. *Clin Chem Lab Med* 2004; 42(11):1256-1264.

[6] Yang Y, Muzny DM, Reid JG, Bainbridge MN, Willis A, Ward PA *et al.* Clinical Whole-Exome Sequencing for the Diagnosis of Mendelian Disorders. *N Engl J Med* 2013; 369(16):1502-1511.

[7] Ahn SJ, Costa J, Rettig Emanuel J. PicoGreen Quantitation of DNA: Effective Evaluation of Samples Pre-or Psost-PCR. *Nucleic Acids Res* 1996; 24(13):2623-2625.

[8] Xie HG, Prasad HC, Kim RB, Stein CM. CYP2C9 allelic variants: ethnic distribution and functional significance. *Advanced Drug Delivery Reviews* 2002; 54(10):1257-1270.

[9] Sullivan-Klose TH, Ghanayem BI, Bell DA, Zhang ZY, Kaminsky LS, Shenfield GM *et al.* The role of the CYP2C9-Leu(359) allelic variant in the tolbutamide polymorphism. *Pharmacogenetics* 1996; 6(4):341-349.

[10] Yasar U, Eliasson E, Dahl ML, Johansson I, Ingelman-Sundberg M, Sjoqvist F. Validation of methods for CYP2C9 genotyping: Frequencies of mutant alleles in a Swedish population. *Biochem Biophys Res Commun* 1999; 254(3):628-631.

[11] Haining RL, Hunter AP, Veronese ME, Trager WF, Rettie AE. Allelic Variants of Human Cytochrome P450 2C9: Baculovirus-Mediated Expression, Purification, Structural Characterization, Substrate Stereoselectivity, and Prochiral Selectivity of the Wild-Type and I359L Mutant Forms. *Arch Biochem Biophys* 1996; 333(2):447-458.

[12] Rettie AE, Wienkers LC, Gonzalez FJ, Trager WF, Korzekwa KR. Impaired (S)-Warfarin Metabolism Catalyzed by the R144C Allelic Variant of Cyp2C9. *Pharmacogenetics* 1994; 4(1):39-42.

[13] King BP, Khan TI, Aithal GP, Kamali F, Daly AK. Upstream and coding region CYP2C9 polymorphisms: correlation with warfarin dose and metabolism. *Pharmacogenetics* 2004; 14(12):813-822.

[14] Aithal GP, Day CP, Kesteven PJ, Daly AK. Association of polymorphisms in the cytochrome P450 CYP2C9 with warfarin dose requirement and risk of bleeding complications. *The Lancet* 1999; 353(9154):717-719.

[15] Kidd RS, Straughn AB, Meyer MC, Blaisdell J, Goldstein JA, Dalton JT. Pharmacokinetics of chlorpheniramine, phenytoin, glipizide and nifedipine in an individual homozygous for the CYP2C9*3 allele. *Pharmacogenetics* 1999; 9(1):71-80.

[16] Sandberg M, Johansson I, Christensen M, Rane A, Eliasson E. The impact of CYP2C9 genetics and oral contraceptives on cytochrome P4502C9 phenotype. *Drug Metab Dispos* 2004; 32(5):484-489.

[17] Gardiner SJ, Begg EJ. Pharmacogenetics, drug-metabolizing enzymes, and clinical practice. *Pharmacol Rev* 2006; 58(3):521-590.

[18] Desta Z, Zhao XJ, Shin JG, Flockhart DA. Clinical significance of the cytochrome P4502C19 genetic polymorphism. *Clin Pharmacokinet* 2002; 41(12):913-958.

[19] Goldstein JA, Ishizaki T, Chiba K, Demorais SMF, Bell D, Krahn PM *et al.* Frequencies of the defective CYP2C19 alleles responsible for the mephenytoin poor metabolizer phenotype in various Oriental, Caucasian, Saudi Arabian and American black populations. *Pharmacogenetics* 1997; 7(1):59-64.

[20] Griese EU, Ilett KF, Kitteringham NR, Eichelbaum M, Powell H, Spargo RM *et al.* Allele and genotype frequencies of polymorphic cytochromes P4502D6, 2C19 and 2E1 in Aborigines from Western Australia. *Pharmacogenetics* 2001; 11(1):69-76.

[21] de Morais SMF, Wilkinson GR, Blaisdell J, Nakamura K, Meyer UA, Goldstein JA. The Major Genetic-Defect Responsible for the Polymorphism of S-Mephenytoin Metabolism in Humans. *Journal of Biological Chemistry* 1994; 269(22):15419-15422.

[22] Ibeanu GC, Goldstein JA, Meyer U, Benhamou S, Bouchardy C, Dayer P *et al.* Identification of new human CYP2C19 alleles (CYP2C19*6 and CYP2C19*2B) in a Caucasian poor metabolizer of mephenytoin. *J Pharmacol Exp Ther* 1998; 286(3):1490-1495.

[23] Ibeanu GC, Blaisdell J, Ferguson RJ, Ghanayem BI, Brosen K, Benhamou S *et al.* A novel transversion in the intron 5 donor splice junction of CYP2C19 and a sequence polymorphism in exon 3 contribute to the poor metabolizer phenotype for the anticonvulsant drug S-mephenytoin. *J Pharmacol Exp Ther* 1999; 290(2):635-640.

[24] Sim SC, Risinger C, Dahl ML, Aklillu E, Christensen M, Bertilsson L *et al.* A common novel CTP2C19 gene variant causes ultrarapid drug metabolism relevant for the drug response to proton pump inhibitors and antidepressants. *Clin Pharmacol Ther* 2006; 79(1):103-113.

[25] Wedlund PJ. The CYP2C19 enzyme polymorphism. *Pharmacology* 2000; 61(3):174-183.

[26] Furuta T, Ohashi K, Kosuge K, Zhao XJ, Takashima M, Kimura M *et al.* C Upsilon P2C19 genotype status and effect of omeprazole on intragastric pH in humans. *Clin Pharmacol Therap* 1999; 65(5):552-561.

[27] Kearns GL, Leeder JS, Gaedigk A. Impact of the CYP2C19*17 Allele on the Pharmacokinetics of Omeprazole and Pantoprazole in Children: Evidence for a Differential Effect. *Drug Metab Dispos* 2010; 38(6):894-897.

[28] Rudberg I, Mohebi B, Hermann M, Refsum H, Molden E. Impact of the ultrarapid CYP2C19*17 allele on serum concentration of escitalopram in psychiatric patients. *Clin Pharmacol Ther* 2008; 83(2):322-327.

[29] Sibbing D, Koch W, Gebhard D, Schuster T, Braun S, Stegherr J *et al.* Cytochrome 2C19*17 Allelic Variant, Platelet Aggregation, Bleeding Events, and Stent Thrombosis in Clopidogrel-Treated Patients With Coronary Stent Placement. *Circulation* 2010; 121(4):512-518.

[30] Simon T, Verstuyft C, Mary-Krause M, Quteineh L, Drouet E, Meneveau N *et al.* Genetic Determinants of Response to Clopidogrel and Cardiovascular Events. *N Engl J Med* 2009; 360(4):363-375.

[31] Mizutani T. PM frequencies of major CYPs in Asians and Caucasians. *Drug Metab Rev* 2003; 35(2-3):99-106.

[32] Teh LK, Bertilsson L. Pharmacogenomics of CYP2D6: Molecular Genetics, Interethnic Differences and Clinical Importance. *Drug Metabolism and Pharmacokinetics* 2012; 27(1):55-67.

[33] Marez D, Legrand M, Sabbagh N, LoGuidice JM, Spire C, Lafitte JJ *et al.* Polymorphism of the cytochrome P450 CYP2D6 gene in a European population: Characterization of 48 mutations and 53 alleles, their frequencies and evolution. *Pharmacogenetics* 1997; 7(3):193-202.

[34] Hanioka N, Kimura S, Meyer UA, Gonzalez FJ. The Human Cyp2D Locus Associated with A Common Genetic-Defect in Drug Oxidation - A G1934-]A Base Change in Intron-3 of A Mutant Cyp2D6 Allele Results in An Aberrant-3' Splice Recognition Site. *Am J Hum Genet* 1990; 47(6):994-1001.

[35] Kagimoto M, Heim M, Kagimoto K, Zeugin T, Meyer UA. Multiple Mutations of the Human Cytochrome-P450Iid6 Gene (Cyp2D6) in Poor Metabolizers of Debrisoquine - Study of the Functional-Significance of Individual Mutations by Expression of Chimeric Genes. *Journal of Biological Chemistry* 1990; 265(28):17209-17214.

[36] Xie HG, Kim RB, Wood AJJ, Stein CM. Molecular basis of ethnic differences in drug disposition and response. *Annu Rev Pharmacol Toxicol* 2001; 41:815-850.

[37] Dahl ML, Johansson I, Palmertz MP, Ingelmansundberg M, Sjoqvist F. Analysis of the Cyp2D6 Gene in Relation to Debrisoquin and Desipramine Hydroxylation in A Swedish Population. *Clin Pharmacol Therap* 1992; 51(1):12-17.

[38] Sachse C, Brockmoller J, Bauer S, Roots I. Cytochrome P450 2D6 variants in a Caucasian population: Allele frequencies and phenotypic consequences. *Am J Hum Genet* 1997; 60(2):284-295.

[39] Johansson I, Oscarson M, Yue QY, Bertilsson L, Sjoqvist F, Ingelmansundberg M. Genetic-Analysis of the Chinese Cytochrome P4502D Locus - Characterization of Variant Cyp2D6 Genes Present in Subjects with Diminished Capacity for Debrisoquine Hydroxylation. *Mol Pharmacol* 1994; 46(3):452-459.

[40] Raimundo S, Toscano C, Klein K, Fischer J, Griese EU, Eichelbaum M *et al.* A novel intronic mutation, 2988G > A, with high predictivity for impaired function of cytochrome P450 2D6 in white subjects. *Clin Pharmacol Ther* 2004; 76(2):128-138.

[41] Toscano C, Klein K, Blievernicht J, Schaeffeler E, Saussele T, Raimundo S *et al.* Impaired expression of CYP2D6 in intermediate metabolizers carrying the *41 allele caused by the intronic SNP 2988G > A: evidence for modulation of splicing events. *Pharmacogenetics and Genomics* 2006; 16(10):755-766.

[42] Rau T, Diepenbruck S, Diepenbruck I, Eschenhagen T. The 2988G > A polymorphism affects splicing of a CYP2D6 minigene. *Clin Pharmacol Therap* 2006; 80(5):555-558.

CHAPTER 6

IN VITRO CYTOCHROME P450 ACTIVITY DECREASES IN CHILDREN WITH HIGH PAEDIATRIC END-STAGE LIVER DISEASE SCORES

This section is a modified version of the publication:

L. De Bock, K. Boussery, M. Van Winckel, P. De Paepe, X. Rogiers, X. Stephenne, E. Sokal, J. Van Bocxlaer. In vitro cytochrome P450 activity decreases in children with high Pediatric End-Stage Liver Disease scores. Drug Metabolism and Disposition, 41 (2013) 390-397.

'A model is a lie that helps you see the truth'

- Howard Skipper

ABSTRACT

To improve modelling and simulation of the pharmacokinetics (PK) in paediatric patients, there is a need for research on developmental and disease-specific determinants. This article describes the evaluation of the in vitro cytochrome P450 activity, an important enzyme family in drug metabolism, in children with hepatic dysfunction. The activity of 6 CYP isoforms, CYP1A2, 2C9, 2C19, 2D6, 2E1, and 3A4 was evaluated in thirty-one patients with different pathologies, mainly biliary atresia (n=23). A hypervariable activity was observed for all the isoforms. Compared to an average adult activity, low activities were seen for CYP1A2, 2C19, 2E1, and 3A4. For CYP3A4, a positive correlation between activity and abundance was observed. For CYP2E1 at the other hand, the correlation was poor. In this population, age, co-medication, and genotype (unless a null-allele was present) could not be used as predictors for the CYP activity. In contrast, the Paediatric End-stage Liver Disease score was negatively correlated with the ln(activity). This suggests a decrease in CYP activity with deteriorating hepatic function. Moreover, the activity of all isoforms was correlated, demonstrating a concomitant decrease of all isoforms in young patients with liver disease. To our knowledge, this is the first study to evaluate CYP activity in children with hepatic impairment. The presented data may provide support in the further optimization of a disease-specific model in this patient population.

1 INTRODUCTION

There is a paucity of approved drugs to treat children and young infants. It is estimated that 50 to 75% of drugs in children are used off-label [1]. Many of the dosing regimens of drugs currently used in children are derived using coarse methodologies, such as linear extrapolations from adult doses on the basis of mere body weight. Information on the behaviour of pharmacology of drugs in children is difficult to acquire, due to the ethical and practical restraints of performing clinical studies in the paediatric population [2]. Modelling and simulation of the pharmacokinetics (PK) and pharmacodynamics (PD) of drugs in children has therefore gained a lot of interest. Many attempts have been made to create models to predict the PK and/or PD of specific drugs in children. These models, integrating information on the paediatric biological system, are predicted to replace the currently used empirical and allometric models [3], albeit that more in-depth model optimization is still an obvious necessity. Determinants of the PK in paediatric patients should be investigated, such as drug absorption in neonates and infants, the ontogeny of transporters, or age-related changes in PD [4,5]. Besides the important developmental changes, variability due to environmental, genetic and disease related factors also need to be incorporated [6]. The latter have been shown to influence both PK and PD of drugs, underlining the need for disease-specific models.

In the development of disease-specific models, there is a particular interest in hepatic failure. Hepatic impairment has been identified as a condition leading to changes in the PK of drugs through various mechanisms, such as changes in the hepatic blood flow (portal-systemic shunts in cirrhosis), or an impaired metabolic clearance (as in biliary obstruction, where hepatocellular damage can be seen) [7]. The specific mechanisms of the alterations in PK can be elucidated through *in vitro* studies each focusing on one particular aspect of the PK, such as e.g. the activity of drug metabolizing enzymes. Johnson et al. recently published a semi-mechanistic model for the prediction of drug clearance in adult patients with liver cirrhosis [8]. The model was based on specific mechanistic data that were previously published, such as the study conducted by George et al. [9]. In this study, the activity and abundance of several cytochrome P450 (CYP) isoforms, the main enzymes involved in drug metabolism, were evaluated in patients with severe chronic liver disease [9]. Based on these *in vitro* data, they concluded that the CYP isoforms were selectively altered in liver disease, with some isoforms showing a profound decrease, whereas others were only slightly or even not affected. Frye et al. demonstrated a similar selective regulation of the various enzymes in liver disease in vivo, with variable and non-uniform alterations in CYP activity [10]. The results from comparable studies were reviewed by Villeneuve and Pichette [11], and Elbekai et al. [12].

In these previously published studies investigating the effect of hepatic impairment on drug PK, no children were included. Moreover, several studies concluded that the changes in PK depend on the aetiology of the disease, as well as the degree of hepatic impairment (as reviewed by Villeneuve and Pichette [11]). The aetiology of severe hepatic dysfunction differs considerably between adults and children. In adults, the main indication for liver transplantation are noncholestatic liver cirrhosis (± 60%) due to alcoholism or hepatitis C, liver cancers (± 10%), cholestatic diseases (± 10%), acute hepatic failure and metabolic disorders [13]. In children however, biliary atresia is the main indication for liver transplantation, followed by fulminant liver failure, other cholestatic diseases, such as progressive familial intrahepatic cholestatis (PFIC) and Alagille syndrome, and other metabolic diseases [13-15].

The available data on the influence of liver disease on CYP activity in adults cannot be extrapolated to the paediatric population, due to the aforementioned ontogeny of several systems, as well as to the differences in aetiology of the liver disease. Information on the influence of liver disease on the CYP activity is pivotal for the development of disease-specific models for this particular patient population. This study therefore aimed to evaluate the in vitro CYP activity in samples of liver explants of children with severe hepatic dysfunction, in view of it being a determinant parameter in such disease-specific physiology based pharmacokinetic (PBPK) models.

2 MATERIALS AND METHODS

This study was approved by the Ethics Committee of Ghent University Hospital (B67020084281) and in accordance with the Ethical Committee approval of Saint-Luc Clinics Brussels.

2.1 Sample collection

Liver samples were obtained from the explanted liver of 31 paediatric patients who underwent liver transplantation at Ghent University Hospital or Saint-Luc Clinics Brussels (see Table 2). The clinical record was consulted for clinical and laboratory data, such as age, gender, weight, pre-operative medication, and relevant liver function tests. Part of this information was used to calculate the Paediatric End-stage Liver Disease (PELD) score [16].

From each liver, small blocks of tissue (1-4 cm^3) were taken on four different sample sites (superficial and central of both right and left lobe). The tissue samples were snap frozen within 15 minutes after explantation of the liver, and were stored at -80°C until processing.

2.2 Preparation of liver microsomes

Part of the liver tissue sample was processed into microsomes using a modification of the method of Wilson et al. [17]. The samples were thawed on iced, rinsed with homogenization buffer (0.25 M phosphate buffer pH 7.25, 1.15% KCl) and blot dried. After weighing (0.6 – 4 g), the tissue was minced with scissors and homogenized in homogenization buffer (4 ml g^{-1} tissue) using an automated Potter-Elvehjem system (VWR, Leuven, Belgium). Tissue homogenates were centrifuged at 10,000 *x g* for 15 minutes at 4°C (Beckman L8-70M Ultracentrifuge, Beckman Coulter Limited, High Wycombe, Buckinghamshire, UK). In order to form a microsomal pellet, the resulting supernatant (S9 fraction) was centrifuged at 100,000 *x g* for 75 minutes at 4°C. The supernatant (cytosolic fraction) was kept aside, and the microsomal pellet was re-suspended and washed using 4 ml homogenization buffer per gram tissue. Centrifugation at 100,000 *x g* was repeated, and the final microsomal pellet was re-suspended in 1.5 volumes of resuspension buffer (homogenization buffer, containing 30% *v/v* glycerol). All fractions were snap frozen in liquid nitrogen and stored at -80°C until analysis. The total protein content of the microsomes was estimated using the method of Bradford [18].

2.3 Cytochrome P450 activity determinations

In order to exclude possible zonal differences in activity, a microsomal pool, consisting of the four different zones according to their relative weight, was prepared for each patient sample. Microsomal activities of CYP1A2, 2C9, 2C19, 2D6, 2E1, and 3A4 were determined by incubating the microsomes with specific probe substrates that are mainly metabolized to marker metabolites by one of the studied isoforms (see Table 1). Incubations were performed in triplicate (with CV% always < 15%), and in parallel with each of the specific substrates individually. The final reaction medium (total volume 250 µl) consisted of 1 mM NADPH, 0.2 M phosphate buffer (pH 7.4), 0.25 mg protein/ml and one of the substrates at its apparent Km [19]. The samples were incubated in a shaking heating block at 37°C for 15 minutes, except for the CYP2C19 assay, which was incubated for 40 minutes. The enzymatic reactions were stopped with 25 µl of ice cold stopreagent containing the internal standard (chlorpropamide), 3% *v/v* formic acid, and 55% *v/v* acetonitrile. The terminated reaction mixtures were vortex mixed and placed on ice. In order to pellet the proteins, samples were centrifuged at 20,000 *x g* for 10 minutes at 4°C. The formed metabolites were quantified with a previously described ultra high performance liquid chromatography – tandem mass spectrometry (UPLC-MS/MS) method [20], and activities were expressed as pmol metabolite formed/(minute x mg protein).

Table 1: Specific substrates and conditions for the incubation experiments. Total microsomal protein content was 0.25 mg/ml.

Enzyme	Substrate	Concentration (µM)	Time (min)	Metabolite
CYP1A2	Phenacetin	50	15	Acetaminophen
CYP2C9	Tolbutamide	100	15	4-Hydroxytolbutamide
CYP2C19	S-Mephenytoin	100	40	4'-Hydroxymephenytoin
CYP2D6	Dextromethorphan	5	15	Dextrorphan
CYP2E1	Chlorzoxazone	50	15	6-Hydroxychlorzoxazone
CYP3A4	Midazolam	5	15	1-Hydroxymidazolam

Besides the incubation of the patient microsomes, two commercially available adult microsomal pools were incubated under the same conditions (Xtreme 200 HLM, Xeno Tech, Lenexa KS, USA, and BD UltraPool HLM 150, BD Biosciences, Bedford MA, USA).

2.4 DNA extraction and genotyping

DNA was extracted from 25 mg of the remaining liver tissue sample using the QIAamp DNA Mini kit from Qiagen (Santa Clarita, CA, USA). The samples were genotyped using TaqMan® Drug Metabolism Genotyping Assays from Applied Biosystems (Life Technologies, Carlsbad, CA, USA) for the following NCBI dbSNP identification numbers: rs3892097 (2D6*4, 1846G>A), rs1065852 (2D6*10, 100C>T), rs28371725 (2D6*41, 2988G>A), rs1799853 (2C9*2, 3608C>T), and rs1057910 (2C9*3, 42614A>C), rs4244285 (2C19*2, 19154G>A), and rs12248560 (2C19*17, -806C>T).

2.5 CYP3A4 and 2E1 abundance determination

The abundance of CYP3A4 was determined in the microsomal suspension using an indirect ELISA [21]. CYP2E1 abundance was determined using a similar method with optimized calibration range (4 – 256 pmol CYP2E1/mg microsomal protein), primary and secondary antibody concentration (1:1,600 and 1:10,000, respectively), and incubation times (secondary antibody incubation at 37°C, and incubation with substrate for 60 minutes) [22].

2.6 Data analysis

The individual activities of the 6 isoforms were compared graphically. Subsequently, the isoform activities were grouped based on their percentage of the mean isoform activity in the adult microsomal pools. Five groups were selected: <25%; 25-55%, 55-85%, 85-115% (considered equal to adult activity, based on the allowed analytical variability of ± 15%), and >115%. The possible influence of age, weight, co-medication, and genotype was also evaluated graphically. The relationship between ln(activity) and ln(abundance),

and ln(activity) and PELD score was evaluated graphically using locally weighted scatter plot smoothing (LOWESS). Moreover, the Pearson's correlation coefficient was calculated for these pairs of variables. Furthermore, the correlation between the activities of the isoforms was evaluated (graphically and with Pearson's correlation). All data analysis was performed using R® v.2.13 (R foundation for statistical computing, Vienna, Austria), except for the influence of co-medication, which was evaluated using ANOVA analysis with post-hoc Bonferoni correction using SPSS Statistics 20 (SPSS Inc, Chicago, IL, USA). For all statistical analyses, $p < 0.05$ was accepted as indicating a significant difference.

3 RESULTS

3.1 Patient characteristics

Thirty-one patients were included in the study. The patients suffered from biliary atresia (BA; n = 23), α–1 antitrypsin deficiency (A1AD; n = 1), a combination of BA and A1AD (n=1), progressive familial intrahepatic cholestasis (PFIC; n = 2), cystic fibrosis (CF; n = 1), Caroli's disease (CAR; n = 1), acute liver failure (ALF; n = 1), or neonatal hemochromatosis (NH; n = 1). A summary of the patient characteristics is given in Table 2.

3.2 Cytochrome P450 enzyme activities

The results of the individual enzyme activity measurements are depicted in Figure 1. Due to the non-normal distribution, activities were ln transformed. The mean adult ln(activity) of the isoforms, determined in the two commercially available adult pools, was added to the graph as a point of reference (dashed line). For all isoforms, a high inter-individual variability was observed. A low ln(activity) of CYP3A4, 1A2, 2E1 and 2D6 was seen in many patients, compared to the adult reference. The CYP2C9 ln(activities) were spread around the adult reference, with both very low and very high activities in some patients.

Figure 2 shows the same activity data after grouping the results into five groups, based on the percentage of the mean reference adult activity. A different pattern can be seen between the different isoforms. Fifty percent or more of the patients show a CYP3A4, 2E1, 1A2 and 2C19 activity below 25% of the adult activity. In contrast, about 50% of the patients had a CYP2C9 activity higher than in adults. As for the CYP2D6 activity, the patients were more or less equally distributed over all five groups.

The evaluation of the relation between the activity and the age and weight of the patients showed no correlation (data not shown).

Table 2: Patient information. Summary of the gender, age at transplantation, diagnosis and PELD of the 31 included patients (M: male; F: female; OLT: orthotopic liver transplantation; PELD: Paediatric End-Stage Liver Disease; A1AD: α-1 antitrypsin deficiency; PFIC: progressive familial intrahepatic cholestasis).

