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Approaches to Studying the Development of Drug Metabolism in Children

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Altered drug disposition in the developing child occurs as a result of both biochemical and physiological changes. The clearance of many drugs is dependent on their biotransformation in the liver and small bowel and consequently is developmentally determined by a number of factors including both the activity and abundance of enzymes involved in Phase 1 & 2 drug metabolism. This paper reviews the *in vitro* and *in vivo* methods used to study the development of drug metabolism in humans with predominant reference to cytochrome P450 and UDP-glucuronyltransferase. The future possibility of *in silico* prediction of drug metabolism in children is also explored.

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Introduction

From birth onwards changes in the pharmacokinetics and pharmacodynamics of administered drugs occur as a consequence of changes in body composition and maturation of organs and enzymes. Accordingly, effective and safe drug therapy in neonates, infants, children and adolescents requires a thorough understanding of human developmental biology and the ontogeny of the processes that govern the absorption, distribution, metabolism, excretion and action of drugs[1].

A number of factors contribute to the developmental changes in metabolic drug clearance in children. These include relative liver size, liver blood flow, extent of protein binding and the maturation of drug metabolising enzymes.

Altered drug metabolism can lead to the development of adverse effects in neonates and small infants that are not generally seen in the adult population. The altered metabolism of sodium valproate in children under 3 years of age is thought to be responsible for a higher incidence of hepatotoxicity[2]. The impaired metabolism of chloramphenicol in neonates can result in the grey baby syndrome (cyanosis and respiratory failure)[3]. Metabolic acidosis following the use of propofol in the critically ill child [4,5] may be due to altered drug metabolism.

This review will focus principally on approaches and problems in determining the development of drug metabolism in humans with particular reference to Phase 1 reactions mediated by cytochromes P450 (CYP) and Phase 2 glucuronidation.

Enzymes involved in drug metabolism

Biotransformation generally converts lipophilic pharmacologically active drug molecules into polar metabolites that are then eliminated by the kidneys or other organs.

The liver is the major site of drug metabolism with intestinal drug extraction playing a secondary yet potentially important role in the pre-systemic elimination of orally administered drugs.

The process of drug metabolism is normally divided into two phases, phase l or functionalisation reactions and phase 2 conjugation reactions. Phase l reactions introduce or expose a chemically reactive group on which the phase 2 reactions can occur. The phase 2 reaction is usually the true detoxification pathway resulting in products that are generally water soluble and easily excreted. CYPs catalysing the mixed-function oxidation of xenobiotics comprise the most important group of enzymes involved in phase l metabolism. Others include the flavin-containing mono-oxygenases (FMO). Common phase 2 or conjugation reactions include glucuronidation, glycosidation, sulphation, methylation, acetylation and glutathione conjugation[6].

Cytochrome P450

The CYP isoenzymes are a superfamily of haemoproteins that are the terminal oxidases of the mixed function oxidase system found in the endoplasmic reticulum. CYPs play two important roles in the living organism. Firstly, in the biosynthesis or biodegradation of endogenous compounds (steroids, fatty acids, prostaglandins) and, secondly in the oxidative biotransformation of exogenous compounds (drugs, environmental pollutants, toxins).

Approximately 500 CYP enzymes have so far been isolated spread across different species and grouped according to their amino acid sequences[7]. An amino acid homology greater than 40% defines a family, and greater than 55% defines a sub-family. The symbol CYP is used to denote both human and rat cytochrome P-450.

CYP is followed by an Arabic numeral denoting the family (CYP2), a capital letter designating the subfamily (CYP2C) and then another Arabic numeral representing the individual gene or enzyme (CYP2C9). The important human isoforms of the enzyme include CYPs 1A1 & 2, 2B6, 2C8–10, 2C19, 2D6, 2E1 and 3A4 & 5. The CYP3A subfamily are the most abundant enzymes in human liver and small intestine and are involved in the metabolism of around 50% of drugs. The principal substrates, inhibitors and inducers of the major human forms of this enzyme are summarised in Table 1.

Glucuronidation

Glucuronidation is an important detoxification pathway in humans. Many therapeutic drugs and their metabolites are substrates for UDP-glucuronyltransferases (UGT) leading to the formation of usually inactive glucuronides (a notable exception is morphine 6 glucuronide which has analgesic activity[9]) which are then eliminated via the bile or urine[10].