Patient ID (n = 31)	Gender	Age at transplantation (months)	Indication for OLT	PELD
ID1	F	6.3	Biliary atresia	23
ID2	M	0.7	Neonatal hemochromatosis	38
ID3	M	7.3	Biliary atresia	13
ID4	M	9	Biliary atresia	26
ID5	M	4.7	Biliary atresia	35
ID6	F	6.5	Biliary atresia	15
ID7	F	9.5	Biliary atresia	7
ID9	F	7.4	Biliary atresia	18
ID10	M	132	Cystic fibrosis with cirrhosis	8
ID11	M	9.2	Biliary atresia	38
ID12	M	15.3	Biliary atresia	20
ID13	M	6.4	Biliary atresia	31
ID14	F	108	Acute liver failure	34
ID15	M	13	Biliary atresia	26
ID16	M	23.7	Biliary atresia	1
ID17	M	11.4	Biliary atresia	19
ID18	M	25.4	Biliary atresia	17
ID19	M	11.5	Biliary atresia	19
ID20	M	11.5	A1AD	32
ID21	F	60.7	Caroli's disease	12
ID22	F	7.8	Biliary atresia	24
ID23	F	9.4	Biliary atresia	18
ID24	M	24.5	Biliary atresia	0
ID25	F	51.4	PFIC II	11
ID26	M	11	Biliary atresia	37
ID27	M	7.1	Biliary atresia	30
ID28	M	8.1	Biliary atresia	19
ID29	M	78.1	Biliary atresia + A1AD	33
ID30	F	11.6	Biliary atresia	10
ID31	F	8.9	Biliary atresia	3
ID32	M	83.8	PFIC III	6

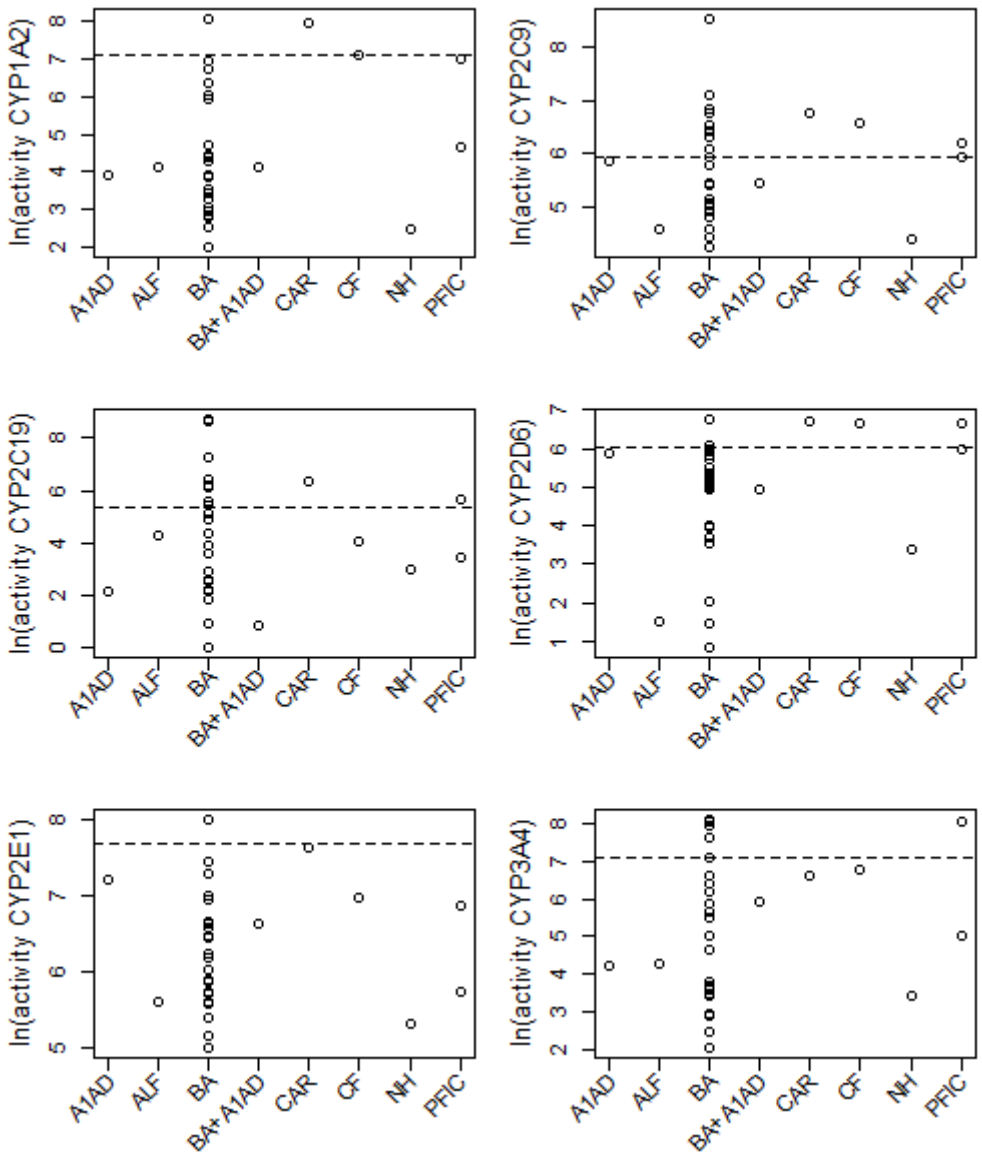

Figure 1: Ln(activity) of the 6 CYP isoforms of the 31 patients. Indications for OLT: A1AD: α-1 antitrypsin deficiency; ALF: acute liver failure; BA: biliary atresia; CAR: Caroli's disease; CF: cystic fibrosis; NH: neonatal hemochromatosis; PFIC: progressive familial intrahepatic cholestasis. The dashed line represents the mean ln(activity) as determined in two commercially available adult microsome pools.

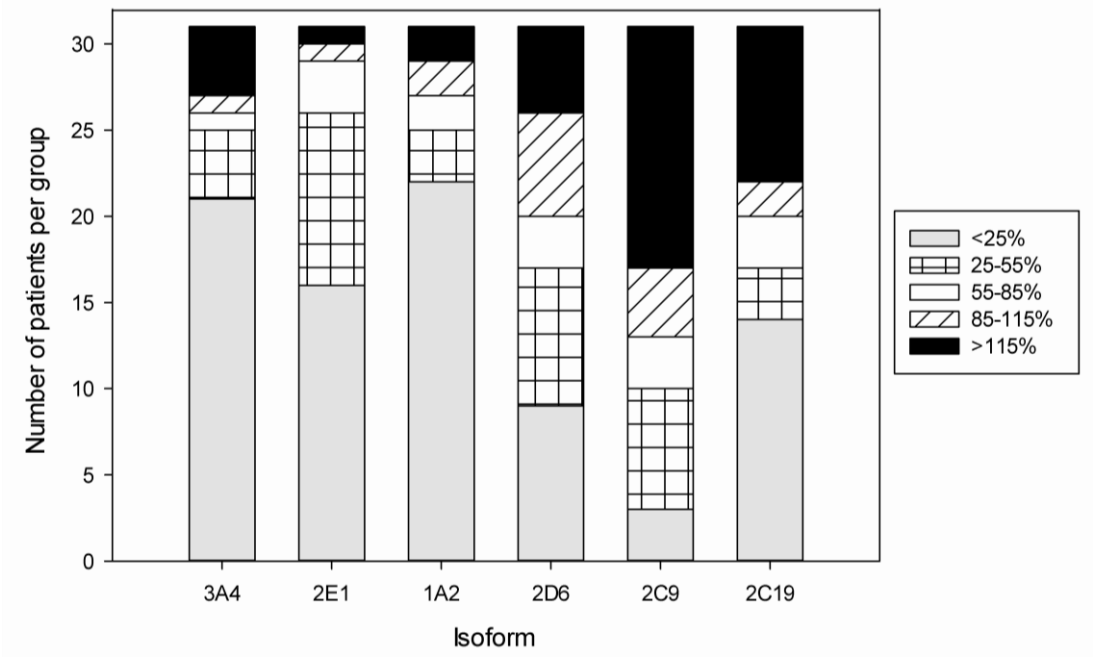

Figure 2: The activity of the CYP isoforms in terms of percentage of the adult activity. The results were classified into 5 groups, and the percentage of the study population (n=31) present in a group is depicted.

3.3 Possible influence of co-medication

A list of the known inducing or inhibiting drugs taken in the 2 weeks prior to the transplantation is presented in Table 3. Graphical comparison of the ln(activity) of the 6 isoforms between patients receiving inhibitors, inducers, both, or no co—medication is depicted in Figure 3. Statistical comparison showed significant differences ($p<0.05$) in ln(activity) of CYP2C9 between the patients receiving no co-medication and those receiving both an inhibitor and an inducer, as well as between the patients receiving an inhibitor and those receiving both. The same results were obtained for CYP2C19. For CYP3A4, significant differences were seen between: (1) no co-medication and inhibitor + inducer; (2) no co-medication and inducer; (3) inhibitor and inhibitor + inducer; (4) inhibitor and inducer.

3.4 Correlation genotype and activity

A comparable graphical analysis was performed for the evaluated polymorphisms. For all SNPs, a reduced activity was expected compared to the wild type, except for CYP2C19*17, where an increased activity was anticipated. As shown in Figure 4, this trend was seen for CYP2D6*4 and *10, 2C9*2 (except for one extreme value), and in the patient homozygous for the CYP2C19*2 null allele. The other expected trends were not reflected in the data.

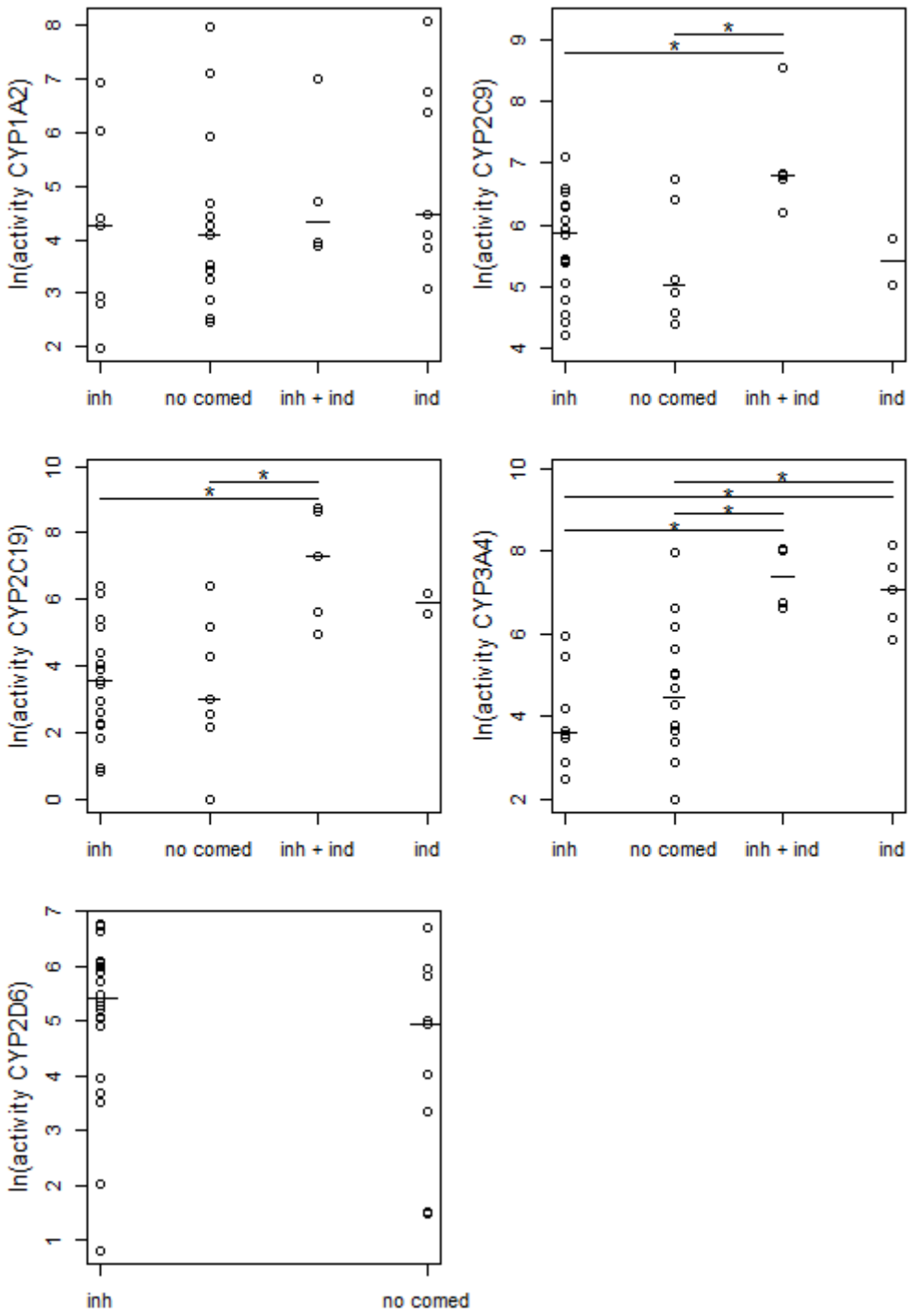

Figure 3: Comparison of the ln(activity) between patients receiving co-medication known to induce or inhibit CYP activity and patients who did not receive these drugs. (Inh: inhibitor; ind: inducer; comed: co-medication). (*p<0.05, –: median ln(activity) within each group)

Table 3: Medication history. Summary of the administered pre-operative medication, known to influence (induce or inhibit) CYP activity.

	Number of patients receiving the drug	Affected CYP isoform(s)	Reference(s)
Inhibiting drugs			
retinol	14	CYP2C9, 2C19	[23,24]
cholecalciferol	13	CYP2C9, 2C19, 2D6	[23]
somatostatin	4	CYP2D6, 3A4	[25]
sulfamethoxazole	1	CYP2C9	[26]
fluconazole	2	CYP2C9, 2C19	[26]
(es)omeprazole	8	CYP2C9, 2C19	[27]
amlodipine	1	CYP2C9, 2D6, 3A4	[28]
ciprofloxacine	1	CYP1A2, 3A4	[29,30]
propranolol	5	CYP1A2, 2D6	[31,32]
Inducing drugs			
(es)omeprazole	8	CYP1A2	[33]
rifampicin	7	CYP2C9, 2C19, 3A4	[34]

3.5 Correlation abundance and activity

In Figure 5, the correlation between the ln(activity) and the ln(abundance) of CYP3A4 and CYP2E1 is shown. For CYP3A4, a clear positive correlation could be observed graphically. This is also reflected in the Pearson's correlation coefficient of 0.869. For CYP2E1, a very weak correlation between activity and abundance could be seen (Pearson's correlation = 0.371).

3.6 Correlation of the CYP enzyme activity with PELD score

The ln(activity) was correlated with the PELD score at time of transplantation. This can be seen graphically (LOWESS line) as depicted in Figure 6. This negative correlation is confirmed by the Pearson's correlation coefficients of -0.769 for CYP1A2, -0.461 for CYP2C9, -0.721 for CYP2C19, -0.492 for CYP2D6, -0.545 for CYP2E1, and -0.643 for CYP3A4.

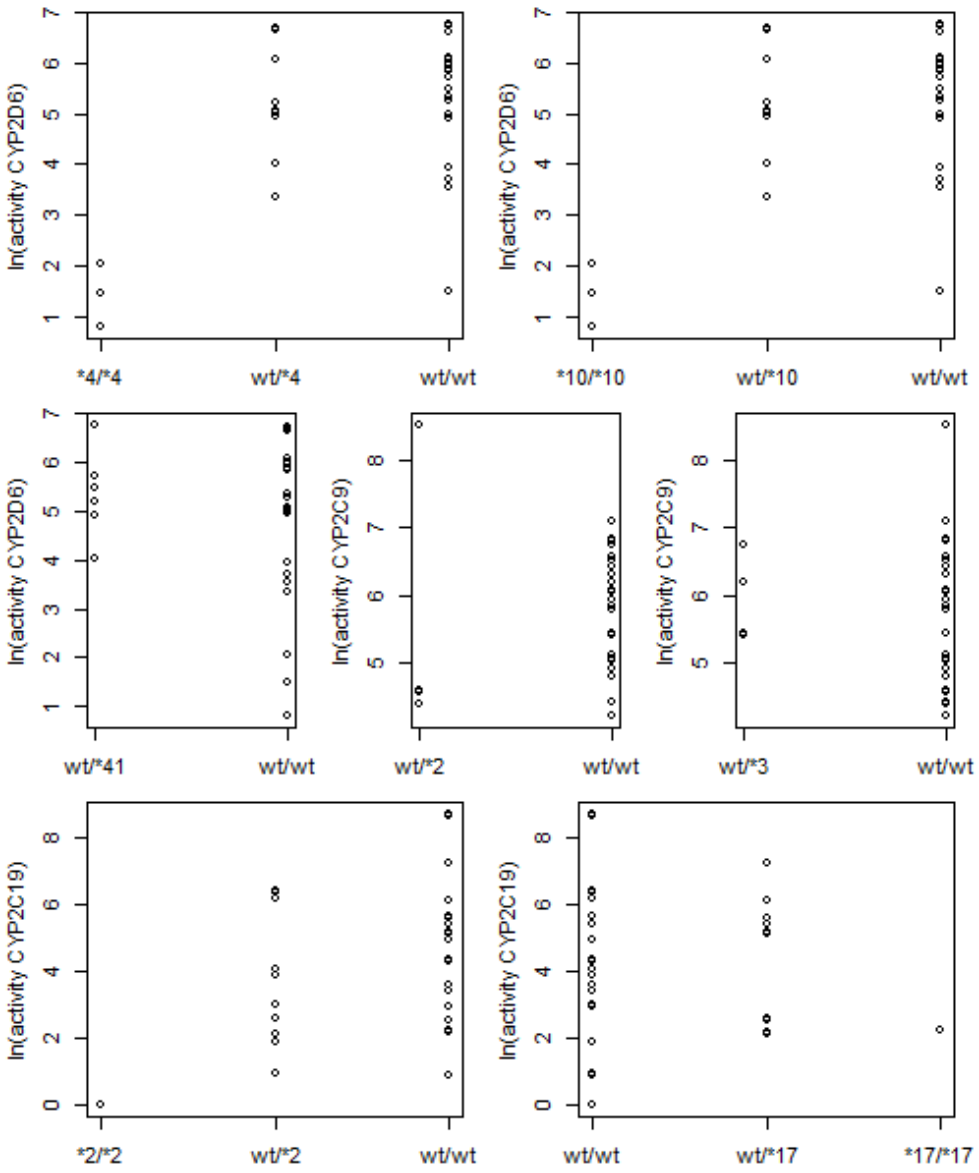

Figure 4: Analysis of the effects of SNPs on the activity of CYP2D6 (*4, *10, *41), CYP2C9 (*2, *3), and CYP2C19 (*2, *17). Allelic combinations are arranged from expected lowest (left) to expected highest (right) activity.

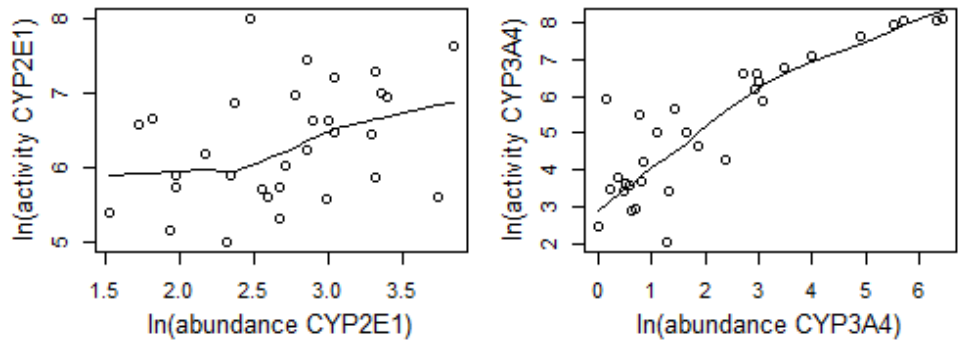

Figure 5: Ln(activity) and ln(abundance) of CYP2E1 and 3A4 were positively correlated.

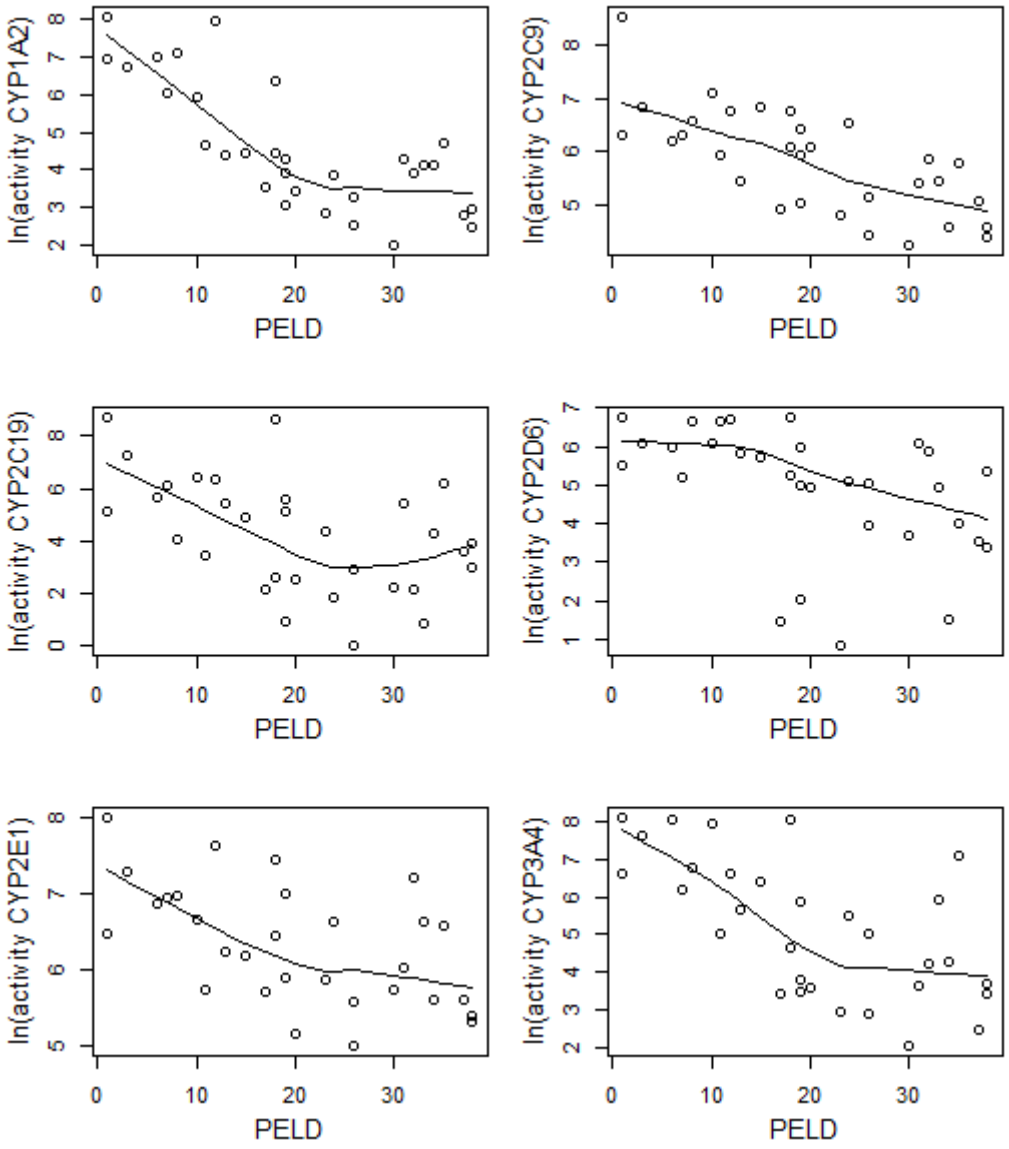

Figure 6: Correlation of ln(activity) and PELD score. A negative correlation was observed for all 6 isoforms.

3.7 Correlation of the 6 isoforms

A possible correlation in ln(activity) between the different isoforms was evaluated. As shown in Figure 7 and Table 4, all isoform activities are positively correlated (see also Table 4).

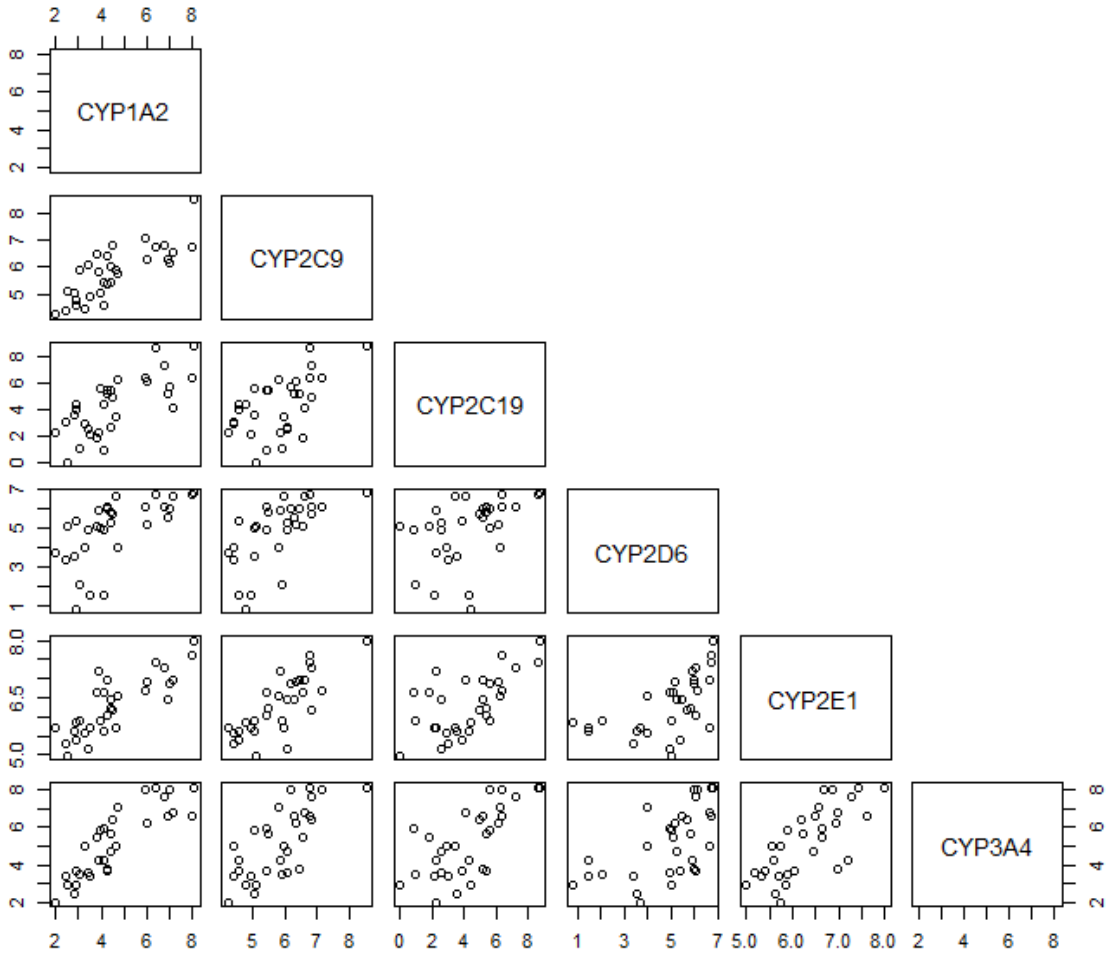

Figure 7: Graphical representation of the positive correlation of the ln(activities) of the 6 isoforms.

Table 4: Pearson's correlation between the CYP isoforms. A positive correlation between the ln(activities) of all six CYP isoforms was observed.

	CYP1A2	**CYP2C9**	**CYP2C19**	**CYP2D6**	**CYP2E1**
CYP2C9	0.756				
CYP2C19	0.581	0.768			
CYP2D6	0.693	0.571	0.603		
CYP2E1	0.846	0.794	0.755	0.738	
CYP3A4	0.594	0.648	0.74	0.594	0.682

4 DISCUSSION

The cytochrome P450 enzyme system has shown to be altered in adults with liver dysfunction [7]. As *in vivo* studies in the paediatric population are fraught with ethical and practical considerations, *in vitro* data can be used to optimize models that may be helpful in determining optimal dosing strategies, in both pre-clinical and clinical settings. This study is the first to describe the CYP activity in children with severe hepatic dysfunction, demonstrating a high inter-individual variability for all studied isoforms. Comparison with the average adult activity showed a mainly lower activity for CYP1A2, 2C19, 2E1, and 3A4, and an activity more spread around adult activity for CYP2C9 and 2C19. Nevertheless, merely relating activities to a "reference activity" obscures the true nature of these physiological findings. Besides the possible influence of liver disease, its aetiology and the degree of impairment, many other factors such as age, inhibiting or inducing co-medication, and genetic polymorphisms, are known to affect enzyme activity, precluding the interpretation of activities as such.

About 75% of the children included in the study suffered from biliary atresia (BA). A large group of patients with this condition was expected, as this is the main cause for OLT in children [13-15]. Contradictory to the expected large group of fulminant hepatic failure, only one patient was included during the time period of collection. This is probably due to the type of recruitment in one of the hospitals, where only planned transplantations by living donor were included. Due to the small number of patients in some groups, the results of patients with different diseases were analysed together. The aetiology of liver disease has been shown to be an important factor in the changes in CYP activity in adults [11]. However, as shown in Figure 1, the variability in ln(activity) in the BA group is larger than the variability between the different groups. Therefore, the patients were considered as one large group for data analysis. Consequently, these results should be interpreted with some caution in respect to these disease types.

The patients included in the study covered a wide age range (from 0.7 months to almost 12 years), with the majority of the patients being age 2 or younger. Age is an important characteristic to take into account in the interpretation of the results, as some of the important CYP isoforms show a certain maturation during development [35-37]. The *in vitro* activity of the 6 enzymes studied, show a gradual increase in activity during the first 2 years of life in healthy children. However, considerable inter-individual differences were observed in the maturation [38]. Nevertheless, a certain correlation between the activity and the age of the patients in our study was expected. It was, however, not observed (data not shown). The lack of correlation between age and activity suggests a disturbance of the normal maturation pattern in children with hepatic dysfunction, or a

stronger influence of another parameter than age. This failure to show a correlation is an important point in the mechanistic elucidation of the influence of hepatic dysfunction on CYP activity in children. Evaluation of the relation between activity and other patient related characteristics, such as weight, also showed no correlation.

Most of the children included in the study are chronically ill and thus often receive chronic medication. Most of the CYPs have been shown to be sensitive to induction or inhibition by several drugs [39]. The influence of inducing or inhibiting pre-operative medication should therefore also be taken into account. Some of the effects of known inducers were observed either graphically or statistically. In contrast, inhibiting effects were not observed. This could be expected, as all the inhibitors that were pre-operatively administered to the patients are competitive, reversible inhibitors. During the preparation of the microsomes the competitive inhibitors are most likely (partially) removed from the microsomal pellet, which may cancel out the influence of (competitive) inhibitors in our experimental set up. This is however an advantage, as this study aims to characterise the activity of the enzymes of the patient as such.

Another issue in the evaluation of CYP activity is the highly polymorphic nature of the CYP enzymes. Three of our studied isoforms, CYP2C9, 2C19 and 2D6 are Class II CYPs, or highly polymorphic CYPs. The other 3 isoforms, CYP3A4, 1A2 and 2E1 are well conserved and do not have important functional polymorphisms, and are therefore Class I CYPs [40]. The presence of one or more polymorphic sites in the genome of the patients may cause significant changes in the observed enzyme activity. The previously mentioned classification could not be perceived in the obtained activities in this study, as the variability in the class I CYPs was similar to that of the Class II CYPs. Moreover, not all the expected effects of the SNPs on the activity were reflected in the data. As shown in Figure 4, this trend was seen for CYP2D6*4 and *10, 2C9*2 (except for one extreme value), and in the patient homozygote for the CYP2C19*2 null allele. The other effects, however, could not be perceived. Despite the observed trends in activity due to some SNPs, the CYP genotype is not the only predictor of the CYP activity in this population.

The analysis of the abundance of the isoforms in the microsomes may give information on the nature of the possible changes in enzyme activity. The positive correlation between activity and abundance of CYP3A4 could infer that the observed changes are due to pre-translational or translational alterations in patients with hepatic dysfunction. The poorer correlation between CYP2E1 activity and abundance could be due to other mechanisms of alterations or to the small range of the observed abundances.

The Paediatric End-Stage Liver Disease (PELD) score was calculated for each patient at the time of transplantation. The PELD score is used in the liver allocation system for

paediatric transplant patients. This severity-of-illness score expresses the urgency for transplantation, based on the objective and measurable elements age (< 1 year), serum albumin and total bilirubin, INR, and growth failure [16]. The diagnosis is not incorporated in the calculation of the PELD score, as it may lead to discrimination of certain subgroups of patients on the transplant waiting list. In this study, the PELD score was used to estimate the degree of hepatic impairment. Evaluation of the relationship between the PELD score (ranging from 0 to 38) and the ln(activity) showed a negative correlation for all six isoforms. The PELD score appears to be the only factor that could give a certain idea on the CYP status of the patient. The observed negative correlation suggests a progressive decrease in enzymatic activity with deteriorating hepatic disease. This negative correlation is in accordance with the *in vitro* findings in adults [11,12], where the CYP activity was shown to decrease with the Child-Pugh score, an indicator for hepatic dysfunction in adults. Moreover, this negative effect of disease on the CYP activity has recently been described in two *in vivo* studies evaluating the midazolam clearance in children. The clearance of midazolam is determined by CYP3A4/5-mediated clearance, uridine diphosphate glucuronosyltransferase (UGT)-mediated clearance, and renal clearance. Ince et al. [41] studied the age-related changes in midazolam clearance in relation to other covariates, such as specific subpopulations or severity of disease. They showed that critical illness is a major determinant for midazolam clearance in children. Simulations using a PBPK model suggested a major impact of reduced CYP3A4 and 3A5 enzyme abundance on the midazolam clearance. These results were confirmed by another study by Vet et al. [42], where a reduced midazolam clearance was observed with increasing critical illness in children on the paediatric intensive care unit. The authors suggested that a reduced CYP3A activity could be the cause for the reduced midazolam clearance. Our *in vitro* data on CYP3A4 activity support this hypothesis.

The activities of all isoforms in our dataset were correlated positively, meaning that a decrease in activity in one isoform often implies a concomitant decrease in the activities of the others. This correlation, however, gives no information on the degree of the change in activity. In previously published studies evaluating the effects of hepatic impairment on CYP activity in adults, some of the CYPs have been shown more sensitive to the effects of hepatic dysfunction than others. Frye et al [10] suggested a sequential progressive model of hepatic dysfunction in adults, as CYP2C19 was shown to be affected very early in the development of liver failure, whereas CYP2E1 activity decreased later in disease progression. Further research should be conducted in order to develop a similar model for the course of CYP decrease in children with hepatic dysfunction.

In order to evaluate the effects on CYP activity of pathology alone, without confounding contribution of the other abovementioned factors except inducers or inhibitors, the

presented activity data could be compared with activity data obtained in healthy children. This would allow a paired comparison between a patient from the study and a healthy child with the same characteristics (such as age, gender, genotype,..). However, the availability of healthy paediatric liver tissue is, for obvious reasons, limited.

5 CONCLUSION

This article is to our knowledge the first to describe the *in vitro* CYP activity in children with hepatic dysfunction. The severity of hepatic dysfunction, expressed as the PELD score, seems to be a major determinant of the *in vitro* CYP activity in children. Although our results show a wide variability in activity of all CYP studied, we believe this study delivers an important contribution to the increase in knowledge on the pharmacokinetics in a particular paediatric population, i.e. children with severe hepatic dysfunction. These results may provide some support in the improvement of paediatric pharmacokinetic models, but also highlight the difficulties in developing such models.

REFERENCES

[1] Rocchi F, Tomasi P. The development of medicines for children: Part of a series on Pediatric Pharmacology, guest edited by Gianvincenzo Zuccotti, Emilio Clementi, and Massimo Molteni. *Pharmacol Res* 2011; 64(3):169-175.

[2] Kauffman RE, Kearns GL. Pharmacokinetic Studies in Pediatric-Patients - Clinical and Ethical Considerations. *Clin Pharmacokinet* 1992; 23(1):10-29.

[3] Knibbe CAJ, Krekels EHJ, Danhof M. Advances in paediatric pharmacokinetics. *Expert Opin Drug Met* 2011; 7(1):1-8.

[4] Johnson TN, Rostami-Hodjegan A. Resurgence in the use of physiologically based pharmacokinetic models in pediatric clinical pharmacology: parallel shift in incorporating the knowledge of biological elements and increased applicability to drug development and clinical practice. *Pediatric Anesthesia* 2011; 21(3):291-301.

[5] Barrett JS, la Casa Alberighi O, Laer S, Meibohm B. Physiologically Based Pharmacokinetic (PBPK) Modeling in Children. *Clin Pharmacol Ther* 2012; 92(1):40-49.

[6] Knibbe CAJ, Danhof M. Individualized dosing regimens in children based on population PKPD modelling: Are we ready for it? *Int J Pharm* 2011; 415(1-2):9-14.

[7] Verbeeck RK. Pharmacokinetics and dosage adjustment in patients with hepatic dysfunction. *Eur J Clin Pharmacol* 2008; 64(12):1147-1161.

[8] Johnson TN, Boussery K, Rowland-Yeo K, Tucker GT, Rostami-Hodjegan A. A Semi-Mechanistic Model to Predict the Effects of Liver Cirrhosis on Drug Clearance. *Clin Pharmacokinet* 2010; 49(3):189-206.

[9] George J, Murray M, Byth K, Farrell GC. Differential Alterations of Cytochrome-P450 Proteins in Livers from Patients with Severe Chronic Liver-Disease. *Hepatology* 1995; 21(1):120-128.

[10] Frye RF, Zgheib NK, Matzke GR, Chaves-Gnecco D, Rabinovitz M, Shaikh OS *et al.* Liver disease selectively modulates cytochrome P450-mediated metabolism. *Clin Pharmacol Ther* 2006; 80(3):235-245.

[11] Villeneuve JP, Pichette V. Cytochrome P450 and liver diseases. *Curr Drug Metab* 2004; 5(3):273-282.

[12] Elbekai RH, Korashy HM, El-Kadi AOS. The effect of liver cirrhosis on the regulation and expression of drug metabolizing enzymes. *Curr Drug Metab* 2004; 5(2):157-167.

[13] Adam R, Hoti E. Liver Transplantation: The current Situation. *Semin Liver Dis* 2009; 29:3-18.

[14] Sokal E, Goldstein D, Ciocca M, Lewindon P, Ni Y, Silveira T *et al.* End-stage liver disease and liver transplant: Current situation and key issues. *J Pediatr Gastroenterol Nutr* 2008; 47(2):239-246.

[15] Muiesan P, Vergani D, Mieli-Vergani G. Liver transplantation in children. *J Hepatol* 2007; 46(2):340-348.

[16] McDiarmid SV, Anand R, Lindblad AS. Development of a pediatric end-stage liver disease score to predict poor outcome in children awaiting liver transplantation. *Transplantation* 2002; 74(2):173-181.

[17] Wilson ZE, Rostami-Hodjegan A, Burn JL, Tooley A, Boyle J, Ellis SW *et al.* Inter-individual variability in levels of human microsomal protein and hepatocellularity per gram of liver. *Br J Clin Pharmacol* 2003; 56(4):433-440.

[18] Bradford MM. Rapid and Sensitive Method for Quantitation of Microgram Quantities of Protein Utilizing Principle of Protein-Dye Binding. *Anal Biochem* 1976; 72(1-2):248-254.

[19] Yuan R, Madani S, Wei XX, Reynolds K, Huang SM. Evaluation of cytochrome P450 probe substrates commonly used by the pharmaceutical industry to study in vitro drug interactions. *Drug Metab Dispos* 2002; 30(12):1311-1319.

[20] De Bock L, Boussery K, Colin P, De Smet J, T'jollyn H, Van Bocxlaer JFP. Development and validation of a fast and sensitive UPLC-MS/MS method for the quantification of six probe metabolites for the in vitro determination of cytochrome P450 activity. *Talanta* 2012; 89:209-216.

[21] De Bock L, Colin P, Boussery K, Van Bocxlaer J. Development and validation of an enzyme-linked immunosorbent assay for the quantification of cytochrome 3A4 in human liver microsomes. *Talanta* 2012;(doi: 10.1016/j.talanta.2012.05.064).

[22] De Bock L, Colin P, Boussery K, Van Bocxlaer J. Quantification of cytochrome 2E1 in human liver microsomes using a validated indirect ELISA. *J Pharm Biomed Anal* 2014; 88(0):536-541.

[23] Yamazaki H, Shimada T. Effects of arachidonic acid, prostaglandins, retinol, retinoic acid and cholecalciferol on xenobiotic oxidations catalysed by human cytochrome P450 enzymes. *Xenobiotica* 1999; 29(3):231-241.

[24] Hamman MA, Thompson GA, Hall SD. Regioselective and stereoselective metabolism of ibuprofen by human cytochrome P450 2C. *Biochem Pharmacol* 1997; 54(1):33-41.

[25] Rasmussen E, Eriksson B, Oberg K, Bondesson U, Rane A. Selective effects of somatostatin analogs on human drug-metabolizing enzymes. *Clin Pharmacol Ther* 1998; 64(2):150-159.

[26] Venkatakrishnan K, Von Moltke LL, Greenblatt DJ. Effects of the antifungal agents on oxidative drug metabolism - Clinical relevance. *Clin Pharmacokinet* 2000; 38(2):111-180.

[27] Li XQ, Andersson TB, Ahlstrom M, Weidolf L. Comparison of inhibitory effects of the proton pump-inhibiting drugs omeprazole, esomeprazole, lansoprazole, pantoprazole, and rabeprazole on human cytochrome P450 activities. *Drug Metab Dispos* 2004; 32(8):821-827.

[28] Ma B, Prueksaritanont T, Lin JH. Drug interactions with calcium channel blockers: Possible involvement of metabolite-intermediate complexation with CYP3A. *Drug Metab Dispos* 2000; 28(2):125-130.

[29] Fuhr U, Anders EM, Mahr G, Sorgel F, Staib AH. Inhibitory Potency of Quinolone Antibacterial Agents Against Cytochrome-P450Ia2 Activity In vivo and In vitro. *Antimicrob Agents Chemother* 1992; 36(5):942-948.

[30] McLellan RA, Drobitch RK, Monshouwer M, Renton KW. Fluoroquinolone antibiotics inhibit cytochrome P450-mediated microsomal drug metabolism in rat and human. *Drug Metab Dispos* 1996; 24(10):1134-1138.

[31] Tassaneeyakul W, Birkett DJ, Veronese ME, McManus ME, Tukey RH, Quattrochi LC *et al.* Specificity of substrate and inhibitor probes for human cytochromes P450 1A1 and 1A2. *J Pharmacol Exp Ther* 1993; 265(1):401-407.

[32] Kudo S, Uchida M, Odomi M. Metabolism of carteolol by cDNA-expressed human cytochrome P450. *Eur J Clin Pharmacol* 1997; 52(6):479-485.

[33] Rost KL, Brosicke H, Brockmoller J, Scheffler M, Helge H, Roots I. Increase of Cytochrome-P450Ia2 Activity by Omeprazole - Evidence by the C-13-[N-3-Methyl]-Caffeine Breath Test in Poor and Extensive Metabolizers of S-Mephenytoin. *Clin Pharmacol Ther* 1992; 52(2):170-180.

[34] Sousa M, Pozniak A, Boffito M. Pharmacokinetics and pharmacodynamics of drug interactions involving rifampicin, rifabutin and antimalarial drugs. *J Antimicrob Chemother* 2008; 62(5):872-878.

[35] Alcorn J, McNamara PJ. Ontogeny of hepatic and renal systemic clearance pathways in infants - Part I. *Clin Pharmacokinet* 2002; 41(12):959-998.

[36] de Wildt SN. Profound changes in drug metabolism enzymes and possible effects on drug therapy in neonates and children
1. *Expert Opin Drug Metab Toxicol* 2011; 7(8):935-948.

[37] Hines RN. The ontogeny of drug metabolism enzymes and implications for adverse drug events. *Pharmacology & Therapeutics* 2008; 118(2):250-267.

[38] Hines RN. Ontogeny of human hepatic cytochromes P450. *J Biochem Mol Toxicol* 2007; 21(4):169-175.

[39] Boobis A, Watelet JB, Whomsley R, Benedetti MS, Demoly P, Tipton K. Drug interactions. *Drug Metab Rev* 2009; 41(3):486-527.

[40] Rodriguez-Antona C, Ingelman-Sundberg M. Cytochrome P450 pharmacogenetics and cancer. *Oncogene* 2006; 25(11):1679-1691.

[41] Ince I, de Wildt SN, Peeters MYM, Murry DJ, Tibboel D, Danhof M *et al.* Critical Illness Is a Major Determinant of Midazolam Clearance in Children Aged 1 Month to 17 Years. *Ther Drug Monit* 2012; 34(4):381-389.

[42] Vet NJ, de Hoog M, Tibboel D, de Wildt SN. The effect of critical illness and inflammation on midazolam therapy in children. *Pediatric Critical Care Medicine* 2012; 13(1):E48-E50.

CHAPTER 7

MICROSOMAL PROTEIN PER GRAM OF LIVER (MPPGL) IN PAEDIATRIC BILIARY ATRESIA PATIENTS

An edited version of this chapter was published:

L. De Bock, K. Boussery, R. De Bruyne, M. Van Winckel, X. Stephenne, E. Sokal, J. Van Bocxlaer. Microsomal protein per gram of liver (MPPGL) in biliary atresia patients. Biopharmaceutics and Drug Disposition, (2014) DOI: 10.1002/bdd.1895.

'If we knew what it was we were doing,

it would not be called research,

would it?'

- Albert Einstein

ABSTRACT

The microsomal protein per gram of liver (MPPGL) is an important scaling factor in the in vitro-in vivo extrapolation of metabolic data obtained in liver microsomes. This study aimed to determine the MPPGL in 4 biliary atresia patients (0.6 - 1.6 years old) undergoing liver transplantation, as it is known that the MPPGL is affected by age and possibly by liver disease. Due to presence of bilirubin in the homogenates and microsomes, the NADPH-cytochrome reductase activity was used to determine the recovery factor, rather than methods using the dithionite difference spectrum. A mean value of 18.73 (± 2.82) mg/g (geometric mean ± SD, n=4) was observed, which is lower than the expected MPPGL based on the age of the patients (26.60 ± 0.40 mg/g). This suggests a decreased amount of microsomal protein in the livers of biliary atresia patients. Moreover, no differences in MPPGL between different zones of the liver could be detected.

1 INTRODUCTION

In the pre-clinical development of drugs, in vitro experiments are performed to obtain meaningful estimates of the in vivo pharmacokinetic behaviour of drugs prior to administration to humans [1]. The in vitro-in vivo extrapolation (IVIVE) procedure can be divided into different components. Firstly, the kinetic parameters are expressed as a rate per functional unit of the in vitro system under which in vitro incubations were performed. Then, a scaling factor is necessary to express the rate in function of the whole liver capacity. This step is followed by multiplication by liver weight (experimentally determined or calculated following Johnson et al [2]). Finally, other physiological parameters, such as hepatic blood flow, drug-protein binding, and hepatic transporters, are taken into account to predict an in vivo clearance [3-5].

The in vitro systems used to investigate drug metabolism are hepatic slices, hepatocytes, liver microsomes and recombinantly expressed enzymes. The prediction of hepatic clearance using hepatocytes was shown to be of high fidelity and very accurate [1]. The downside of this in vitro system is the limited availability of suitable fresh human tissue for hepatocyte preparation, especially in specific populations [1,6]. An advantage of the use of hepatocytes is the straightforward scaling factor that can be used to scale the results from the in vitro incubation to the whole liver, by just multiplying the data/million cells with the hepatocellularity [4]. As an alternative, hepatic microsomes are often used, as they have a high stability for years of storage at -80°C [7]. However, a more complicated scaling factor is needed. Joly et al. were the first to investigate the loss of microsomal enzymes during the destructive process of differential centrifugation to isolate the microsomal fraction [8]. The microsomal protein per gram liver (MPPGL) is the theoretical estimate that accounts for the inefficiency of the fractionation process in recovering the microsomal protein from liver samples [3]. This factor is necessary to scale to units of liver weight with sufficient accuracy.

In 2007, Barter et al reviewed the reporting of MPPGL data since the 1970's [3]. In many published studies, researchers used estimations of the MPPGL obtained in laboratory animals, despite their smaller diversity in genetics and environment compared to the variation in humans [9]. Human MPPGL values of 40 mg/g were assumed as being correct for healthy adults [4], whereas others established a value of 33 mg/g (with a CV% of 25%) [9]. The latter result was also used by Johnson et al. in a paediatric physiologically based pharmacokinetic (PBPK) model, due to the absence of a clear paediatric MPPGL value [10], irrespective of the results published by Pelkonen et al. in 1973, i.e. 26 mg/g, in 11 foetal liver samples [11]. Johnson et al. justified the use of this value by suggesting a similar MPPGL in children at different ages, as the smooth

endoplasmatic reticulum is fully developed in the stage of late gestation. However, multiple linear regression analysis of the pooled data of previously reported MPPGL values identified age as a significant covariate of the MPPGL [3], with a decrease in MPPGL with increasing age. Further extension of the obtained log-linear model with additional paediatric information (4 Caucasian livers and the results reported by Pelkonen et al [11]) leads to an equation (Eq. 1) to describe the nonmonotonic relationship between age and MPPGL [12]:

MPPGL= 10^(1.407+0.0158*age-0.00038*age²+0.0000024*age³) (age in years)(Eq. 1)

In short, MPPGL increases from birth (with a value about 36% lower than in 25 year old adults) to reach a maximum around the age of 28 of 40 mg/g. Afterwards, a decreasing trend is observed with values of 29 mg/g at the age of 65. The above mentioned equation has been used in several studies to calculate the MPPGL of the used liver samples [13,14].