Individual UGT enzymes are defined according to the family (1 or 2 in humans), subfamily (A or B) and an arabic numeral representing the individual gene product. The liver represents the major site of glucuronidation in humans where five UGT1A and five UGT2B genes are expressed and define capacity[11,12]. A number of UGT enzymes are expressed in extrahepatic sites[12,13]. The important human hepatic enzymes are indicated in Table 2.

Approaches to studying the development of drug metabolism in humans

1. Human *in vivo* approaches

Probe substrates for specific CYP enzymes

A number of *in vivo* substrate probes for specific CYPS have been used and are summarised in Table 3.

One of the main problems with many of the tests relate to difficulties in their application for use in paediatric patients. Many of the tests involve the administration of radioactive substrates or intravenous drugs followed by the collection of multiple blood samples. This makes it more difficult to gain ethical approval for their use in children. Even when this is not the case such as with the caffeine breath test and urinary 6β hydroxycortisol:cortisol ratio, there is usually a lack of validation of the tests for use in children. A summary of the consensus view as to the most appropriate *in vivo* probe for a specific CYP are shown in Table 3 with particular reference to those applicable to a paediatric population.

Caffeine breath test

The application of this test to assessing CYP1A2 activity in children has recently been reviewed[32]. Briefly, the caffeine breath test involves the oral administration of a non-radioactive stable isotope of caffeine (^{13}C on the 3-methyl group). The exhaled labelled $^{13}CO_2$ correlates with CYP1A2 activity[33]. The test is relatively non invasive and suitable for use in young children[15,20]. A number of investigators have used this test in children to study the effects of disease states and drug interactions[15–18]. Pons *et al.*[19] have demonstrated no detectable changes in expired CO_2 from basal levels in neonates and young infants, whereas changes were measurable in all infants older than

Table 1. Substrates, inducers and inhibitors of each of the major human CYP isozymes[8]

Enzyme	Substrate	Inducer	Inhibitor
CYP1A1 (Extra-hepatic)	Chlorinated benzenes	Polycyclic hydrocarbons	Propofol
CYP1A2	Caffeine Erythromycin Haloperidol Paracetamol R-Warfarin Theophylline Tricyclic antidepressants	Hydrocarbons Omeprazole Phenobarbitone, Phenytoin, Polycyclic	Quinolone antibiotics
CYP2B6	Cyclophosphamide Mianserin Testosterone	None known	Orphenadrine
CYP2C8	Carbamazepine Diazepam Tricyclic antidepressants.	Phenobarbitone Rifampicin	Cimetidine (Not selective)
CYP2C9/10	Diclofenac Phenytoin S-Warfarin Tolbutamide	Rifampicin	Sulfaphenazole
CYP2C19	Diazepam Mephenytoin Tricyclic antidepressants	Phenobarbitone, Rifampicin	Fluconazole Omeprazole Warfarin
CYP2D6	Amitriptyline β-blockers Clozepine Codeine Debrisoquine SSRIs Tricyclic antidepressants	None Known	Amiodarone Flecainide Fluoxetine Quinidine Trifluperidol
CYP2E1	Caffeine Chlorzoxazone Isoflurane Paracetamol	Benzene Ethanol Isoniazid	Dimethyl sulphoxide Disulfiram
CYP3A4	Carbamazepine Cisapride Clonazepam, Cyclosporin Diazepam Erythromycin Ethosuximide Etoposide Fentanyl Ketoconazole Lignocaine, Midazolam Nifedipine Ondansetron Rifampicin Ritonavir	Carbamazepine Glucocorticoids Omeprazole Phenobarbitone Phenytoin Rifampicin	Grapefruit juice Propofol Naringenin Triazole antifungals Troleandomycin
CYP3A5	Caffeine Cyclosporin Diltiazem Testosterone,	Dexamethasone	Troleandomycin
CYP3A7 (Fetal form)	Midazolam Testosterone		

Table 2. Endogenous and exogenous substrates of the major human UGT isozymes (de Wildt *et al.*[14] with modifications)

UGT isoform	Endogenous substrate	Exogenous substrate	Comment
1A1*	Bilirubin	Ethinyloestradiol	↓ in Gilberts syndrome
1A3	Estrone	Norbuprenorphine	
1A4	Androstanediol	Amitriptyline Imipramine	
1A6*		1-Napthol 2-Napthol Naftazone Naproxen Paracetamol	
1A9	Estrone Phenol Propofol	Paracetamol	
1A10		Mycophenolic acid	
2B			Delayed onset associated with grey baby syndrome
2B4	Hyodeoxyicholic		
2B7	Androsterone Epitestosterone	Buprenorphine Codeine Ibuprofen Morphine Naproxen Propranolol Valproic acid	
2B15*	Androgen steroids 4-Hydroxy-biphenyl	Eugenol	
2B17	Androsterone Dihydrotestosterone Testosterone		