Besides the influence of age, liver disease might also affect the MPPGL. Hakooz et al. stated that it cannot be assumed that the values obtained in previous studies will also apply to patients with compromised liver function, for example in hepatitis [4]. In some studies, the expected change in MPPGL does not have to be specified as such, as it will possibly be subsumed in the altered liver size and decreased functional hepatocyte volume [15]. However, when the functional hepatocyte volume is not determined, the need for a reliable value of the MPPGL remains.

Additionally, it is possible that the MPPGL differs across the lobes of a human liver [9]. No information is available on the zonal distribution of the MPPGL, but in rat, a significant zonal difference in CYP450 content per mg microsomal protein has been reported by Sumner et al [16]. In contrast, Joly et al. reported no difference in CYP450 content in homogenate between different parts of the rat liver [8]. Another study reported the CYP450 content per mg microsomal protein on 10 different locations in one human liver [17], where no zonal differences were observed.

Variance in both the content and activity of drug-metabolizing enzymes of the liver may have a direct effect on risk-relevant PK outcomes [18]. In that context, we recently published a study on the metabolic capacity of the cytochrome P450 (CYP) enzyme system in children with severe hepatic dysfunction [19]. The current follow up study aimed to determine the MPPGL in liver samples from children with biliary atresia, the most common indication for paediatric liver transplantation [20], in order to provide a useful scaling factor for in vitro experiments in this specific population. Moreover,

different zones of the liver were compared in order to map out possible zonal differences in MPPGL.

2 MATERIALS AND METHODS

2.1 Chemicals and reagents

Equine heart cytochrome c and potassium cyanide were obtained from Sigma-Aldrich (Buchs, Switserland), NADPH from Biopredic International (Rennes, France). All other chemicals were purchased from VWR International (Leuven, Belgium).

2.2 Liver samples: collection and processing

The collection of liver samples was executed according to the recommendations of the Ethical Committee of Ghent University Hospital (B67020084281), and in accordance with approval of the ethical committee of Saint-Luc Clinics Brussels. Samples were taken from the explanted livers of children undergoing liver transplantation due to biliary atresia in one of the aforementioned university hospitals. The intact livers were weighed, and subsequently blocks of approximately 1 cm^3 were taken from 4 different zones from the liver (right lobe and left lobe, deep and peripheral). Samples were snap frozen in liquid nitrogen within 15 minutes after explantation and stored at -80°C until preparation of microsomes. The patient information of the 4 included biliary atresia patients is summarized in Table 1.

Table 1: Patient information

Patient	Age (years)	PELD
1	0.66	22
2	1.64	21
3	0.99	17
4	1.39	13

Hepatic microsomes were prepared from each separate zone as previously described [19]. In short, the weighed samples were minced with scissors and homogenized in homogenization buffer (0.25 M phosphate buffer, pH 7.25, 1.15% KCl) using an automated Potter-Elvehjem system (VWR, Leuven, Belgium). An aliquot of the tissue homogenate was kept aside, while the remaining homogenates were centrifuged at 10,000g for 15 minutes at 4°C (Beckman L8-70M Ultracentrifuge, Beckman Coulter Limited, High Wycombe, Buckinghamshire, UK). Subsequently, the resulting supernatant (S9 fraction) was centrifuged at 100,000g for 75 minutes at 4°C, followed by a washing step of the microsomal pellet. Centrifugation at 100,000g was repeated, and the final

microsomal pellet was resuspended in 1.5 volumes of resuspension buffer (homogenization buffer, containing 30% v/v glycerol). Both homogenate and microsomal suspension were snap-frozen in liquid nitrogen and stored at -80°C until analysis. The total protein content of the microsomes was estimated using the method of Bradford [21].

2.3 Determination of the NADPH-cytochrome reductase activity and calculation of the microsomal protein per gram of liver (MPPGL)

The microsomal recovery factor was estimated through the analysis of the NADPH-cytochrome P450 reductase (NCR) activity in homogenate and microsomes according to Guengerich et al. [22]. The NCR activity was determined on the homogenates and microsomes from each zone separately, as well as on pools of homogenate and microsomes, prepared for each patient, by mixing the four different zones according to their relative weight. Homogenate and microsomes were diluted to contain the same amount of liver tissue (minimum 50 mg/ml). Equine heart cytochrome c stock solution was prepared (0.5 mM in 10 mM potassium phosphate buffer pH 7.7) and frozen until use. Eighty µl of cytochrome c (0.5 mM), 900 µl of 0.3 M potassium phosphate buffer (pH 7.7) containing 1 mM KCN and 10 µl of the diluted homogenate or microsomes were mixed. Ten µl of reduced NADPH solution (10 mM in water, prepared daily) was added to initiate the cytochrome c reduction. After mixing of the sample, the absorbance at 550 nm was monitored for 4 minutes. The rate of cytochrome c reduction was calculated following Eq. 2:

ΔA550/min/0.021 = nmol of cytochrome c reduced per minute in the cuvette (1 mL)(Eq. 2)

with 0.021 mM-1 cm-1 being the extinction coefficient of reduced cytochrome c. The microsomal recovery factor was provided by the ratio of the reductase activity in the microsomes and in the homogenate. The MPPGL was consequently calculated by the following equation (Eq. 3):

MPPGL (mg g^{-1}) = yield of microsomal protein (mg g^{-1}) / microsomal recovery factor (Eq.3)

Comparison of the observed and expected MPPGL was done using a paired t-test. The MPPGL values of the different zones were compared using ANOVA (SPSS). P-values equal to or below 0.05 were considered significant.

3 RESULTS AND DISCUSSION

In vitro - in vivo extrapolations require scaling factors for the specific in vitro system that was used. Besides the known influence of age on the MPPGL, it is not clearly known whether the values or equations suggested in the literature are applicable to samples from diseased livers. Therefore, this study aimed to determine the MPPGL in children with biliary atresia, in support of the study attempting to characterize CYP activity in this patient population.

3.1 Determination of the recovery factor in cholestatic livers: methodological considerations

In order to calculate the MPPGL, the amount of microsomal protein needs be quantified. Also, a microsome specific marker needs to be determined in order to calculate the microsomal recovery factor. Up until now, 2 methods were generally used for this purpose: the spectrophotometric determination of the total CYP450 content in homogenate and microsomes through the dithionite difference spectrum at 450 nm [23,24], and a method based on the determination of the NADPH-cytochrome P450 reductase (NCR) activity [3,25]. Another method that was applied in some studies was based on the immunoquantification of total CYP levels or of one or more individual CYP isoforms [26]. This method, however, may not be accurate, as the antibodies may not asses active (holo-) protein alone, but also apo-protein [9]. The comparison of the 2 frequently used methods showed that the resulting scaling factors were comparable [4,9,11,27]. Therefore, the choice of method does not affect the final calculated scaling factor.

In both methods, some assumptions should be made [4,9]. In the spectrophotometric determination, it is assumed that all the CYP450 enzymes in the homogenate are microsomal in origin. This was confirmed by Guengerich et al., who showed that over 96% of the CYPs in the liver are microsomal [28]. As for the determination of the NCR activity, a comparable assumption should be made, i.e. that all reductase activity is microsomal. Paine et al. [29] showed the existence of a cytosolic reductase, which was found to be 5.8% of the total homogenate reductase activity by Wilson et al. [9]. However, Hakooz et al. concluded that no correction for nonmicrosomal expression of the enzyme was necessary [4]. Nevertheless, potassium cyanide was added to the reaction medium in order to inhibit other reductases, and thus prevent interference of other reductases [22].

The methods described by Omura [23] and Matsubara [24] were not suitable for the type of samples used in this study, i.e. liver samples originating from diseased and cholestatic

livers. The included patients typically have (very) high serum total bilirubin levels (patients 1-4: 15.4 – 38.3 mg/dL – reference interval 0.3 – 1.2 mg/dL). The large amount of bilirubin still present in the samples (as seen by the yellow colour of the homogenates and microsomes) is believed to interfere with the dithionite difference method (λ 450 nm and 490 nm), as bilirubin shows an absorption maximum at 453 nm (see Figure 1). Despite the use of a reference sample, the interference by bilirubin could not be eliminated. Moreover, the obtained spectral differences between the reference and sample cuvettes were too small to be reliable. This method was therefore not applicable for the samples from diseased, often cholestatic, livers. Also, to our impression, the spectrophotometric analysis of any sample with that much turbidity is not reliable.

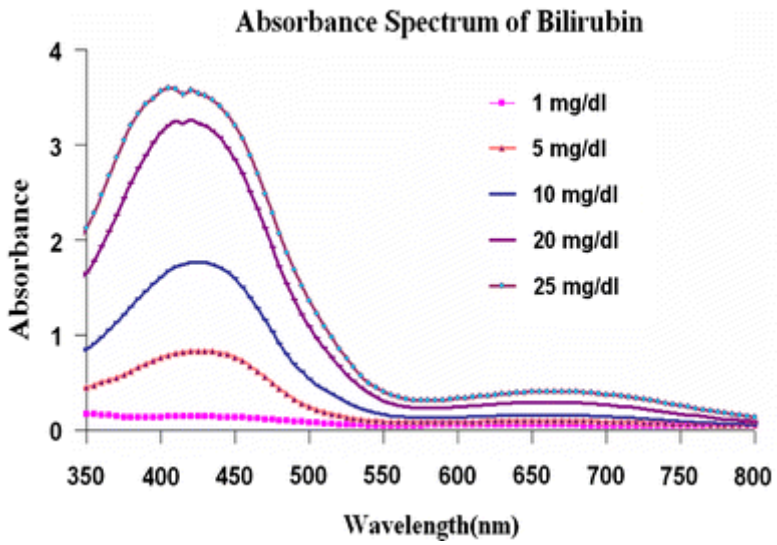

Figure 1: Absorbance spectrum of bilirubin (adapted from Bhadri et al. [30])

The NCR method is based on the catalysis of electron transfer from NADPH to cytochrome c [25]. The increased amount of reduced cytochrome c increases the absorption at 550 nm, a wavelength at which much less interference of bilirubin is expected. Moreover, due to the small amount of sample (10 µl) in the total reaction volume (1000 µl), less interference due to turbidity is encountered. The method was optimized following the recommendations of Guengerich et al. [22]. Achour et al. described a protocol in which the addition of 1 µg liver tissue was sufficient [31]. However, no increase in absorbance was observed when using this amount. According to the recommendations of Guengerich et al., more of the biological sample was added, as this probably meant the concentration of reductase was too low. The analysis of several samples showed that a concentration of at least 50 mg liver tissue/ml was necessary to obtain the recommended 0.02 increase in absorbance unit per minute. Moreover, the assay was carried out in the recommended absorbance range of 0.05 – 1.0 in order to

have sufficient absorbance to allow accurate measurements. A representative dynamic spectrum is shown in Figure 2.

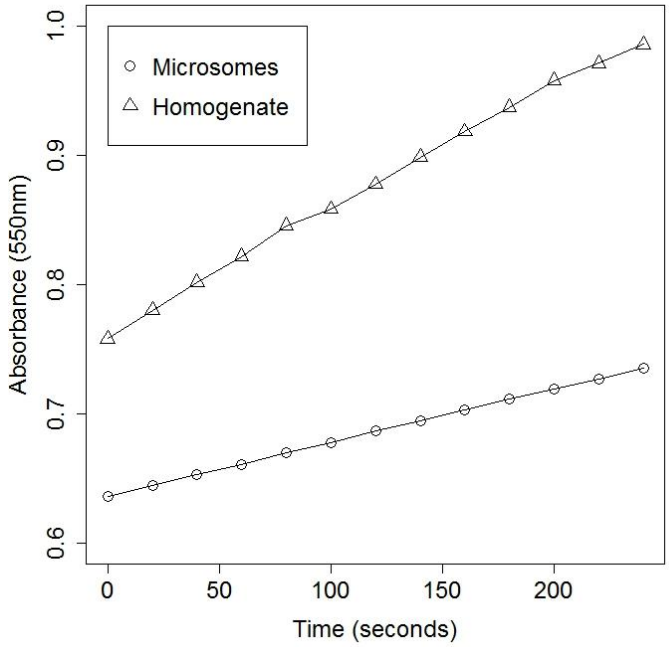

Figure 2: Dynamic spectra of the determination of the NCR activity in the homogenate and microsomes from the LP (left peripheral) sample from patient 3. 66 mg liver tissue/ml was added to the reaction medium. NADPH was added at t = 0 seconds. The recovery factor was calculated based on the slopes of the graphs.

The above described methods for the determination of the MPPGL both rely on the mixed function oxidase system enzymes, i.e. cytochrome P450 and NADPH-cytochrome P450 reductase. Miyagi et al. suggested that possibly a different scalar for the MPPGL may be needed in studies evaluating enzymes other than CYP/NCR [14].

3.2 Estimation of the MPPGL in pre-transplant biliary atresia patients: results

In accordance to the incubation experiments previously performed in this study [19], pools were made based on the amount of liver, in order to rule out possible zonal differences. The NCR activity was determined in the homogenates and microsomes, and the recovery factor was calculated. The results are summarized in Table 2. Low recovery factors were seen for some samples, probably due to the cirrhotic nature of the samples and the consequent longer and stronger homogenization needed. A mean (±SD) MPPGL of 18.73 (±2.82) mg/g was determined in patients with biliary atresia. According to the equation reported by Barter et al. (Eq. 1) [12], however, a mean (±SD) MPPGL of 26.60 (±0.40) mg/g was expected based on the age of the patients (see Table 2). The

significant difference between the observed and the expected MPPGL ($p < 0.01$), suggests a decreased MPPGL in biliary atresia patients.

Table 2: Total microsomal protein content before and after correction. The mean MPPGL was estimated at 18.73 mg/g liver tissue. The expected MPPGL was calculated based on Eq. 1, as previously reported by Barter et al. [12].

Patient	Uncorrected microsomal protein content per gram liver (mg/g liver)	Recovery factor	Corrected MPPGL (mg/g liver)	Expected MPPGL (mg/g liver)
1	6.10	0.28	21.81	26.13
2	7.63	0.50	15.26	27.04
3	9.65	0.53	18.34	26.44
4	4.27	0.21	20.28	26.81
			geometric mean	
			18.73 (± 2.82)	26.60 (± 0.40)

3.3 Evaluation of the zonal differences in MPPGL

The possible difference in MPPGL between different sampling locations of the liver was investigated through the comparison of 4 zones (left lobe deep (LD) – left lobe peripheral (LP) – right lobe deep (RD) – right lobe peripheral (RP)). The same methods were applied as for the pooled samples and the MPPGL was calculated for each zone separately (Table 3). No significant difference was seen between the 4 zones (ANOVA, $p>0.05$). Due to the high variability, a high number of samples would be needed to detect a possible zonal difference. Therefore, it is recommended to keep using pooled samples from different zones of the liver in characterization experiments, such as the determination of the MPPGL or enzyme phenotyping reactions.

Table 3: MPPGL measured in 4 zones of the liver, in 4 different patients (left lobe deep (LD) – left lobe peripheral (LP) – right lobe deep (RD) – right lobe peripheral (RP)).

	MPPGL (mg/g liver)				
Patient	**RD**	**RP**	**LD**	**LP**	**POOL**
1	19.02	16.90	26.09	27.54	21.81
2	17.69	15.67	13.61	14.31	15.26
3	13.62	13.25	16.24	20.19	18.34
4	28.67	16.89	12.60	19.05	20.28

4 CONCLUSION

In conclusion, the MPPGL in biliary atresia patients undergoing liver transplantation should be determined by using the NADPH-cytochrome reductase activity rather than methods using the dithionite difference spectrum, due to the presence of bilirubin. A mean value of 18.73 mg/g was observed in 4 patients, which was shown to be lower than the expected MPPGL. This suggests a decreased amount of microsomal protein in these pathological livers. Moreover, no zonal differences in MPPGL could be detected.

The presented results have some limitations due to which they should be interpreted with caution. Firstly, the results are based on a low number of included patients (n = 4). This is due to the dependency of available samples in the cooperating transplantation centres. Also, the comparison of the observed MPPGL with the expected MPPGL does not take into account the inter-individual variability of the MPPGL. Despite these limitations, the findings are considered relevant in the light of filling the knowledge gap on specific paediatric patient populations.

5 REFERENCES

[1] Houston JB, Carlile DJ. Prediction of hepatic clearance from microsomes, hepatocytes, and liver slices. *Drug Metab Rev* 1997; 29(4):891-922.

[2] Johnson TN, Tucker GT, Tanner MS, Rostami-Hodjegan A. Changes in liver volume from birth to adulthood: A meta-analysis. *Liver Transplantation* 2005; 11(12):1481-1493.

[3] Barter ZE, Bayliss MK, Beaune PH, Boobis AR, Carlile DJ, Edwards RJ *et al.* Scaling factors for the extrapolation of in vivo metabolic drug clearance from in vitro data: Reaching a consensus on values of human microsomal protein and hepatocellularity per gram of liver. *Curr Drug Metab* 2007; 8(1):33-45.

[4] Hakooz N, Ito K, Rawden H, Gill H, Lemmers L, Boobis AR *et al.* Determination of a human hepatic microsomal scaling factor for predicting in vivo drug clearance. *Pharm Res* 2006; 23(3):533-539.

[5] Ito K, Houston JB. Comparison of the use of liver models for predicting drug clearance using in vitro kinetic data from hepatic microsomes and isolated hepatocytes. *Pharm Res* 2004; 21(5):785-792.

[6] Carlile DJ, Hakooz N, Bayliss MK, Houston JB. Microsomal prediction of in vivo clearance of CYP2C9 substrates in humans. *Br J Clin Pharmacol* 1999; 47(6):625-635.

[7] Pearce RE, McIntyre CJ, Madan A, Sanzgiri U, Draper AJ, Bullock PL *et al.* Effects of freezing, thawing, and storing human liver microsomes on cytochrome P450 activity. *Arch Biochem Biophys* 1996; 331(2):145-169.

[8] Joly JG, Doyon C, Pesant Y. Cytochrome-P-450 Measurement in Rat-Liver Homogenate and Microsomes - Its Use for Correction of Microsomal Losses Incurred by Differential Centrifugation. *Drug Metab Dispos* 1975; 3(6):577-586.

[9] Wilson ZE, Rostami-Hodjegan A, Burn JL, Tooley A, Boyle J, Ellis SW *et al.* Inter-individual variability in levels of human microsomal protein and hepatocellularity per gram of liver. *Br J Clin Pharmacol* 2003; 56(4):433-440.

[10] Johnson TN, Rostami-Hodjegan A, Tucker GT. Prediction of the clearance of eleven drugs and associated variability in neonates, infants and children. *Clin Pharmacokinet* 2006; 45(9):931-956.

[11] Pelkonen O, Kaltiala EH, Larmi TKI, Karki NT. Comparison of Activities of Drug-Metabolizing Enzymes in Human Fetal and Adult Livers. *Clin Pharmacol Therap* 1973; 14(5):840-846.

[12] Barter ZE, Chowdry JE, Harlow JR, Snawder JE, Lipscomb JC, Rostami-Hodjegan A. Covariation of human microsomal protein per gram of liver with age: Absence of influence of operator and sample storage may justify inter laboratory data pooling. *Drug Metab Dispos* 2008; 36(12):1-5.

[13] Miyagi SJ, Collier AC. The Development of UDP-Glucuronosyltransferases 1A1 and 1A6 in the Pediatric Liver. *Drug Metab Dispos* 2011; 39(5):912-919.

[14] Miyagi SJ, Milne AM, Coughtrie MWH, Collier AC. Neonatal Development of Hepatic UGT1A9: Implications of Pediatric Pharmacokinetics. *Drug Metab Dispos* 2012; 40(7):1321-1327.

[15] Johnson TN, Boussery K, Rowland-Yeo K, Tucker GT, Rostami-Hodjegan A. A Semi-Mechanistic Model to Predict the Effects of Liver Cirrhosis on Drug Clearance. *Clin Pharmacokinet* 2010; 49(3):189-206.

[16] Sumner IG, Lodola A. Total Cytochrome-P-450, But Not the Major Phenobarbitone Or 3-Methylcholanthrene Induced Isoenzyme, Is Differentially Induced in the Lobes of the Rat-Liver. *Biochem Pharmacol* 1987; 36(3):391-393.

[17] Watkins PB, Murray SA, Thomas PE, Wrighton SA. Distribution of Cytochromes-P-450, Cytochrome-B5, and Nadph-Cytochrome-P-450 Reductase in An Entire Human Liver. *Biochem Pharmacol* 1990; 39(3):471-476.

[18] Lipscomb JC, Teuschler LK, Swartout JC, Striley CAF, Snawder JE. Variance of microsomal protein and cytochrome P450 2E1 and 3A forms in adult human liver. *Toxicology Mechanisms and Methods* 2003; 13(1):45-51.

[19] De Bock L, Boussery K, Van Winckel M, De Paepe P, Rogiers X, Stephenne X *et al.* In Vitro Cytochrome P450 Activity Decreases in Children with High Pediatric End-Stage Liver Disease Scores. *Drug Metab Dispos* 2013; 41(2):390-397.

[20] Sokal E, Goldstein D, Ciocca M, Lewindon P, Ni Y, Silveira T *et al.* End-stage liver disease and liver transplant: Current situation and key issues. *J Pediatr Gastroenterol Nutr* 2008; 47(2):239-246.

[21] Bradford MM. Rapid and Sensitive Method for Quantitation of Microgram Quantities of Protein Utilizing Principle of Protein-Dye Binding. *Anal Biochem* 1976; 72(1-2):248-254.

[22] Guengerich FP, Martin MV, Sohl CD, Cheng Q. Measurement of cytochrome P450 and NADPH-cytochrome P450 reductase. *Nat Protocols* 2009; 4(9):1245-1251.

[23] Omura T, Sato R. Carbon Monoxide-Binding Pigment of Liver Microsomes .I. Evidence for Its Hemoprotein Nature. *Journal of Biological Chemistry* 1964; 239(7):2370-&.

[24] Matsubara T, Koike M, Touchi A, Tochino Y, Sugeno K. Quantitative determination of cytochrome P-450 in rat liver homogenate. *Anal Biochem* 1976; 75(2):596-603.

[25] Strobel HW, Dignam JD. Purification and properties of NADPH-Cytochrome P-450 reductase. In: Sidney Fleischer and Lester Packer, ed. *Methods in Enzymology Biomembranes - Part C: Biological Oxidations*. Academic Press. 1978: 89-96.

[26] Lipscomb JC, Fisher JW, Confer PD, Byczkowski JZ. In vitro to in vivo extrapolation for trichloroethylene metabolism in humans. *Toxicol Appl Pharmacol* 1998; 152(2):376-387.

[27] Pelkonen O, Kaltiala EH, Larmi TKI, Karki NT. Cytochrome P-450-Linked Monooxygenase System and Drug-Induced Spectral Interactions in Human Liver-Microsomes. *Chem Biol Interact* 1974; 9(3):205-216.

[28] Guengerich FP. Similarity of Nuclear and Microsomal Cytochromes-P-450 in the Invitro Activation of Aflatoxin-B-1. *Biochem Pharmacol* 1979; 28(19):2883-2890.

[29] Paine MJI, Garner AP, Powell D, Sibbald J, Sales M, Pratt N *et al.* Cloning and characterization of a novel human dual flavin reductase. *Journal of Biological Chemistry* 2000; 275(2):1471-1478.

[30] Bhadri PR, Kumar SA, Salgaonkar VA, Beyette FR, Clark JF. Development of an integrated hardware and software platform for the rapid detection of cerebral aneurysm rupture. *Analog Integrated Circuits and Signal Processing* 2008; 56(1-2):127-134.

[31] Achour B, Barber J, Rostami-Hodjegan A. Cytochrome p450 pig liver pie: determination of individual cytochrome p450 isoform contents in microsomes from two pig livers using liquid chromatography in conjunction with mass spectroscopy. *Drug Metab Dispos* 2011; 39(11):2130-2134.

CHAPTER 8

PREDICTION OF THE PHARMACOKINETICS OF OMEPRAZOLE AND MIDAZOLAM IN CHILDREN WITH LIVER DISEASE: ARE WE THERE YET?

'Science never solves a problem

without creating ten more'

- George Bernard Shaw

ABSTRACT

The effects of liver disease on the PK of children can be predicted using population-specific PBPK models. This, however, requires the incorporation of disease related physiological changes into the model. A decrease in cytochrome P450 activity with deteriorating liver disease, as expressed by an increasing paediatric end-stage liver disease (PELD) score, was observed in a previous study. By introducing several assumptions, the activity results from this previous study were transformed into abundance data after dividing the patients in 4 categories based on the PELD score (A: 0-10, B: 11-20, C: 21-30, D: 31-40). The PK of the model compounds omeprazole and midazolam was then predicted using the SimCYP® PBPK model. Large increases in the AUC of omeprazole were predicted in patients with a high PELD score (C or D). The same trend was observed for the plasma concentration of midazolam, where increases up to 5 fold compared to healthy children were predicted in patients with a PELD score of 10 or higher. The adjustments done to the available paediatric model in order to create a diseased population are however not sufficient to capture the complex combination of physiological changes in liver disease. Nevertheless, the sole influence of changes in CYP abundance on the PK already provides valuable information, not in the least regarding more future (data)requirements to bridge the knowledge gap.

1 CASE

T., a boy with biliary atresia, underwent living donor liver transplantation from his mother at the age of 8 months. After undergoing a Kasai portoenterostomy at a younger age, his condition deteriorated and a transplant was considered the only treatment option. On the admission for the surgery, the patient's weight was 6.4 kg and his length 64 cm. His albumin level was 3 g/dl (3.4-4.8 g/dl), INR 1.6, bilirubin 15.1 mg/dl (0.3-1.2 mg/dl), ALT 235 IU/l (14-63 IU/l), and AST 568 (6-33 IU/l). At the time of transplantation he had a PELD score of 19. Prior to the transplantation, the boy needed several drugs to relieve symptoms associated with common complications of liver disease: omeprazole 5 mg once daily to treat reflux symptoms, furosemide (6 mg twice daily) and spironolactone (30 mg per day) to treat ascites and portal hypertension, and vitamin E and K to avoid hypovitaminosis due to the fat-malabsorption associated with intestinal bile acid deficiency. The boy also received co-trimoxazole (trimethoprim 40 mg and sulfamethoxazole 8 mg, twice a day) to treat cholangitis.

2 INTRODUCTION

T. is only one of the children being treated for biliary atresia and its complications. In Western Europe, biliary atresia occurs in 1/18000 live births [1]. The extent of the disease is different among patients [2]. The initial management consists of a surgical intervention (the Kasai portoenterostomy), in order to restore some bile flow [3]. However, due to the progressive nature of the disease, all children will develop portal fibrosis, cirrhosis and portal hypertension, to a greater or lesser extent [4]. They need pharmacological treatment for the associated complications, such as hypovitaminosis, portal hypertension and ascites.

Liver disease is known to influence drug PK in adults [5], and dose adjustments are recommended in several cases, depending on the PK and toxicity properties of the drug, as well as the severity of liver dysfunction [5,6]. In paediatrics, however, similar dose adjustments are not yet common practice. The dosing regimens used in these children are generally based on the bodyweight or body surface area of the child, as applied for all children, with or without co morbidity. In the cooperating centres of our study, currently only the dose of paracetamol is consistently lowered (10 mg/kg instead of 15 mg/kg). Based on the information in adults, and on the results of our study indicating a considerable influence of liver disease on the CYP activity in children (see Chapter 6 and [7]), we believe dose adjustments could be necessary for other drugs to avoid toxic reactions in or mistreatment of children with liver diseases. Therefore, we would like to investigate whether these patients actually receive the optimal dose to treat their

symptoms or to conquer the infection and to avoid possible adverse or toxic reactions of the drug.

Due to the many practical and ethical difficulties encountered in all paediatric clinical trials [8,9], obtaining this information by performing an actual trial in children with liver disease is not obvious. Physiologically based pharmacokinetic (PBPK) modelling could offer an *in silico* approach to predict the exposure to drugs in this vulnerable population [10]. PBPK models are multi-scale models containing both system-specific (patient-specific) and drug-specific properties. A specific target population can be modelled when specificities linked to particular organs and to a given population are used to build the model. In order to develop disease-specific models, disease-related physiological changes need to be accurately identified, as PBPK models rely on accurate and consistent information on physiological, biochemical and physicochemical processes [11,12]. A PBPK model for the prediction of the effect of liver cirrhosis on the PK of IV drugs in adults was successfully developed by Edginton and Willmann [13], and extended for oral drugs by Johnson et al. [14]. The model contains the physiological changes in liver cirrhosis, such as organ blood flows, cardiac index, plasma binding proteins, hematocrit, functional liver volume, hepatic enzyme activity, and glomerular filtration rate. These models focus on an adult patient population, and are therefore not suitable for predictions in children, as the changes in children may not be comparable to those in adults. Moreover, the ontogeny information also needs to be taken into account when performing predictions in a paediatric population.

To our knowledge, the paediatric PBPK models published up until now incorporated the maturation profiles of the enzymes or other systems, but no study was found in which a disease state, and more particularly liver disease, was implemented. Therefore, this exercise aimed to use the available algorithm for paediatric predictions, and to extend it with the obtained in vitro data on CYP activity in children with liver disease [7]. The model was used for the prediction of the PK of two model compounds, omeprazole and midazolam. Additionally, due to the exploratory nature of this pilot study, it is expected that the information gaps that hamper further optimization of the model will be revealed.

3 METHODS

3.1 SimCYP® Population Based ADME simulator: substrate parameters and clinical trial settings

The Paediatric module of the SimCYP® ADME simulator [15] was used to simulate clinical trials. The prediction of the pharmacokinetics in healthy children of the model compounds midazolam (CYP3A4) and omeprazole (CYP2C19 and 3A4) by SimCYP® was previously

validated by Johnson et al. [10]. The incorporation of developmental changes in physiology and in CYP450 expression led to the development of the paediatric module within the software. The substrate information, as incorporated in SimCYP® is described in Table 1. Simulated median clearance values were within 2- fold of the observed values.

Table 1: Substrate information required to predict the PK of midazolam and omeprazole with SimCYP®, adapted from Johnson et al. [10]. Abbreviations: 1-HMDZ = 1-hydroxymidazolam; 4-HMDZ =4-hydroxymidazolam; 5-HOME = 5-hydroxyomeprazole; B/P = blood plasma ratio; CL_R = renal clearance;; f_a = fraction absorbed; f_u = fraction unbound in plasma; f_{uGut} = unbound fraction in gut (enterocytes); f_{uMic} = unbound fraction in microsomes; HLM = human liver microsomes; Km = Michaelis-Menten constant; MW = molecular weight; OME-S = omeprazole sulphone; Q_{Gut} = drug specific nominal blood flow/permeability descriptor; V_{max} = maximum rate.

	Midazolam		**Omeprazole**			
MW (g/mol)	325.8		345.4			
f_a	1		1			
f_u	0.034		0.065			
f_{uMic}	0.922		0.729			
B/P	0.55		0.6			
CL_R adult (L/h)	0.085		0.034			
CYP involved	**3A4**	**3A4**	**3A4**	**2C8**	**2C19**	**3A4**
Metabolite	1-HMDZ	4-HMDZ	OME-S	5-HOME	5-HOME	5-HOME
Km (µmol/L)	6.35	56.7	71	300	12.2	50
V_{max} ((pmol/min/pmol CYP) or (pmol/min/mg protein))	20.6	20.7	18.2	3.3	10.8	10.9
System [a]	HLM		Yeast [b]			
Q_{Gut}	20		/			
f_{uGut}	1		/			

[a] Microsomal system used to generate the enzyme PK parameters
[b] *Saccaromyces Cerevisiae* cells

In order to predict the PK, a virtual clinical study was performed in 1000 virtual patients aged 0-2 years, for each PELD category, as well as for healthy children. Dosing of midazolam was set at an intravenous bolus of 0.1 mg/kg, followed by a 24 h infusion of 0.05 mg/kg/h (dose regimen for postoperative sedation [16]). The plasma concentration – time profiles and the concentration at steady state were evaluated, as this PK parameter is correlated to the degree of sedation. For omeprazole, a single dose of 1 mg/kg of an oral solution was virtually administered. As the area under the plasma concentration – time curve (AUC) is correlated with the acid suppression [17], this parameter was compared between the groups.

3.2 SimCYP® Population Based ADME simulator: population parameters

In order to evaluate the difference between the healthy paediatric population and the target population, i.e. children with liver disease, several 'what if' scenarios were created at the level of the abundance of the CYP isoforms. All the other population parameters defined in SimCYP® were left unchanged.

The results from a previous study were used to adjust the population parameters [7]. The in vitro CYP activity of the six most important CYP isoforms (CYP1A2, 2C9, 2C19, 2D6, 2E1, and 3A4) was determined in microsomes from paediatric patients undergoing liver transplantation for various indications [7]. Additionally, the abundance of CYP2E1 and 3A4 was determined. In order to obtain a more homogenous data set, only the results of the 23 patients diagnosed with biliary atresia were selected for the clinical trial simulations. Due to the small number of patients, 4 categories (A to D) were created based on the PELD score, as depicted in Table 2.

Table 2: Grouping of the biliary atresia patients into 4 categories following PELD score.

Category	PELD score	n	Mean age (years)
A	0 - 10	5	1.30
B	11 - 20	8	0.95
C	21 - 30	5	0.72
D	31 - 40	5	0.71

As information on the abundance, rather than activity, is needed to insert into the software for the PBPK model, and abundance information was experimentally obtained only for CYP2E1 and 3A4, a strategy was developed to convert the available activity data into usable abundance data for the model. Several assumptions were made during this process, thus necessitating caution in the interpretation of the final results. Figure 1 shows a summary of the assumptions and the calculations, as described hereafter.

Due to the high variability of the activities of the isoforms, the percentage of the mean adult activity was calculated for each of the patients, as described in De Bock et al. [7]. These data show a relative result, instead of an absolute activity, and create a more uniform result for all isoforms.

For CYP3A4, and in a lesser extent for CYP2E1, a positive correlation between the activity and abundance was observed (Figure 2). As there is no experimentally obtained information on the abundance of the other 4 studied isoforms (CYP1A2, 2C9, 2C19 and 2D6), the same mechanism of changes was assumed as for CYP3A4 (and 2E1). Consequently, *assumption 1 states that the abundance and the activity are correlated for*

all isoforms. Following this assumption, *assumption 2* can be made. This states that *the calculated fraction of the adult activity can be considered equal to the fraction of the adult abundance*. As such, we can convert the available results (activity) into the needed information (abundance).

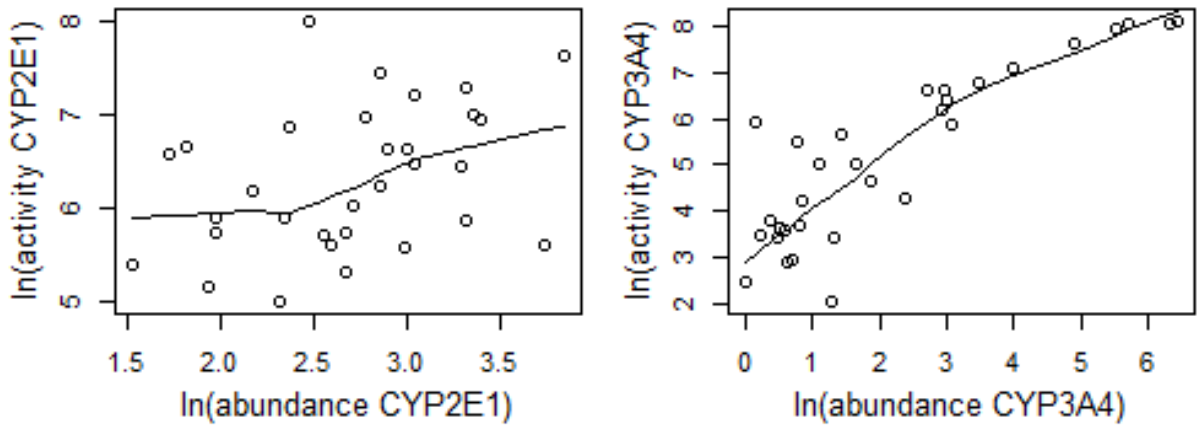

Figure 2: Positive correlation between the activity and the abundance of CYP3A4, and to a lesser extent of CYP2E1 (Pearson correlation coefficient of 0.869 and 0.371, respectively).

The currently available information on the ontogeny of the abundance of the CYP isoforms is mainly expressed as the fraction of the mean adult abundance at a certain age. The most specific information, which is currently used in the SimCYP® model, was described by Johnson et al. [10]. The functions published in this article, can be used to calculate the predicted fraction of the adult abundance at a specific age. *This point estimate is assumed to be correct (assumption 3*), despite the known variability in enzyme expression.

After application of assumptions 1 to 3, an observed and a predicted fraction of adult abundance of each isoform are available for each patient. The percentage difference between the observed and predicted fraction represents the net effect of disease on the abundance in the patient. The important effect of age is already taken into account when calculating the predicted fraction by using the ontogeny functions of Johnson et al. As such, *assumption 4 states that the isolated effect of disease can be determined*. This is important, as the SimCYP® simulations apply the ontogeny functions as programmed in the software. If the effects of disease would not be isolated from the effect of age, the age associated effects would be applied twice: once in the determination of the abundance in the patient, as this contains an effect of age per definition, and once during simulation by applying the ontogeny profiles of the single isoforms.

Subsequently, the CYP enzyme abundances as used in SimCYP® (Table 3) were adjusted following the percentage difference as calculated after the implementation of assumption 4, in order to obtain an absolute abundance value (pmol/mg), usable for the execution of the 'what if' scenarios. The abundances incorporated in SimCYP® are based on a meta-analysis of 42 to 241 liver samples [18].

Table 3: Default CYP isoform abundance (pmol per mg microsomal protein) in healthy adults in SimCYP®. Abbreviations: PM: poor metabolizers – UM: ultra rapid metabolizers.

	Abundance (pmol/mg)
CYP1A2	52
CYP2C9	73
CYP2C9 PM	29
CYP2C19	14
CYP2D6	8
CYP2D6 UM	16
CYP2E1	61
CYP3A4	137

'What if' scenarios were performed for each PELD category. *The mean effect of the disease* was simulated by using the geometric mean of the obtained abundances within each category. Also, the minimal and maximal abundance of the isoforms within each category were used for a simulation, in order to provide information on the extremes of the population, i.e. what if the abundance is well conserved or induced, and what if the abundance is (extremely) low*?* Moreover, the individual variability of the abundance was excluded by setting the coefficient of variation (CV) on zero. As such, *exclusive information on the effect of the disease on the abundance is provided by the simulations (Assumption 5)*.

Finally, these 'what if' scenarios suggest that *only the abundance is affected by the disease* (*Assumption 6)*. Other expected effects of the disease are ignored, such as potential changes in protein binding, MPPGL, and/or blood flow, on the one hand due to lack of data, and on the other hand for reasons of limiting the complexity.

The obtained data after using this strategy on the available data are summarized in Tables 3 and 4. Table 4 illustrates, as an example, the calculations performed in order to obtain the geometric mean abundance of CYP3A4 of the patients in PELD category D. The final abundances of all the isoforms, as used for the simulations, are summarized in Table 5.

Table 4: Calculations performed in order to obtain the geometric mean abundance of CYP3A4 of the patients in PELD category D. The average adult activity determined in pooled human liver microsomes was 2020 pmol HMDZ/ (min x mg pr), the abundance used in SimCYP® is 137 pmol CYP3A4/mg. Abbreviations: y: years - PELD: paediatric end-stage liver disease score - HMDZ: 1-OH-midazolam – pr: protein.

Patient	Age (y)	PELD	Observed absolute activity (pmol HMDZ/ (min x mg pr))	Observed fraction of adult activity (%) ≈ Observed fraction of adult abundance (%)	Predicted fraction of adult abundance (%) (using Johnson et al. [9])	Percentage difference (%) (= 100 - net effect of disease)	Calculated abundance of CYP3A4 (pmol/mg)
1	0.5	31	38.55	1.91	65.6	2.91	4
2	1	32	67.57	3.34	75.7	4.42	6.1
3	0.4	35	1188	58.8	59.5	98.77	135.3
4	0.9	37	11.96	0.59	75	0.79	1.1
5	0.8	38	39.62	1.96	72.1	2.72	3.7
Geometric mean	0.7		68.11	3.37	69.3	4.86	**6.7**

Table 5: Calculated abundances as used in the simulations.

	PELD A			**PELD B**		
	Low	Mean	High	Low	Mean	High
CYP1A2	35.5	73.1	199.9	2.1	7.3	81.5
CYP2C9	104.6	215.6	982.0*	26.1	73.0	176.6
CYP2C9 PM	41.6	85.6	390.1*	10.4	29.0	70.2
CYP2C19	13.7	79.0	497.2*	0.3	7.0	653.8*
CYP2D6	3.7	7.4	16.5	0.1	2.4	17.7
CYP2D6 UM	7.4	14.7	33.0	0.2	4.8	35.4
CYP2E1	32.0	66.2	149.2	9.6	30.2	111.1
CYP3A4	45.7	130.1	272.3	2.4	13.6	313.8
	PELD C			**PELD D**		
	Low	Mean	High	Low	Mean	High
CYP1A2	1.1	2.3	6.2	1.6	5.5	26.0
CYP2C9	13.0	29.0	131.1	18.3	39.8	65.9
CYP2C9 PM	5.2	11.5	52.1	7.3	15.8	26.2
CYP2C19	0.0**	0.5	9.7	0.9	7.9	66.9
CYP2D6	0.0**	0.8	3.3	0.7	3.0	9.4
CYP2D6 UM	0.1	1.7	6.6	1.4	6.0	18.8
CYP2E1	9.1	20.0	48.7	13.7	30.5	79.4
CYP3A4	0.7	3.8	23.4	1.1	6.7	135.3

* SimCYP® does not accept values above 300, therefore 300 was used if the abundance exceeded 300
** SimCYP® does not accept values of 0, therefore 0.01 (the minimal Accepted value) was used

3.3 Data analysis

The results of the predictions were graphically analysed using R® v.2.13 (R foundation for statistical computing, Vienna, Austria).

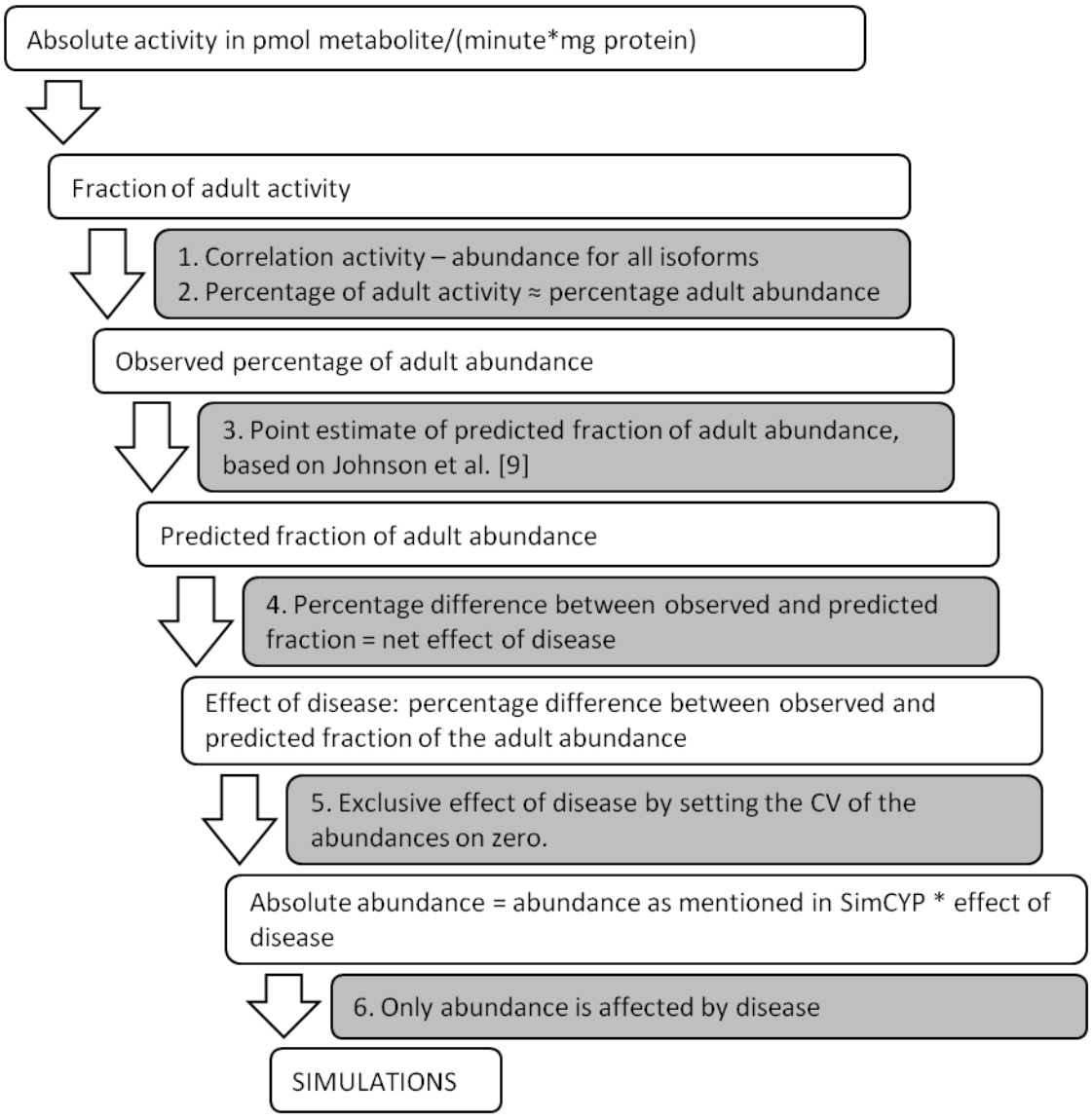

Figure 1: Flow chart of the generation of usable abundance data for the incorporation in the PBPK model, by stating several assumptions (grey areas).

4 RESULTS

The simulated AUC after administration of 1 mg/kg omeprazole in oral solution in healthy children from 0 to 2 years old was 1.44 mg/L.h (geometric mean; 1.39 – 1.50 mg/L.h 95% confidence interval; median 1.35 mg/L.h). As shown in Figure 2a, significantly higher AUCs (15 to 65-fold higher) were predicted in the average patient in PELD category C, as well as in the patients in category B, C and D with the lowest CYP abundances. In order to be able to see the differences between healthy children and the other categories, Figure 2b shows the same results, but with the AUC in log scale. The patients in PELD category A have a similar or lower AUC than the healthy children. A lower or rather comparable AUC (max 2 fold higher) is to be expected in the average

patients in PELD category B or D, as well as in the patients in category B, C and D with the highest CYP abundances.

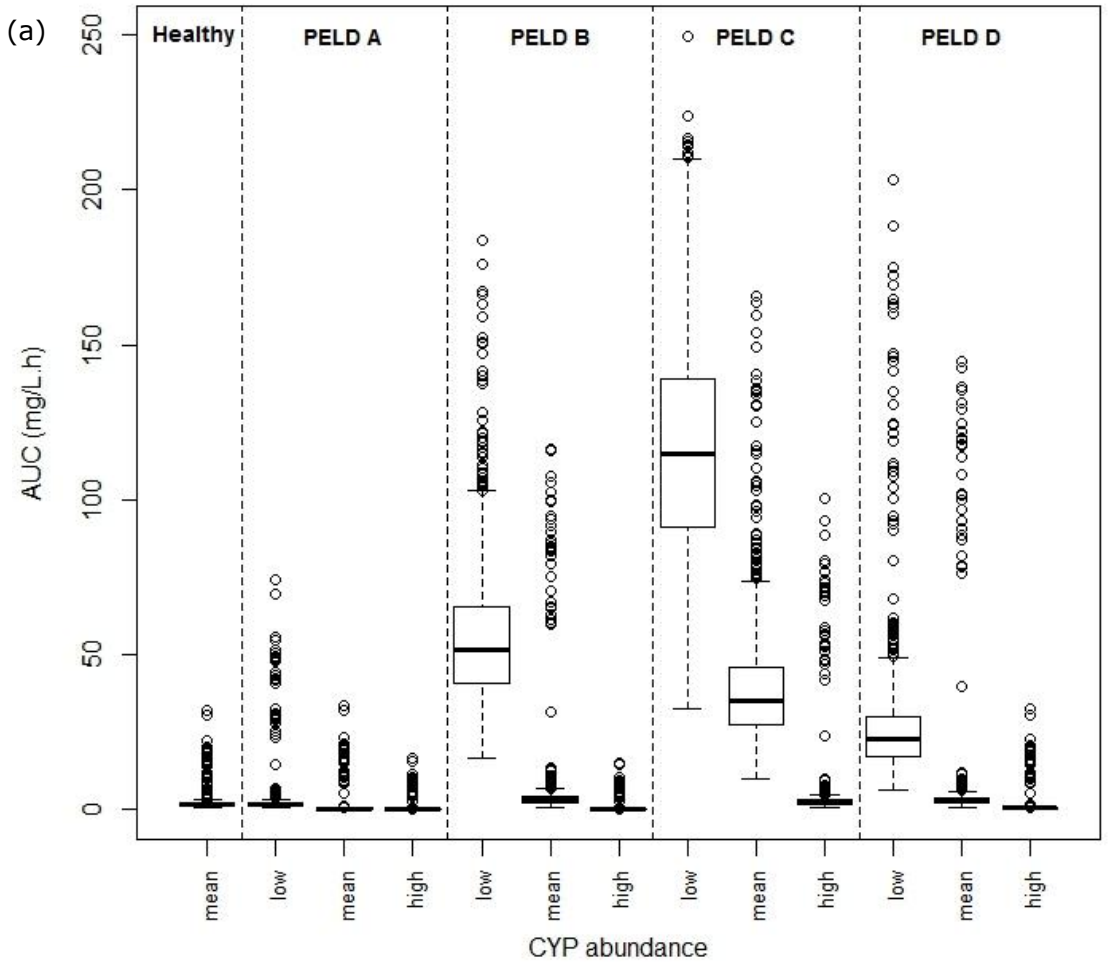

Figure 2 a and b: Simulated AUCs of omeprazole for healthy children, and children in the 4 PELD categories, each time using the geometric mean value (mean) and the extremes (low: lowest CYP abundances, high: highest CYP abundances). Each box represents the median of the 1000 patients with 25 and 75% quartiles, whiskers show the minimum and maximum values, whereas the dots represent outliers within each group. (a) = linear y-axis.

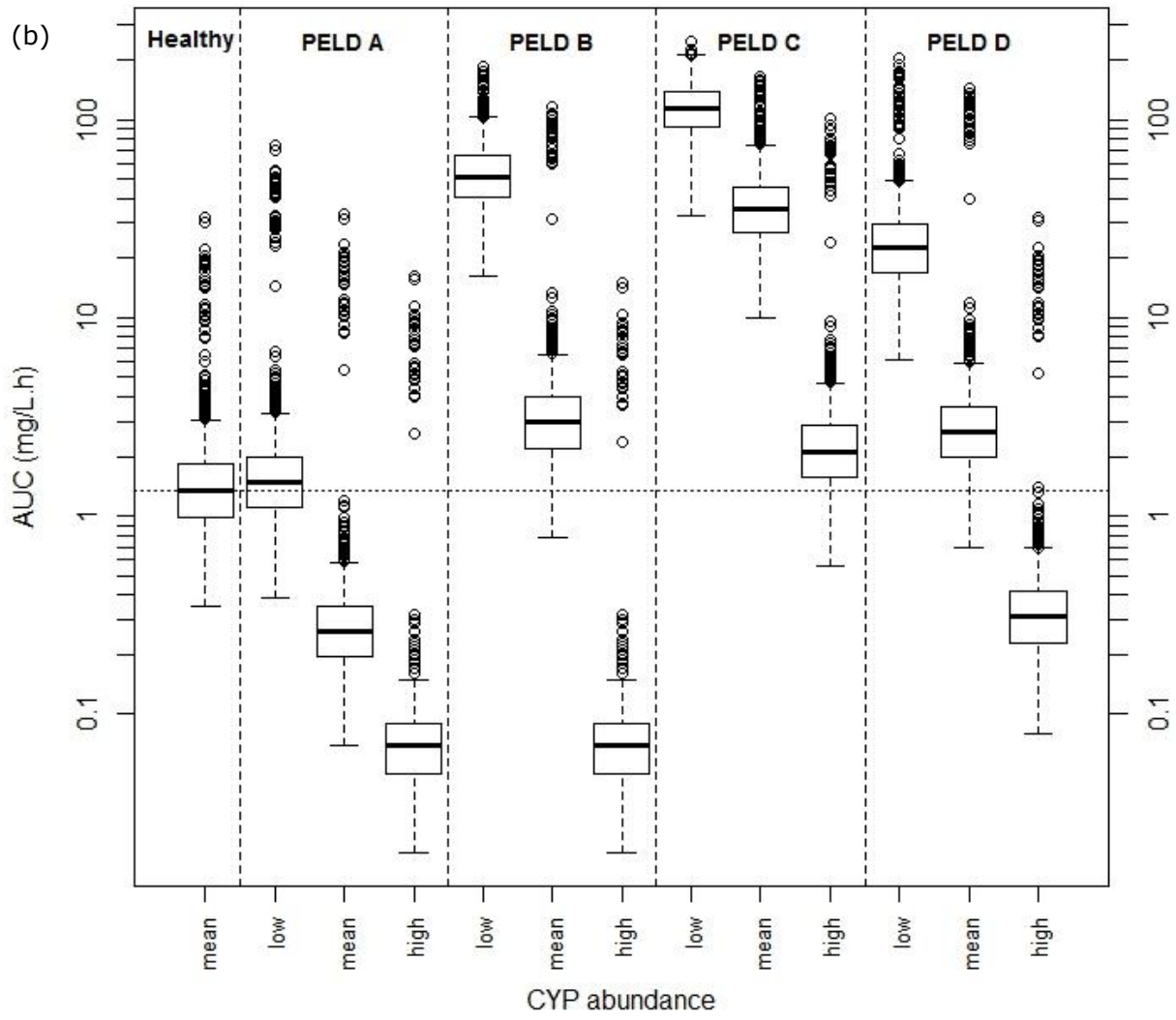

Figure 2 a and b (continued): Simulated AUCs of omeprazole for healthy children, and children in the 4 PELD categories, each time using the geometric mean value (mean) and the extremes (low: lowest CYP abundances, high: highest CYP abundances). Each box represents the median of the 1000 patients with 25 and 75% quartiles, whiskers show the minimum and maximum values, whereas the dots represent outliers within each group. (b) = log scale y-axis.

The predicted plasma concentration – time profiles of midazolam of the patients in PELD category A were comparable to those predicted in the healthy children (see Figure 3). Patients with the highest abundance in category B and D also show a comparable profile to healthy children. In the average patient in category B, C, and D, as well as in the patients with lowest expected CYP abundances in these categories, a significant higher plasma concentration was predicted. Maximum plasma concentrations of about 5 times higher than in the average healthy child can be expected (Figure 3).

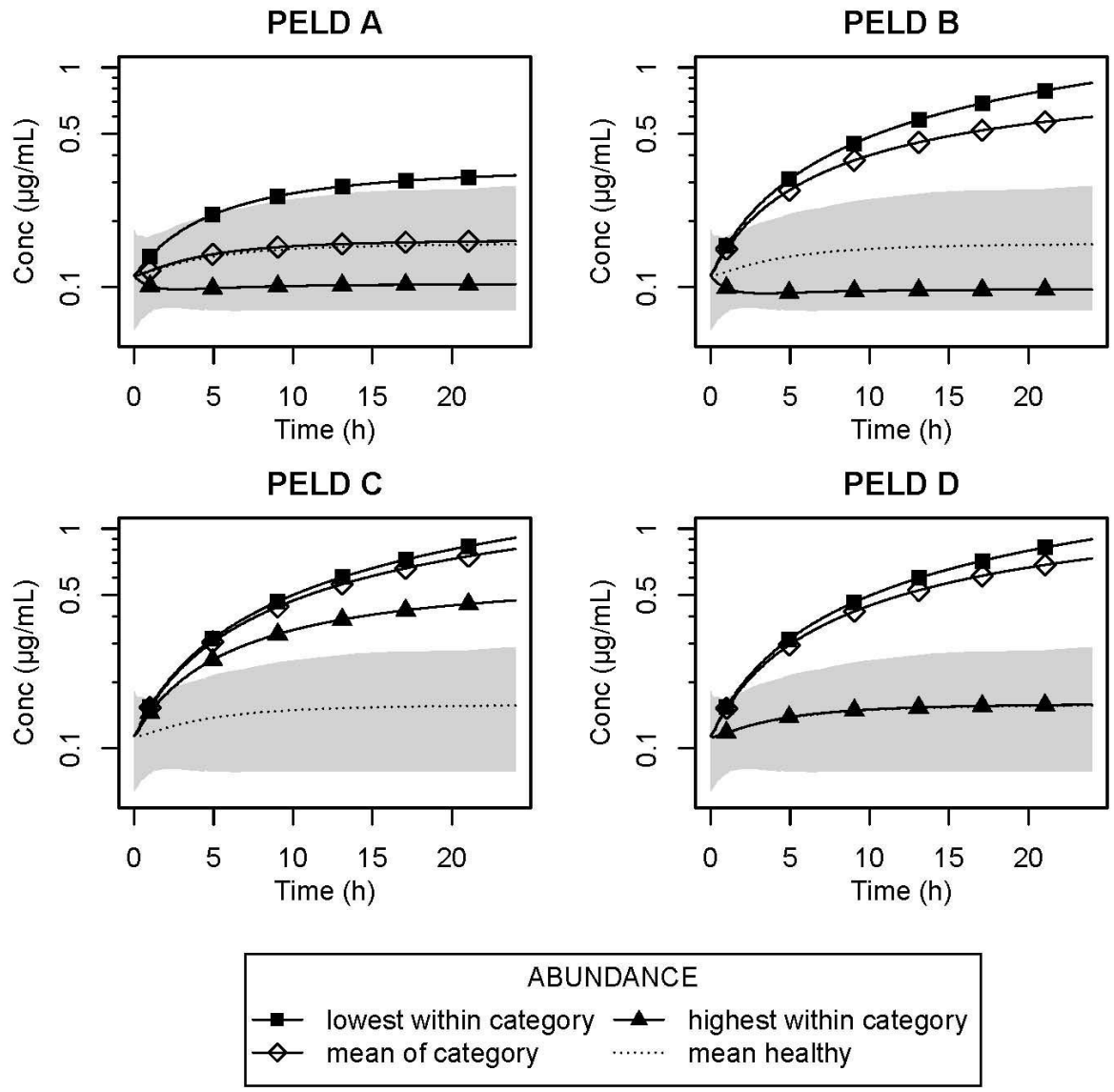

Figure 3: Simulated plasma concentration – time profiles of midazolam. The grey area represents the 5 to 95% confidence interval of the simulations in healthy children. In each category, the lowest, mean and highest abundance were used to predict the PK. Compared to the healthy children, patients with PELD scores in the categories B, C, and D are likely to have an increased plasma concentration. (conc = concentration)

5 DISCUSSION

Drug disposition is expected to be altered in children with liver disease. However, the influence of liver dysfunction in children on the PK of drugs has not yet been extensively studied mechanistically. A recent *in vitro* study showed a decrease in absolute activity of the six most important CYP isoforms (CYP1A2, 2C9, 2C19, 2D6, 2E1, and 3A4) with deteriorating liver dysfunction, as expressed by the PELD (paediatric end-stage liver disease) score [7]. PBPK modelling and simulation provides a useful tool to gain insight in

the (changing) PK in these patients. By means of "what if" scenarios, the effect of the change in enzyme activity or abundance on the PK of the model compounds omeprazole and midazolam can be evaluated. These compounds were selected as they are often used in the patients to relieve reflux symptoms and in (post-operative) sedation, respectively. Moreover, the compound information of these molecules available in SimCYP® is extensive and has been used before in paediatric PBPK models [10].

The results from the study investigating *in vitro* CYP activity in the target population could not be used as such in the PBPK modelling software (SimCYP®). The absolute activity data were transformed into enzyme abundances, a process that needed the adoption of several assumptions. These assumptions oblige us to be careful with the conclusions drawn from the results. Nevertheless, by combining previously published ontogeny functions of the enzyme abundance [10], and the available data, the effect of the disease could be separated from the effect of the maturation of the enzyme expression. As such, the abundance in patients with a PELD score in a certain range could be estimated.

The suppression of gastric acid secretion by omeprazole is correlated with the area under the plasma concentration – time curve (AUC) [17,19,20]. As omeprazole depends significantly on CYP2C19 for its metabolism, changes in the level of CYP2C19 activity will affect its clearance and thus the systemic exposure to the drug. A reduced clearance of omeprazole has been described in adults with cirrhosis, necessitating dose reductions up to 50% [21-25]. In children, however, the impact of liver disease on the disposition of omeprazole has not yet been studied. The elimination in one 5-year-old patient with liver disease was shown to be delayed [26]. Compared to healthy children, simulations of the oral administration of omeprazole 1 mg/kg to virtual populations of 1000 patients predicted higher AUCs of omeprazole in paediatric patients with a high PELD score (category C = 20-30), as well as in patients with a possible low CYP abundance and an intermediate to high PELD score (categories B, C, D = 10-40). These observations were as expected, as the patient groups in which the highest AUCs were predicted, also showed very low CYP2C19 abundances (even down to virtually zero). Moreover, the predicted AUCs within the same PELD category often showed large variability (up to 50 or even 500-fold), as the predictions were performed on the mean (n = 5 or 8), as well as on the extreme abundance values as observed in the patients within each category. This variability should be taken into account in further studies.

It should be noted that CYP2C19 shows important polymorphisms which are known to affect enzyme activity [27], resulting in either ultra rapid metabolizers (CYP2C19*17) [28], or poor metabolizers (CYP2C19*2, amongst others) [29]. The genotype of the patients included in the study was determined, but was shown to be a poor predictor of

the CYP activity, as no reduced or increased activity was seen in patients carrying a SNP known to affect enzyme activity [7]. Therefore, the genotype was not taken into account when calculating the abundances necessary for this pilot study. Nevertheless, the known genetic variation of the different CYPs is incorporated in the SimCYP® algorithm, and is thus inserted in the diseased population.

The effect of the increased AUC of omeprazole is difficult to predict, as the therapeutic target AUC in children is not known [19]. A larger increase in intra-gastric pH is unlikely, as an increased AUC has been shown to not necessarily result in a larger effect in adults [30]. The low risk (1-3%) of minor adverse events, such as headache, diarrhoea, and nausea [31] could increase due to the significant increase in predicted AUC (up to 65 fold). Moreover, the risk of serious adverse events could become more real. Costa-Rodrigues et al., for example, reported a dose dependent effect on the *in vitro* cellular function of osteoclasts and osteoblasts [32], with possible deleterious effects on bone density, and consequently a higher risk of fractures. Spontaneous bacterial peritonitis associated with incorrect omeprazole dosing was also observed in adults [33]. These examples show the importance of correct dosing of omeprazole in order to avoid possible severe adverse reactions. As such, a large number of paediatric patients with liver disease is more likely to experience adverse events when using omeprazole.

Midazolam, a drug often used in the sedation of paediatric patients, is a widely used model compound to investigate CYP3A4/5 mediated drug metabolism [34,35]. The PK parameters were estimated after the virtual administration of the generic dose for paediatric post-operative monitoring, i.e. a bolus of 0.1 mg/kg, followed by an infusion of 0.05 mg/kg/h. It should be noted that in paediatric clinical practice, the dosing is continuously adjusted based on the COMFORT sale, a score comprising both physiological and behavioural items to specifically asses sedation in paediatric intensive care patients and pain in postoperative infants [36,37]. Nevertheless, the simulations using the generic dosing give a valuable insight in the mechanisms of the changes in midazolam PK in children with liver disease. In children with a PELD score between 1 and 10 (category A), and thus with only mild liver dysfunction, plasma concentration – time profiles similar to those in healthy children were predicted. A distinct difference to healthy children was however seen when the abundances in children with higher PELD scores were implemented in the PBPK model (categories B, C, D = 10-40). Due to the major role of CYP3A4 in the metabolism of midazolam, it is not surprising that higher plasma concentrations are observed in children with impaired liver function and the associated low CYP3A4 abundance. Plasma-concentrations of up to 5-fold of the mean healthy concentration were predicted, due to the low CYP3A4 abundance, and the consequent lower midazolam clearance.

A similar observation was previously described by Ince et al. in children on the PICU during an in vivo trial, where a reduced midazolam clearance was observed due to inflammation. A simulation in which the known reduction in CYP3A4 abundance due to inflammation was applied, showed that this reduction by 80% was the major determinant of the reduced midazolam clearance [34]. The large variability in the PK of midazolam, together with the observed influence of liver disease on the PK, stresses the relevance of a dynamic dosing of midazolam, as over sedation is expected in children with moderate to severe liver disease (PELD score of 11 and higher).

The unusual trend of a higher plasma concentration in PELD C than in PELD D for midazolam, and a higher AUC in PELD C than PELD D for omeprazole, is probably a result of the low number of patients within each group, combined with the high variability in activity. Moreover, by chance there is a patient with (very) high activity of all isoforms in PELD category D, despite his severe liver disease. This causes the strange deviation of the expected trend.

6 PBPK MODELLING IN CHILDREN WITH LIVER DISEASE: UNCOVERING THE NEEDS

As assumptions were needed due to incompatibilities between the collected information and the data needed in the simulation algorithm, continuation of this research is needed. Based on the predictions reported here, several options for the continuation of this research can be explored.

In our opinion, increasing the number of samples would not drastically change the trend of the results, but could decrease the uncertainty related to the high inter-individual variability. The available microsomes could be further characterized. Determination of the abundance of all the isoforms could possibly evaluate the assumption stating that the abundance and the activity are correlated in all the isoforms.

The physiological changes due to liver disease also comprise more than just altered CYP450 concentration and/or function. For example, changes in albumin concentrations, the MPPGL, liver blood flow, amongst others, were reported in adults with liver dysfunction [5]. As the MPPGL is an important scaling factor, we focused on the MPPGL in children with biliary atresia in chapter 7 of this thesis. However, additional information is needed before the changes in MPPGL due to liver disease could be incorporated in the PBPK model. Firstly, despite the observed decreased MPPGL in the patients, these results are based on only 4 samples. Therefore, a similar study set up should focus on the storage of the homogenates, in order to be able to determine the MPPGL. As such, more reliable information on the effect of liver disease on this scaling factor could be

determined. Additionally, the PBPK model adjusts the MPPGL of the virtual patients according to the model defined by Barter et al. [38] A correct way to change the coefficients of this model in function of the degree of severity of liver disease should be determined.

The possible role of inflammatory cytokines, such as interleukin-6 (IL-6) in the regulation of the CYP activity in seriously ill paediatric patients was previously described [34,39]. Therefore, besides the standard tests, such as albumin, bilirubin and INR, amongst others, extra parameters, such as IL-6, should be investigated [34]. Hereto, a more profound pre-operative protocol should be developed in cooperation with the treating doctors, in order to fix which clinical biology tests should be performed prior to the transplantation. Additionally, a standardized test to determine the liver blood flow in the patients could provide valuable information in the investigation of drug metabolism, especially when the PK of high extraction ratio drugs (blood flow limited) is predicted.

PBPK simulation results can be assessed by performing sensitivity analyses, identifying the key dependencies of the model [40]. Moreover, although there are no generally accepted criteria to validate a model, a visual predictive check can be performed to compare the predicted results with observed results from in vivo studies [41]. The lack of comprehensive in vivo results from the PK of drugs, and more specifically of the used model compounds omeprazole and midazolam, in children with liver disease has been one of the major limitations of the current study. Consequently, the execution of a clinical trial to provide this in vivo information should have great priority in a follow up study.

7 CONCLUSION

This study was a first attempt to incorporate *in vitro* obtained disease related changes into PBPK models. Predictions of the PK of 2 model compounds, omeprazole and midazolam, showed that liver disease in children may have a (large) effect on the PK of drugs which depend on CYP450 for their metabolism. However, the extent of the changes as predicted by the PBPK model should be interpreted with caution due to the many assumptions that were made. Moreover, the adjustments done to the available paediatric model (i.e. changing the absolute abundance of the CYP isoforms) in order to create a diseased population are not sufficient to capture the complex combination of physiological changes in liver disease. The information from this pilot study is nevertheless valuable as a starting point in guiding follow up studies in this research field.

REFERENCES

[1] Chardot C. Biliary atresia. *Orphanet J Rare Dis* 2006; 1:28.

[2] Gallo A, Esquivel CO. Current options for management of biliary atresia. *Pediatr Transplant* 2013; 17(2):95-98.

[3] Greenberger NJ, Paumgartner G. Diseases of the Gallbladder and Bile Ducts. In: Fauci AS, Kasper DL, Longo DL, Braunwald E, Hauser SL, Jameson JL *et al.*, eds. *Harrison's Principles of Internal Medicine*. McGraw-Hill Medical. 2008: 1991-2001.

[4] Kelly DA, Davenport M. Current management of biliary atresia. *Arch Dis Child* 2007; 92(12):1132-1135.

[5] Verbeeck RK. Pharmacokinetics and dosage adjustment in patients with hepatic dysfunction. *Eur J Clin Pharmacol* 2008; 64(12):1147-1161.

[6] Delco F, Tchambaz L, Schlienger R, Drewe J, Krahenbuhl S. Dose adjustment in patients with liver disease. *Drug Saf* 2005; 28(6):529-545.

[7] De Bock L, Boussery K, Van Winckel M, De Paepe P, Rogiers X, Stephenne X *et al.* In Vitro Cytochrome P450 Activity Decreases in Children with High Pediatric End-Stage Liver Disease Scores. *Drug Metab Dispos* 2013; 41(2):390-397.

[8] Rocchi F, Tomasi P. The development of medicines for children: Part of a series on Pediatric Pharmacology, guest edited by Gianvincenzo Zuccotti, Emilio Clementi, and Massimo Molteni. *Pharmacol Res* 2011; 64(3):169-175.

[9] Kauffman RE. Clinical Trials in Children: Problems and Pitfalls. *Pediatric Drugs* 2000; 2(6):411-418.

[10] Johnson TN, Rostami-Hodjegan A, Tucker GT. Prediction of the clearance of eleven drugs and associated variability in neonates, infants and children. *Clin Pharmacokinet* 2006; 45(9):931-956.

[11] Khalil F, Läer S. Physiologically Based Pharmacokinetic Modeling: Methodology, Applications, and Limitations with a Focus on Its Role in Pediatric Drug Development. *Journal of Biomedicine and Biotechnology* 2011.

[12] Knibbe CAJ, Danhof M. Individualized dosing regimens in children based on population PKPD modelling: Are we ready for it? *Int J Pharm* 2011; 415(1-2):9-14.

[13] Edginton AN, Willmann S. Physiology-Based Simulations of a Pathological Condition Prediction of Pharmacokinetics in Patients with Liver Cirrhosis. *Clin Pharmacokinet* 2008; 47(11):743-752.

[14] Johnson TN, Boussery K, Rowland-Yeo K, Tucker GT, Rostami-Hodjegan A. A Semi-Mechanistic Model to Predict the Effects of Liver Cirrhosis on Drug Clearance. *Clin Pharmacokinet* 2010; 49(3):189-206.

[15] Jamei M, Marciniak S, Feng KR, Barnett A, Tucker G, Rostami-Hodjegan A. The Simcyp (R) Population-based ADME Simulator. *Expert Opin Drug Met* 2009; 5(2):211-223.

[16] Peeters MYM, Prins SA, Knibbe CAJ, DeJongh J, Mathot RAA, Warris C *et al.* Pharmacokinetics and pharmacodynamics of midazolam and metabolites in nonventilated infants after craniofacial surgery. *Anesthesiology* 2006; 105(6):1135-1146.

[17] Stedman CAM, Barclay ML. Review article: comparison of the pharmacokinetics, acid suppression and efficacy of proton pump inhibitors. *Alimentary Pharmacology & Therapeutics* 2000; 14(8):963-978.

[18] Rowland-Yeo K, Rostami-Hodjegan A, Tucker GT. Abundance of cytochromes P450 in human liver: a meta-analysis. *Br J Clin Pharmacol* 2004; 57(5):687-688.

[19] Litalien C, Théorêt Y, Faure C. Pharmacokinetics of Proton Pump Inhibitors in Children. *Clin Pharmacokinet* 2005; 44(5):441-466.

[20] Shin JM, Kim N. Pharmacokinetics and Pharmacodynamics of the Proton Pump Inhibitors. *Journal of Neurogastroenterology and Motility* 2013; 19(1):25-35.

[21] Andersson T, Olsson R, Regardh CG, Skanberg I. Pharmacokinetics of [C-14] Omeprazole in Patients with Liver-Cirrhosis. *Clin Pharmacokinet* 1993; 24(1):71-78.

[22] Sjovall H, Bjornsson E, Holmberg J, Hasselgren G, Rohss K, Hassan-Alin M. Pharmacokinetic study of esomeprazole in patients with hepatic impairment. *European Journal of Gastroenterology & Hepatology* 2002; 14(5):491-496.

[23] Pique JM, Feu F, de Prada G, Rohss K, Hasselgren G. Pharmacokinetics of omeprazole given by continuous intravenous infusion to patients with varying degrees of hepatic dysfunction. *Clin Pharmacokinet* 2002; 41(12):999-1004.

[24] Rinetti M, Regazzi MB, Villani P, Tizzoni M, Sivelli R. Pharmacokinetics of Omeprazole in Cirrhotic-Patients. *Arzneimittel-Forschung/Drug Research* 1991; 41-1(4):420-422.

[25] Kumar R, Chawla YK, Garg SK, Dixit RK, Satapathy SK, Dhiman RK *et al.* Pharmacokinetics of omeprazole in patients with liver cirrhosis and extrahepatic portal venous obstruction. *Methods Find Exp Clin Pharmacol* 2003; 25(8):625-630.

[26] Jacqzaigrain E, Bellaich M, Faure C, Andre J, Rohrlich P, Baudouin V *et al.* Pharmacokinetics of Intravenous Omeprazole in Children. *Eur J Clin Pharmacol* 1994; 47(2):181-185.

[27] Ingelman-Sundberg M. Human drug metabolising cytochrome P450 enzymes: properties and polymorphisms. *Naunyn Schmiedebergs Arch Pharmacol* 2004; 369(1):89-104.

[28] Sim SC, Risinger C, Dahl ML, Aklillu E, Christensen M, Bertilsson L *et al.* A common novel CTP2C19 gene variant causes ultrarapid drug metabolism relevant for the drug response to proton pump inhibitors and antidepressants. *Clin Pharmacol Ther* 2006; 79(1):103-113.

[29] Desta Z, Zhao XJ, Shin JG, Flockhart DA. Clinical significance of the cytochrome P4502C19 genetic polymorphism. *Clin Pharmacokinet* 2002; 41(12):913-958.

[30] Pounder RE, Sharma BK, Walt RP. 24 Hour Intragastric Acidity During Treatment with Oral Omeprazole. *Scand J Gastroenterol* 1986; 21:108-117.

[31] Thomson AB, Sauve MD, Kassam N, Kamitakahara H. Safety of the long-term use of proton pump inhibitors. *World Journal of Gastroenterology* 2010; 16(19):2323-2330.

[32] Costa-Rodrigues J, Reis S, Teixeira S+, Lopes S, Fernandes MH. Dose-dependent inhibitory effects of proton pump inhibitors on human osteoclastic and osteoblastic cell activity. *FEBS J* 2013; 280(20):5052-5064.

[33] Franz C, Hildbrand C, Born C, Egger S, R+ñtz Bravo A, Kr+ñhenb++hl S. Dose adjustment in patients with liver cirrhosis: impact on adverse drug reactions and hospitalizations. *Eur J Clin Pharmacol* 2013; 69(8):1565-1573.

[34] Ince I, de Wildt SN, Peeters MYM, Murry DJ, Tibboel D, Danhof M *et al.* Critical Illness Is a Major Determinant of Midazolam Clearance in Children Aged 1 Month to 17 Years. *Ther Drug Monit* 2012; 34(4):381-389.

[35] Li AP, Kaminski DL, Rasmussen A. Substrates of human hepatic cytochrome P450 3A4. *Toxicology* 1995; 104(1-3):1-8.

[36] de Wildt SN, de Hoog M, Vinks AA, Joosten KEM, van Dijk M, van den Anker JN. Pharmacodynarnics of midazolarn in pediatric intensive care patients. *Ther Drug Monit* 2005; 27(1):98-102.

[37] van Dijk M, de Boer JB, Koot HM, Tibboel D, Passchier J, Duivenvoorden HJ. The reliability and validity of the COMFORT scale as a postoperative pain instrument in 0 to 3-year-old infants. *Pain* 2000; 84(2-3):367-377.

[38] Barter ZE, Chowdry JE, Harlow JR, Snawder JE, Lipscomb JC, Rostami-Hodjegan A. Covariation of human microsomal protein per gram of liver with age: Absence of influence of operator and sample storage may justify inter laboratory data pooling. *Drug Metab Dispos* 2008; 36(12):1-5.

[39] Vet NJ, de Hoog M, Tibboel D, de Wildt SN. The effect of critical illness and inflammation on midazolam therapy in children. *Pediatric Critical Care Medicine* 2012; 13(1):E48-E50.

[40] Barrett JS, Alberighi OD, Läer S, Meibohm B. Physiologically Based Pharmacokinetic (PBPK) Modeling in Children. *Clin Pharmacol Therap* 2012; 92(1):40-49.

[41] Jiang XL, Zhao P, Barrett JS, Lesko LJ, Schmidt S. Application of physiologically based pharmacokinetic modeling to predict acetaminophen metabolism and pharmacokinetics in children. *CPT Pharmacometrics Syst Pharmacol* 2013; 2:e80.

GENERAL CONCLUSION

AND

FUTURE PERSPECTIVES

"The important thing is to not stop questioning."

— Albert Einstein

The general aim of this thesis was to investigate whether CYP450 characteristics could bridge the present knowledge gap on drug disposition in children with liver disease. Due to their important role in drug metabolism, the isoforms CYP1A2, 2C9, 2C19, 2D6, 2E1 and 3A4 were selected for investigation. In order to determine several characteristics, the necessary methods were developed. For the determination of the enzyme activity in human liver microsomes, 2 methods were successfully developed and validated: (1) a derivatization-HPLC-MS/MS method and (2) a UPLC-MS/MS method. The main advantage of the derivatization method with pyridine-3-sulfonyl chloride was the simultaneous quantification of all metabolites in a single analytical run with positive ESI for mass spectrometric detection. Nevertheless, the UPLC-MS/MS method was eventually chosen to analyze the patient samples, as it showed a better sensitivity and shorter time of analysis than the derivatization method. Moreover, experiments showed that incubations of the microsomes needed to be performed with each probe substrate individually, rather than in cocktail. As the aim of this study is to determine absolute activity of the CYPs, such interactions between probe substrates could confound the results. Some other analytical and practical problems were encountered when performing the incubations in cocktail. The results of these incubations showed a larger imprecision. Moreover, the preparation of the cocktail of the probes in the correct concentration was difficult, as some probes have a poor solubility, and the maximum solvent concentration within the incubation should not exceed 1%. As only one metabolite of interest is present in each incubation sample, the correct ESI mode could be chosen for each sample individually. Pooling of the samples after incubation was also not possible, as this would dilute the samples and would probably lead to sensitivity issues for the detection of some of the metabolites. A possible solution to this would be the use of a mass spectrometer capable of in-run ESI mode switching.

For the quantification of CYP2E1 and 3A4 in the microsomes, 2 indirect ELISAs were developed and validated. This type of method was preferred above a western blotting method, as the latter is more time-consuming and relatively prone to technical difficulties. Moreover, ELISAs have a higher sample throughput. Other techniques that can be used for the quantification of CYP enzymes are proteomics approaches or the use of AQUA (Absolute Quantification) probes. These MS based techniques have a high throughput and very good sensitivity and selectivity, but require a more difficult optimization procedure, as well as specialized equipment. After the fit-for-purpose optimization, the developed ELISAs proved to be usable for the quantification of the study samples. Despite the advantages over other techniques, it should be noted that the method for the quantification of CYP3A4 could lack specificity and could possibly also detect CYP3A5, but this was not evaluated during the optimization.

In order to determine the genotype of the patients, a workflow was implemented to analyse important SNPs of the highly polymorphic CYP2C9, 2C19 and 2D6 using TaqMan® genotyping assays. Seven SNPs (CYP2C9*2 and *3, CYP2C19*2 and *17, CYP2D6*4, *10 and *41) were selected based on their prevalence in the Caucasian population and on their significant effect on enzyme activity. Despite their high prevalence, these 7 SNPs are only a small fraction of the known SNPs (44, 48, +80 known haplotypes for CYP2C9, 2C19, and 2D6, respectively). In order to obtain a more complete genotype, other genotyping methods, such as microarrays or next-generation sequencing should be considered in future experiments or studies.

A clinical study was set up in order to collect liver samples from paediatric patients undergoing liver transplantation. The total number of samples collected in the study was only 32 (one patient was excluded due to resistant gram negative sepsis at the time of operation). This low number reflects the (luckily) low prevalence of end-stage liver disease in children, but also impeded the study as the sample size, in combination with the high variability of the results, was too low for a thorough statistical analysis of the results.

The developed bio-analytical methods were applied to analyse the patient samples. After determination of the enzyme activity, the determinants of the CYP activity in this patient population were derived. No significant correlation of the activity with age, weight, co-medication or genotype (despite the observation of some expected trends) was found. However, a clear negative correlation between the CYP activity and the Paediatric End-Stage Liver Disease (PELD) score was observed, suggesting a decrease in activity with deteriorating liver function. This negative correlation is in accordance with the findings in adults where the CYP activity was shown to decrease with the Child-Pugh score, an indicator for hepatic dysfunction in adults. Moreover, the decrease in activity occurred in all isoforms concomitantly. Dose adjustments of drugs which depend on the CYPs for their metabolism seems therefore as important in children as in adults. Currently, only paracetamol is systematically administered in a lower dose in the paeditric patients, but no other dose adjustments are carried out. This is in striking contrast with the large list of dose recommendations for adult with liver dysfunction.

The MPPGL (microsomal protein per gram liver) is an important scaling factor in *in vitro-in vivo* extrapolation of metabolic data. The determination of the MPPGL requires the quantification or estimation of the CYP content of both homogenates and microsomes. During the project, the widely used method of Omura or Matsubara, based on the dithionite-difference spectrum, proved to be unusable for the study samples. The presence of high concentrations of bilirubin in the cholestatic samples interfered with spectrophotometric detection. The publication of the NCR (NADPH-cytochrome P450

reductase activity) method in the course of the project, in combination with the first article in which the method was successfully applied, provided a new possibility to determine the MPPGL. As the method was optimized only near the end of the project, the MPPGL was determined in only 4 patient samples. The results showed a decrease in MPGGL in paediatric patients with biliary atresia, compared to the expected value based on the age of the patients. In order to obtain sufficient information on the MPPGL to be able to adjust the age-related function in the PBPK model of SimCYP®, the MPPGL should be determined in patient samples over the entire age-range (from neonates to elderly) and in patients with different degrees of liver dysfunction.

The generated CYP characteristics were implemented in a PBPK model in order to create a virtual specific population of paediatric patients with liver disease. The drug disposition of the model compounds omeprazole and midazolam was predicted in patients with varying degrees of liver dysfunction, as expressed by the PELD score. Prior to the predictions, several assumptions had to be made, due to missing information or incompatibilities between the available data and the data needed to be implemented in the model. Moreover, the performed adjustments to the available paediatric model in order to create a diseased population are not sufficient to capture the complex combination of physiological changes in liver disease Therefore, the results should be interpreted with caution. Nevertheless, alarming trends were predicted. For both model compounds, a large increase in exposure to the drug was predicted, starting in patients with moderate liver disease (PELD score >10), and reaching alarmingly high levels in patients with severe to very severe (PELD >20) liver dysfunction. These predictions provide another argument for the need for dose adjustments in children with liver disease.

Despite the valuable information obtained in this study, many other aspects of the physiological changes in paediatric liver disease should be investigated. Or, to say it metaphorically: "*we believe the construction works have been started, the contractor arrived at the construction site and the first layers of concrete to build the bridge were poured. However, in order to cross the huge knowledge gap, many more bricks will need to be produced before the bridge can be built and can be used with full confidence.*"

The further optimization of the PBPK model requires more information on the changes of other physiological parameters, such as protein binding or liver blood flow. Also, information on the influence of liver disease on creatinine clearance could be interesting in order to optimize the renal compartment of the PBPK model. This will however be an intricate task, as renal function also shows a certain ontogeny, and has been shown to often be compromised in patients with advanced liver disease (hepato-renal syndrome).

In our study, the absolute CYP activity was determined by using specific probe substrates. Another way to study the changes in metabolic capacity would be the *in vitro* determination of the intrinsic clearance of a certain model compound in the collected paediatric samples. This compound is ideally already defined in SimCYP (as the physicochemical characterization of the drug would be beyond the scope of the study), and preferably used in the regular clinical treatment of the patients. If the selected drug meets the latter requirement, the collection of *in vivo* data needed for the validation of the model would be less complicated, as clinical trials in children are hindered with many ethical and practical hurdles. If the administration of the drug is already part of the regular treatment, one of the main problems can be eliminated. Other considerations, such as sampling, still need to be taken into account. The availability of *in vivo* data is of great importance for the validation of the PBPK model. Consequently, to our knowledge, follow up research should most definitely focus on the design and execution of an informative clinical trial that provides information to validate the PBPK model.

PBPK models can be used to help in the design of clinical trials, by providing information on the optimal sampling times, number of subjects and number of samples per subject. As many drugs are used off label, and very little information is available on drug dosing in children with liver disease, clinical trials in this population are needed. After the optimization and validation of a PBPK model for paediatric patients with liver disease, it could be used for this purpose.

The optimal end point of PBPK based predictions would be the development of dose changing algorithms in children with liver disease. If a validated PBPK model would be able to accurately predict the PK in children with a certain degree of liver dysfunction, for example based on the PELD scores as done in this study, decreases (or increases) in dose could be advised. Consequently, adverse events or toxic effects could be avoided in case of possible overdosing, whereas the therapeutic efficiency could be increased if the drug dose would be too low.

All the previously described research topics focus on the pre-transplant patients. However, during transplantation, many children receive (parts of) an organ originating from an adult donor. As such, another question arises: what is the metabolic capacity of the transplanted organ and how does it develop? Should we consider the age of the receiving patient and the known maturation of the enzymes? Or should we consider them as adult metabolizers? This could be important for the post-transplant treatment, for example for the correct dosing of the immunosuppressive drugs these patients receive.

In summary, we successfully developed several methods for the characterization of CYP450 enzymes in human liver microsomes originating from diseased paediatric livers.

The extensively validated methods provided information on the activity and the abundance of (some of) the studied isoforms CYP1A2, 2C9, 2C19, 2D6, 2E1, and 3A4. Moreover, the most important SNPs from CYP2C9, 2C19, and 2D6 were evaluated in the patients. Combination of all the results leads to the identification of the paediatric end-stage liver disease (PELD) score, a measure for the degree of liver disease, as a determinant of the CYP activity in children with liver disease. In contrast, no correlation between the activity and other patient characteristics, such as age, weight, or the administration of inducing or inhibiting co-medication, could be demonstrated. The MPPGL, an important scaling factor in *in vitro-in vivo* extrapolation, was shown to be lower in patients with biliary atresia than in healthy children. A first attempt to use a PBPK-model for the prediction of the drug disposition of 2 model compounds, omeprazole and midazolam, showed a large effect of moderate to severe liver dysfunction on the exposure to the drug. These predictions, however, were based on a number of assumptions, which make the results somewhat tricky to interpret. Nonetheless, this study identified the changes in CYP activity in children with liver disease and provides an interesting starting point to guide further studies on the pharmacokinetics in this patient population.

SUMMARY

Very little is known on the influence of liver disease on the pharmacokinetics (PK) of drugs in paediatric patients. Due to many practical and ethical issues in the set up and execution of clinical trials in children, the use of modelling and simulation techniques, such as physiologically-based pharmacokinetic (PBPK) models, has gained interest. Disease-specific PBPK models require adequately obtained in vitro information on the physiological processes that are influenced by the disease. Therefore, this thesis aimed to characterize the CYP activity in children with hepatic dysfunction, in order to optimize a PBPK model specifically for this patient population. This research can be roughly divided in two parts: (1) a major part consisting of the development of several methods for the characterization of CYP450 enzymes in human liver microsomes originating from diseased paediatric livers, and (2) a part in which these methods were applied on patient samples, and in which the obtained results were used to perform a pilot-study to predict the drug disposition in the target population using a PBPK model.

In the **introduction** the problem of off-label drug use in children is discussed. **The administration of drugs to children without a sound knowledge on a correct dosing** was the main reason to start up this study. One of the problems encountered in paediatric drug dosing is the change in drug disposition with age due to the maturation of the underlying physiological processes. On top of these changes with age, pathological conditions can also alter drug disposition. Liver disease is known to alter the drug disposition in adults, and recommendations for dose adjustments are available for these patients. However, the combination of the maturational changes in drug disposition and the different aetiology of liver disease in children, make it impossible to extrapolate these recommendations to children. After the explanation of the structure of PBPK models, their applications are discussed.

The determination of the activity of the most important CYP450 isoforms CYP1A2, 2C9, 2C19, 2D6, 2E1, and 3A4 is done through the incubation of hepatic microsomes with specific probe substrates, phenacetin, tolbutamide, S-mephenytoin, dextromethorphan, chlorzoxazone, and midazolam, respectively. The formed metabolites need to be quantified in order to calculate the enzyme activity. Due to insufficient sensitivity of a previously developed high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) method for some of the metabolites, **a fast and easy derivatization method with pyridine-3-sulfonyl chloride (PS)** is described in **Chapter 1**. Acetaminophen (CYP1A2), dextrorphan (CYP2D6), hydroxy-chlorzoxazone (CYP2E1) and hydroxy-mephenytoin (CYP2C19) can be derivatized because of the presence of a phenolic OH, whereas hydroxy-midazolam (CYP3A4) and hydroxy-tolbutamide (CYP2C9) remain unchanged. As PS improves the ionization efficiency in the positive electrospray ionization (ESI) mode, the sensitivity of the detection is improved

significantly and meets the requirements for the activity determination. Native negative electrospray type molecules, moreover, become positive ESI candidates. The direct derivatization in the aqueous incubation medium using 20 s microwave irradiation, without any other sample pre-treatment steps, such as evaporation or extraction, makes this procedure easy to perform. Collision induced fragmentation of the derivatives resulted in at least one native compound, rather than derivative, specific product ion, thereby improving the selectivity of the method in the multiple reaction monitoring mode. The **HPLC-MS/MS method was validated**, and was demonstrated to be sensitive, selective, precise and accurate. The absence of a relative matrix effect was established, notwithstanding that an absolute matrix effect was observed.

Despite the improved sensitivity and the necessity of only 1 generic method in the ESI + mode after derivatization of the metabolites with PS, the analytical run time was 14 minutes. In order to increase the throughput of the method, **an ultra high performance LC-MS/MS (UPLC-MS/MS) method was developed** (**Chapter 2**). The metabolites, together with the internal standard chlorpropamide, were separated on a UPLC BEH C18 column with pre-column. A short gradient elution (total run time of 5.25 minutes), using water with 0.1% formic acid (eluent A) and acetonitrile with 0.1% formic acid (eluent B) at a flow rate of 400 µl/min, was used. The metabolites were detected with a triple quadrupole mass spectrometer in the multiple reaction monitoring mode. Two runs, one in the positive ionization mode and one in the negative mode, were necessary for the detection of all metabolites. The necessity of two ionization modes did not derogate the high throughput of the method, as the incubations were performed for each probe individually, rather than in a cocktail, in order to avoid interactions between probes. As such, the ESI mode needed for the metabolite of interest can be selected for each incubation individually. The method was selective and showed good accuracy and between-day and within-day precision. The LOQ was lower than the method described in chapter 1 and was in full accordance with the intended application. Also, no relative matrix effects were observed, and the sample incubation extracts were stable after three freeze-thaw cycles.

The determination of the abundance of the different isoforms in paediatric microsomes may provide valuable information on the mechanisms of possible changes in CYP activity in liver disease. Until now, western blotting was mostly used for abundance measurements, but this technique only provides semi-quantitative data. Therefore, this study aimed to **develop and validate an indirect ELISA for the quantification of the most important CYP isoform, CYP3A4**, in human liver microsomes, using commercially available reagents (**Chapter 3**). Samples, calibrators and validation samples were diluted to a final concentration of 10 µg microsomal protein/ml. A

polyclonal antibody raised against the full length human protein was used as primary antibody, horseradish peroxidase conjugated secondary antibodies were used for detection. The assay was validated for sensitivity, working range and calibration, accuracy and precision. Amounts of CYP3A4 between 2 and 300 pmol/mg microsomal protein could be quantified with a 5-parameter logistics function with 1/x weighting factor. Coefficients of variation of intra and inter assay variability, as well as the relative error and the total error, were acceptable. The cross-reactivity of the method with human CYP2E1 showed to have no significant effect on the accuracy of the results.

A similar approach was used to **develop an indirect ELISA for CYP2E1, an important isoform** in many endogenous processes and in the metabolism of organic solvents (**Chapter 4**). In accordance to the CYP3A4 assay, a 5-parameter logistics function with 1/x weighting was used for quantification. The concentration range of this assay was 4-256 pmol CYP2E1/mg microsomal protein. The method showed acceptable intra- and inter-assay precision, relative error and total error. No significant cross reactivity with other abundant CYP isoforms was observed.

Chapter 5 describes the **workflow for genotyping of the patient samples**. The polymorphic nature of many of the CYP isoforms leads to large interindividual differences in drug clearance and respons. Single nucleotide polymorphisms (SNPs) are the most common genetic mutations and may lead to altered activity, either abolished or reduced, or increased. CYP2C9, 2C19, and 2D6 belong to the Class II CYP isoforms, which are highly polymorphic. Based on the frequency in the Caucasian population, and on the significant effect on the enzyme activity, the following SNPs were studied: CYP2C9*2, 2C9*3, 2C19*2, 2C19*17, 2D6*4, 2D6*10, 2D6*41. After the extraction of the DNA from liver tissue samples with a Qiagen QIAamp DNA Mini kit, it was quantified using the Picogreen® assay. TaqMan® genotyping assays were performed in order to detect the presence of the SNPs, and the patients were consequently characterized as homozygous wild type, heterozygous or homozygous SNP. The analysis of the patient samples was shown to be reliable, as the observed minor allele frequencies were similar to previously reported frequencies based on the analysis of large populations.

A study was set up to collect samples from the explanted livers from children undergoing liver transplantation for various indications, such as biliary atresia, α1–antitrypsin deficiency, and cystic fibrosis, amongst others. **Chapter 6** describes the analysis of the patient samples using the previously described developed methods, in order to obtain information on several CYP characteristics. A hypervariable activity was observed for all the isoforms. Compared to an average adult activity, low activities were seen for CYP1A2, 2C19, 2E1 and 3A4. For CYP3A4, and to a lesser extent for CYP2E1, a positive correlation between activity and abundance was observed. In this population, age, co-medication,

and genotype (unless a null-allele was present) could not be used as predictors for the CYP activity. In contrast, the Paediatric End-stage Liver Disease (PELD) score was negatively correlated with the ln(activity). This suggests **a decrease in CYP activity with deteriorating hepatic function. Moreover, the activity of all isoforms was correlated, demonstrating a concomitant decrease of all isoforms in young patients with liver disease**. To our knowledge, this is the first study to evaluate CYP activity in children with hepatic impairment. The presented data may provide support in the further optimization of a disease-specific model in this patient population.

In **Chapter 7**, the determination of the microsomal protein per gram of liver (MPPGL) in 4 biliary atresia patients undergoing liver transplantation is described. The MPPGL is an important scaling factor in the *in vitro-in vivo* extrapolation (IVIVE) of metabolic data obtained in liver microsomes. It is known to be affected by age and possibly by liver disease. An important methodological consideration is discussed, as the presence of bilirubin in the homogenates and microsomes makes it impossible to use the dithionite difference spectrum. Therefore, the NADPH-cytochrome reductase activity was used to determine the recovery factor. A mean value of 18.73 (± 2.82) mg/g (geometric mean ± SD, n=4, 0.6-1.6 years old) was observed, which is lower than the expected MPPGL based on the age of the patients (26.60 ± 0.40 mg/g). This suggests **a decreased amount of microsomal protein in the livers of biliary atresia patients**. Moreover, no differences in MPPGL between different zones of the liver could be detected.

The population-specific information on the CYP characteristics was subsequently implemented in a paediatric PBPK model (SimCYP®) for the prediction of the drug disposition of the model compounds omeprazole and midazolam (**Chapter 8**). The patients were divided in 4 categories based on the PELD score (A: 0-10, B: 11-20, C: 21-30, D: 31-40), as previously a decrease in cytochrome P450 activity with deteriorating liver disease, as expressed by an increasing PELD score, was observed. The activity results within each category were transformed into abundance data, by introducing 5 assumptions. The predictions of the drug disposition showed a large increase in the AUC of omeprazole in patients with a high PELD score (C or D). Moreover, the results within each category were highly variable. The same trend was observed for the plasma concentration of midazolam, where increases up to 5 fold compared to healthy children were predicted in patients with a PELD score of 10 or higher. We acknowledge however that **the performed adjustments to the available paediatric model in order to create a diseased population are not sufficient to capture the complex combination of physiological changes in liver disease**. Nevertheless, the sole influence of changes in CYP abundance on the drug disposition already provides valuable information to start filling the knowledge gap.

SAMENVATTING

Er is weinig bekend over de invloed van leverlijden op de farmacokinetiek (PK) van geneesmiddelen in pediatrische patiënten. Het opzetten en uitvoeren van klinische studies in kinderen brengt verschillende praktische en ethische hindernissen met zich mee. Daardoor groeit de interesse in modellerings en simulatie (M&S) technieken, zoals fysiologisch-gebaseerde farmacokinetische (physiologically-based pharmacokinetic; PBPK) modellen. PBPK modellen die specifiek voor een bepaalde pathologie gebruikt worden, vereisen adequaat verkregen *in vitro* informatie omtrent de fysiologische processen die beïnvloed worden door de betrokken ziekte. Het doel van deze thesis was het karakteriseren van de cytochroom P450 (CYP450) activiteit in kinderen met leverlijden om het PBPK model specifiek voor deze populatie (verder) te optimaliseren. Het project kan opgedeeld worden in 2 delen: (1) een groot deel bestaat uit de ontwikkeling van diverse methodes voor de karakterisatie van de CYP450 enzymen in humane levermicrosomen afkomstig van pediatrische leverpatiënten, gevolgd door (2) het toepassen van deze methodes op de patiëntstalen. De bekomen resultaten werden vervolgens gebruikt in een pilootstudie, waarin de farmacokinetiek in de doelpopulatie werd voorspeld met behulp van het PBPK model.

De **introductie** beschrijft het probleem van off-label gebruik van geneesmiddelen in kinderen. Het **frequent gebruik van geneesmiddelen aan kinderen zonder uitgebreide kennis over correcte dosering** was de drijfveer van deze studie. Een belangrijk aspect van dit probleem vindt zijn oorsprong in de verandering van de geneesmiddeldispositie met de leeftijd door de ontwikkeling van de onderliggende fysiologische processen. Het is bovendien gekend dat leverlijden ook de PK in volwassenen beïnvloedt en bijgevolg zijn er aanbevelingen voor doseringsaanpassingen beschikbaar voor gebruik in deze patiënten. De combinatie van de ontwikkelingsgerelateerde veranderingen en de andere etiologie van leveraandoeningen in kinderen, zorgt ervoor dat deze doseringsaanpassingen niet zomaar geëxtrapoleerd kunnen worden naar kinderen. Verder wordt in de introductie de structuur van PBPK modellen besproken, alsook hun applicaties.

De activiteit van de belangrijkste CYP450 isovormen CYP1A2, 2C9, 2C19, 2D6, 2E1 en 3A4 kan worden bepaald door incubaties van levermicrosomen met specifieke probes (respectievelijk fenacetine, tolbutamide, S-mefenytoïne, dextromethorfan, chlorzoxazone, en midazolam). Om de enzymactiviteit te berekenen moeten de gevormde metabolieten gekwantificeerd worden. Aangezien een vorige hogedruk vloeistof chromatografie-tandem massa spectrometrische (HPLC-MS/MS) methode onvoldoende gevoelig was voor de kwantificatie van sommige metabolieten, werd een **snelle en makkelijke derivatizatiemethode met pyridine-3-sulfonyl chloride (PS)** ontwikkeld (**Hoofdstuk 1**). De aanwezigheid van een fenolische hydroxylfunctie zorgde

ervoor dat de metabolieten acetaminofen (CYP1A2), dextrorfan (CYP2D6), hydroxy-chlorzoxazone (CYP2E1) en hydroxy-mefenytoïne (CYP2C19) gederivatiseerd worden; hydroxy-midazolam (CYP3A4) en hydroxy-tolbutamide (CYP2C9) bleven onveranderd. De sensitiviteit van de elektrospray ionizatie (ESI) detectie werd significant verhoogd door de aanwezigheid van de PS-groep en voldeed aan de eisen voor de activiteitsbepalingen. Bovendien werd het mogelijk moleculen met intrinsieke negatieve ESI eigenschappen toch in de positieve ESI modus te detecteren. De directe derivatizatie in waterig milieu werd uitgevoerd door 20 s microgolf bestraling, zonder voorafgaande staalvoorbereiding zoals evaporatie of extractie. Bijgevolg is dit een snelle en makkelijk uitvoerbare procedure. Na fragmentatie van de gederivatiseerde moleculen werd een product-ion gevormd specifiek aan de oorspronkelijke molecule, waardoor de selectiviteit van de methode in de 'multiple reaction monitoring' modus verhoogd werd. De **HPLC-MS/MS methode werd gevalideerd** en was sensitief, selectief, precies en accuraat. Er werd een absoluut matrix effect geconstateerd, maar geen relatief matrix effect.

De derivatizatiemethode leidde tot een verbeterde gevoeligheid en slechts 1 generische methode in de ESI+ modus, maar had een analysetijd van 14 minuten. Om de performantie te verhogen werd een **ultra high performance LC-MS/MS (UPLC-MS/MS) methode ontwikkeld (Hoofdstuk 2)**. De metabolieten en de interne standaard werden gescheiden op een UPLC BEH C18 kolom met pre-kolom. Een korte gradiëntelutie (totale analysetijd 5,25 minuten) werd toegepast met water met 0,1% mierenzuur (eluens A) en acetonitrile met 0,1% mierenzuur (eluens B) aan een vloeisnelheid van 400 µL/min. De metabolieten werden gedetecteerd met een triple quadrupool massaspectrometer in de 'multiple reaction monitoring' modus. Detectie van alle metabolieten vereiste 2 analysegangen: één in de positieve ESI modus, en één in de negatieve ESI modus. De noodzaak voor 2 analysegangen bracht de performantie van de methode echter niet in het gedrang, aangezien de incubaties steeds werden uitgevoerd met slechts 1 probe en niet in een cocktail, en dit om interacties tussen de probes te vermijden. Bijgevolg kon telkens voor de gewenste metaboliet de aangepaste ESI modus geselecteerd worden. De methode was selectief en had een goede accuraatheid en precisie. De kwantificatielimiet lag lager dan bij de derivatizatiemethode uit hoofdstuk 1 en voldeed aan de noden voor de applicatie. Er werd eveneens geen relatief matrixeffect geconstateerd, en de stalen waren stabiel na 3 vries-dooi cycli.

De abundantie van de verschillende CYP isovormen in pediatrische microsomen kan waardevolle informatie aanleveren m.b.t. de mechanismen van de mogelijke veranderingen in CYP activiteit ten gevolge van leverlijden. Western blotting werd tot op heden het vaakst gebruikt voor de bepaling van de abundantie, maar deze techniek levert enkel semi-kwantitatieve resultaten op. Daarom werd met behulp van commercieel

beschikbare reagentia een **indirecte ELISA ontwikkeld en gevalideerd voor de kwantificatie van de belangrijkste isovorm CYP3A4** in humane levermicrosomen (**Hoofdstuk 3**). Stalen, kalibratoren en validatiestalen werden verdund tot een finale concentratie van 10 µg microsomaal proteïne/mL. Een polyclonaal antilichaam gericht tegen het volledige humaan proteïne werd gebruikt als primair antilichaam. Het secundaire detectieantilichaam was geconjugeerd met mierikswortelperoxidase voor detectie. De methode werd gevalideerd voor sensitiviteit, bereik en kalibratiecurve, accuraatheid en precisie. Concentraties CYP3A4 tussen 2 en 300 pmol/mg microsomaal proteïne konden gekwantificeerd worden met een 5-parameter logistische functie met een 1/x weegfactor. De variatiecoëfficiënten van de intra- en inter-assay variabiliteit waren aanvaardbaar goed, net zoals de relatieve fout (accuraatheid) en de totale fout. Er was geen significante kruisreactiviteit met CYP2E1.

Een gelijkaardige benadering werd gebruikt voor de ontwikkeling van **een indirecte ELISA voor de kwantificatie van CYP2E1**, een isovorm met een belangrijke rol in verschillende endogene processen en in de metabolisatie van organische solventen (**Hoofdstuk 4**). Net zoals in de CYP3A4 methode werd een 5-parameter logistische functie met 1/x weegfactor gebruikt voor de kwantificatie. De methode had een goede intra- en inter-assay precisie, relatieve fout en totale fout. De methode vertoonde geen kruisreactiviteit met andere abundante CYP isovormen.

In **hoofdstuk 5** wordt de **workflow voor het genotyperen van de patiënten** besproken. Er ontstaat een grote interindividuele variabiliteit in geneesmiddelklaring en -respons door de polymorfismen van sommige CYP isovormen. Single nucleotide polymorphismen (SNPs) zijn de meest voorkomende genetische mutaties en kunnen leiden tot veranderde activiteit, zowel verlaagd, onbestaande, als verhoogd. Doordat ze belangrijke polymorfismen bevatten, zijn CYP2C9, 2C19 en 2D6 zgn. Klasse II CYPs. Op basis van voorkomen in de Kaukasische populatie en significant effect op de enzymactiviteit werden volgende SNPs bestudeerd: CYP2C9*2, 2C9*3, 2C19*2, 2C19*17, 2D6*4, 2D6*10, 2D6*41. Na extractie van DNA met behulp van een Qiagen QIAamp DNA Mini kit uit de leverstalen, werd het DNA gekwantificeerd met de Picogreen® assay. TaqMan® genotyping assays werden gebruikt om de aanwezigheid van de SNPs te detecteren. Vervolgens werden de patiënten gekarakteriseerd als homozygoot wild type, heterozygoot of homozygoot voor de SNP. De analyse van de patiëntstalen bleek betrouwbaar, aangezien de gevonden allelefrequenties gelijkaardig waren aan deze bekomen na analyse van grotere populaties.

Er werd een studie opgezet om leverstalen te collecteren van de geëxplanteerde levers van kinderen die een levertransplantatie ondergaan voor verschillende indicaties, zoals o.a. biliaire atresie, α1–antitrypsine deficiëntie of mucoviscidose. In **hoofdstuk 6** wordt

de analyse van de patiëntstalen om informatie over de CYP eigenschappen te bekomen met behulp van de hierboven beschreven methodes besproken. Voor alle isovormen werd een 'hypervariabele' activiteit geobserveerd. Vergeleken met de activiteit in volwassenen, vertoonden vooral CYP1A2, 2C19, 2E1 en 3A4 een lage activiteit. Voor CYP3A4, en in mindere mate voor CYP2E1, werd een positieve correlatie tussen de activiteit en de abundantie gezien. Leeftijd, co-medicatie of genotype (tenzij er een nul-allele aanwezig is) waren geen goede predictoren van de CYP activiteit in deze populatie. De Paediatric End-stage Liver Disease (PELD) score was wel negatief gecorreleerd met de ln(activiteit), wat suggereert dat de **CYP activiteit daalt wanneer de leverfunctie slechter wordt. Bovendien was de activiteit van alle isovormen gecorreleerd, wat wijst op een gelijktijdige daling in de activiteit van alle isovormen in jonge patiënten met leverlijden**. Dit is de eerste studie die de CYP activiteit evalueert in kinderen met leverlijden. De bekomen resultaten kunnen ondersteuning bieden in de verdere optimalisatie van een pathologie-specifiek model in deze patiënten populatie.

De bepaling van de MPPGL (microsomaal proteïne per gram lever) in 4 patiënten met biliaire atresie wordt beschreven in **hoofdstuk 7**. De MPPGL is een belangrijke schalingsfactor in de *in vitro-in vivo* extrapolatie (IVIVE) van metabole gegevens verkregen in microsomen. Het is gekend dat de MPPGL afhankelijk is van leeftijd en mogelijk van leverziekte. Enkele belangrijke methodologische overwegingen worden besproken, zoals de aanwezigheid van bilirubine die de analyse met het 'dithioniet-difference spectrum' onmogelijk maakt. Bijgevolg wordt de recoveryfactor bepaald d.m.v. de NADPH-cytochroom reductase activiteit. Een gemiddelde waarde van 18,73 (±2,82) mg/g (geometrisch gemiddelde ± SD, n=4, 0,6-1,6 jaar oud) werd bepaald in de patiënten, een resultaat lager dan de verwachte MPPGL op basis van hun leeftijd (26,60 ± 0,40 mg/g). Dit suggereert een gedaalde hoeveelheid microsomaal proteïnen in de lever van patiënten met biliaire atresie. Bovendien werden er geen zonale verschillen in MPPGL gedetecteerd.

De verkregen populatiespecifieke informatie over de CYP eigenschappen werd vervolgens geïmplementeerd in een pediatrisch PBPK model (SimCYP®). De PK van de modelcomponenten omeprazole en midazolam werd voorspeld (**hoofdstuk 8**). De patiënten werden verdeeld in 4 categorieën op basis van de PELD score (A: 0-10, B: 11-20, C: 21-30, D: 31-40), aangezien aangetoond werd dat de CYP activiteit gecorreleerd is met de PELD score. De activiteitsdata werden getransformeerd in informatie m.b.t. de abundantie aan de hand van verschillende assumpties. De voorspelde PK toonde een grote stijging in de plasma AUC van omeprazole in de patiënten met een hoge PELD score (C of D). Bovendien waren de resultaten binnen elke categorie ook zeer variabel. Dezelfde trend werd voorspeld voor de plasmaconcentraties van midazolam, waarvoor

stijgingen tot 5-voud werden voorspeld t.o.v. gezonde kinderen in patiënten met een PELD score van 10 of hoger. We benadrukken echter wel dat de bovenstaande **aanpassingen aan het pediatrische PBPK model om deze pathologie specifieke populatie te creëren, onvoldoende zijn om de complexe combinatie van fysiologische veranderingen in de lever volledig te omvatten**. Desondanks bezorgt de invloed van enkel de verandering in CYP abundantie op de farmacokinetiek in kinderen met leverlijden ons reeds waardevolle informatie om het opvullen van de huidige kenniskloof aan te vatten.

CURRICULUM VITAE - LIES DE BOCK

PERSONALIA

Name:	Lies De Bock	Date of Birth:	14/11/1985
Address:	Georges Dumontstraat 29 9600 Ronse	Place of Birth: Nationality:	Ronse Belgian

PHD PROJECT

Pharmacokinetics in children with liver disease: can population-specific ex vivo obtained CYP450 characteristics bridge the knowledge gap?

Department of Bioanalysis, Faculty of Pharmaceutical Sciences, Ghent University
Prof. Dr. Jan Van Bocxlaer and Prof. Dr. Koen Boussery
Start September 2008 - Expected graduation: June 2014

This study aimed to characterise the cytochrome P450 enzymes in children with liver disease. After the development of the necessary analytical methods, the evaluation of the information on the activity, abundance, and genotype of the 6 most important isoforms showed that the CYP activity decreases with deteriorating liver function. The obtained in vitro results are valuable for the optimization of a disease-specific PBPK model.

Educational activities

- Guidance of Master thesis students (MSc in Pharmaceutical Care and MSc in Drug Development):

    - De invloed van leverlijden op de metaboliserende capaciteit van de lever bij kinderen: bereiding, karakterisatie en kwalitatieve controle van microsomen (The influence of liver disease on the metabolic capacity of the liver in children: preparation, characterization and qualitative control of microsomes). Els Dumoulin, 1st Master Pharmaceutical care (2008-2009).

    - Optimalisatie en validatie van een LC-MS methode voor gederivatiseerde metabolieten m.b.t. de in vitro activiteitsbepaling van CYP450 bij kinderen met ernstig leverlijden (Optimization and validation of an LC-MS method for derivatized metabolites in the in vitro activity determination of CYP450 in children with liver disease). Sylvie Mulliez, 1st Master in Drug Development (2009-2010).

    - Optimalisatie en validatie van een enzyme-linked immunosorbent assay methode voor de kwantificatie van CYP2E1 in microsomen (Optimization and validation of an enzyme-linked immunosorbent, assay for the quantification of CYP2E1 in microsomes). Michiel Beckers, 1st Master Pharmaceutical care (2010-2011).

    - Invloed van T-2 toxine, al dan niet in combinatie met een mycotoxine binder, in varkensvoeder op de activiteit van CYP3A bij het varken (The influence of T-2 toxin in pig feeds, with or without the addition of a mycotoxin binder, on the activity of CYP3A in pigs). Tine De Vleeschouwer, 1st Master Pharmaceutical care (2011-2012).

- Organization and guidance of the practical exercises of the course "Medical Biochemistry" (3rd BSc in Pharmaceutical Sciences)

- Guidance of student groups in the Pharmaceutical Bachelor projects of the BSc in Pharmaceutical Sciences

Extra training

- *Workshop on Pharmacokinetic and Pharmacodynamic Modelling using The Population Approach*. By Prof. Dr. Nick Holford (University of Auckland). July, 6-7, 2009. Woluwe, Belgium.

- *Pharmacokinetics*. Advanced Course organized by the Leiden/Amsterdam Centre for Drug Research (LACDR). September 27-29, 2010. Oegstgeest, the Netherlands.

- *Clinical studies: study design, implementation and reporting*. August 29-30, 2012. Ghent, Belgium.

- *Project Management.* May 6-8, 2014. Ghent, Belgium.

Memberships

- Belgian Society for Mass Spectrometry (BSMS): 2009–2014
- International Society for the Study of Xenobiotics (ISSX): 2012–2013
- American Society for Pharmacology and Experimental Therapeutics (ASPET): 2012-2013

EDUCATION

Master in Pharmaceutical Sciences

Ghent University
Graduated June 2008 with distinction

Master Thesis: *Mimicking the lipid composition, organization and the properties of the unique skin-surface biofilm vernix caseosa*.
Leiden/Amsterdam Centre for Drug Research (LACDR), Department of Drug Delivery Technology, Prof. Dr. Joke Bouwstra (**Erasmus Exchange Program**, 2006-2007)

Student delegate (3rd BSc, 1st and 2nd MSc), graduation speech on the solemn proclamation of the MSc in Pharmaceutical Sciences, 2008.

Specific Teachers training

CVO De Vlaamse Ardennen
Graduated June 2012 with great distinction

Latin-Mathematics (8h)

Koninklijk Atheneum Ronse
Graduated June 2003

PUBLICATIONS

Papers published in peer reviewed journals

L. De Bock, S. Vande Casteele, S. Mulliez, K. Boussery, J. Van Bocxlaer. *In vitro cytochrome P450 activity: Development and validation of a sensitive high performance liquid chromatography-tandem mass spectrometry method for the quantification of six probe metabolites after derivatization with pyridine-3-sulfonyl chloride*. Journal of Chromatography A, 1218 (**2011**) 793-801.

L. De Bock, K. Boussery, P. Colin, J. De Smet, H. T'jollyn, J. Van Bocxlaer. *Development and validation of a fast and sensitive UPLC-MS/MS method for the simultaneous quantification of six probe metabolites for the in vitro determination of cytochrome P450 activity*. Talanta, 89 (**2012**) 209-216.

L. De Bock, P. Colin, K. Boussery, J. Van Bocxlaer. *Development and validation of an enzyme-linked immunosorbent assay for the quantification of cytochrome 3A4 in human liver microsomes*. Talanta, 99 (**2012**) 357-362.

P. Colin, **L. De Bock**, H. T'jollyn, K. Boussery, J. Van Bocxlaer. *Development and qualification of a fast and uniform approach to quantify β-lactam antibiotics in human plasma by solid phase extraction-liquid chromatography-electrospray-tandem mass spectrometry*. Talanta, 103 (**2013**) 285-293.

L. De Bock, K. Boussery, M. Van Winckel, P. De Paepe, X. Rogiers, X. Stephenne, E. Sokal, J. Van Bocxlaer. *In vitro cytochrome P450 activity decreases in children with high Paediatric End-Stage Liver Disease scores*. Drug Metabolism and Disposition, 41 (**2013**) 390-397.

L. De Bock and A. Osselaere, V. Eeckhoudt, P. De Backer, J. Van Bocxlaer, K. Boussery, S. Croubels. *Hepatic and intestinal CYP3A expression and activity in broilers*. Journal of Veterinary Pharmacology and Therapeutics, 36 (**2013**) 588-593.

A. Osselaere, S.J. Li, **L. De Bock**, M. Devreese, J. Goossens,V. Vandenbroucke, J. Van Bocxlaer, K. Boussery, F. Pasmans, A. Martel, P. De Backer, S. Croubels. Toxic *Effects of Dietary Exposure To T-2 Toxin On Intestinal And Hepatic Biotransformation Enzymes and Drug Transporter Systems In Broilers*. Food and Chemical Toxicology, 55 (**2013**) 150-155.

L. De Bock and J. Goossens, A. Osselaere, E. Verbrugghe, M. Devreese, K. Boussery, J. Van Bocxlaer, P. De Backer, S. Croubels. *The mycotoxin T-2 inhibits hepatic cytochrome P4503A activity in pigs*. Food and Chemical Toxicology, 57 (**2013**) 54-56.

K. Cattoor, M. Dresel, **L. De Bock**, K. Boussery, J. Van Bocxlaer, Remon J-P, D. De Keukeleire, D. Deforce, T. Hofmann, A. Heyerick. *Metabolism of Hop-Derived Bitter Acids*. Journal of Agricultural and Food Chemistry, 61 (**2013**) 7916–7924.

L. De Bock, P. Colin, K. Boussery, J. Van Bocxlaer. *Quantification of cytochrome 2E1 in human liver microsomes using a validated indirect ELISA*. Journal of Pharmaceutical and Biomedical Analysis, 88 (**2014**) 536-541.

L. De Bock, K. Boussery, R. De Bruyne, M. Van Winckel, X. Stephenne, E. Sokal, J. Van Bocxlaer. *Microsomal protein per gram of liver (MPPGL) in paediatric biliary atresia patients*. Journal of Biopharmaceutics and Drug Disposition, (**2014**) DOI: 10.1002/bdd.1895.

CONFERENCE PARTICIPATIONS

Oral presentations

Annual BSMS Meeting on Mass spectrometry. April 29, **2011**. Le Grand Hornu, Belgium.

In vitro cytochrome P450 activity: Development and validation of a sensitive high performance liquid chromatography-tandem mass spectrometry method for the quantification of six probe metabolites after derivatization with pyridine-3-sulfonyl chloride.

L. De Bock, S. Vande Casteele, S. Mulliez, K. Boussery and J. Van Bocxlaer.

Poster presentations

Annual BSMS Meeting on Mass spectrometry. April 16, **2010**. Woluwe, Belgium.

In vitro cytochrome P450 activity in children with severe hepatic dysfunction: a UPLC-MS/MS method for the simultaneous quantification of six probe metabolites.

L. De Bock, K. Boussery, M. Van Winckel, P. De Paepe, E. Sokal, X. Stephenne, J. Van Bocxlaer.

Annual Pharmacokinetics United Kingdom (PKUK) Meeting. November 9-11, **2011**. Durham, United Kingdom.

The influence of severe hepatic dysfunction on the metabolic capacity of the liver in children: Composition and characterization of a liver tissue bank.

L. De Bock, K. Boussery, M. Van Winckel, P. De Paepe, X. Stephenne, E. Sokal, J. Van Bocxlaer.

International Conference on Mass spectrometry NVMS-BSMS. March 29-30, **2012**. Kerkrade, the Netherlands.

Cytochrome P450 activity in children with severe hepatic dysfunction: application of a UPLC-MS/MS method.

L. De Bock, K. Boussery, M. Van Winckel, P. De Paepe, X. Stephenne, E. Sokal, J. Van Bocxlaer.

19th MDO and 12th European ISSX Meeting. June 17-21, **2012**. Noordwijk-aan-Zee, the Netherlands.

Hypervariable cytochrome P450 activity in children with severe hepatic dysfunction.

L. De Bock, K. Boussery, M. Van Winckel, P. De Paepe, X. Stephenne, E. Sokal, J. Van Bocxlaer.

Knowledge for growth. May 30, **2013**. Ghent, Belgium.

CYP450 activity in children decreases with high pediatric end-stage liver disease scores.

L. De Bock, K. Boussery, M. Van Winckel, P. De Paepe, X. Rogiers, X. Stephenne, E. Sokal, J. Van Bocxlaer.

Other attended conferences

International Conference on Mass spectrometry NVMS-BSMS. March 26-27, **2009**. Kerkrade, the Netherlands.

Joint Beltox-Invitrom Congress: "*Liver toxicity: from in vitro to man*". November 25, **2009**. Edegem, Belgium.

Recent progress in drug disposition science: let's think mechanisms. Mini symposium organized by the Department of Pharmaceutical Sciences of the Katholieke Universiteit Leuven. March 25, **2010**. Leuven, Belgium.

20 years of hepato-pancreato and biliary surgery at UZ Ghent. September 9-10, **2010**. Ghent, Belgium.

First Symposium of the BPCRN (Belgian Pediatric Clinical Research Network). September 9, **2011**. Brussels, Belgium.

Opening lecture of EDAN: *The role of PBPK modelling in drug development*, by Malcolm Rowland. March 25, **2012**. Leuven, Belgium.

Annual BSMS Meeting on Mass Spectrometry. May 3, **2013**. Antwerp. Belgium.

I may not have gone

where I intended to go,

but I think I have ended up

where I needed to be

- Douglas Adams