* Polymorphic expression

33 days postnatal age and 45 weeks post-conceptional age, in agreement with *in vitro* findings. Lambert *et al.*[20] have demonstrated an increased 2 hour expired CO_2 in pre-pubertal children compared to adults which is contrary to the *in vitro* findings for the development of CYP1A2 in children and may reflect changes in relative liver size[34]. There may be a number of potential problems with the caffeine breath test in the *in vivo* assessment of CYP1A2 activity. Children should be kept still during the test to avoid increased CO_2 production, which may have a dilutional effect on the test[17].

The N3- and N7-demethylation of caffeine are the two mayor pathways of caffeine metabolism in young infants and maturation of N1-demethylation occurs after 19 months[35]. In the fetus the N3 and N7- reactions appear to be mediated by CYP3A rather than CYP1A2[36] with a switch over to CYP1A2 in the neonatal-infant stage. Full caffeine 3-demethylation is not observed until between 4 to 6 months of age[37] Thus, the changes in the pattern of metabolite formation and enzyme involvement in caffeine metabolism with age have to be considered when using this test for the determination of *in vivo* CYP1A2 development in children. Parker *et al.*[18] suggest that the caffeine breath test is unsuitable for use in infants less than 6 months of age. The stable isotope is expensive.

Table 3. Recommended in vivo probe for CYPs (Tucker et al.[31] with modifications)			
CYP	**Probe substrate(s)**	**Comments**	**Reference (paediatric studies)**
1A2	Caffeine	Caffeine breath performed in children. Results only apply to a small number of drugs used in children	15–20
2B6	Bupropion	More validation required. No studies performed in children	
2C8	Unclear – Paclitaxel?	Cannot be given to healthy subjects.	
2C9	Tolbutamide	No studies performed in children using tolbutamide some evidence from placental transfer and prolonged half-life during first 2 days of postnatal life.	22
	Diclofenac		21
2C19	Omeprazole	Potential contamination from 3A4 pathway	23, 24
	Mephenytoin	Availability? No studies performed in children	
2D6	Debrisoquine	Availability and suitability for use in children?	
	Dextromethorphan	Alternative has been used in children but potential contamination from 3A4 pathway and urine pH-dependent renal excretion	25–27
2E1	Chlorzoxazone		
3A4	Midazolam (oral)	Not selective for 3A4 vs 3A5	28
	Midazolam (oral and iv)	Separates liver vs gut contributions; need for stable isotope labelling for concurrent oral and iv administration; staggered oral and iv dosing may avoid use of labelled drug	28
	Erythromycin	Erythromycin breath test marks 3A4 referentially to 3A5 in liver only. Results may be influenced by activity of P-glycoprotein transporter. Use of radioactive compound may be an issue especially in children	
	Midazolam (Oral) + Erythromycin (iv)	Suggested as a combined measure of both intestinal and hepatic CYP3A activity	
	6β hydroxycortosol : cortisol	Has been used in children. Non invasive but results influenced by renal CYP3A5	29, 30

Diclofenac

Diclofenac is metabolised by CYP2C9 to 4-hydroxydiclofenac. Diclofenac is routinely used for analgesia in children. In a pharmacokinetic study of 500 mcg/kg diclofenac administered intravenously the plasma weight-corrected clearance was higher in children aged 4–6 years (7.7 ml/min/kg) compared to adults[21] (3.3 ml/min/kg).

Omeprazole

Omeprazole is metabolised to 5-hydroxyomeprazole an index of CYP2C19 activity and to omeprazole sulphone an index of CYP3A4 activity *in vivo*[38]. Few studies have been undertaken in children. However, the clearance of intravenous omeprazole has been shown to be similar to adults in paediatric patients aged 4 months to 19 years[23]. The oral clearance of omeprazole has been shown to be increased in children age 1–6 years compared to adults[24].

Mephenytoin

Mephenytoin has been used as a probe substrate to determine CYP2C19 phenotype in humans. (S)-(R)-mephenytoin undergoes rapid hydroxylation to form (S)-4 hydroxy mephenytoin (SOHP) with the slower formation of (R)- ethylhydantoin (PEH). The ratio of SOHP to PEH is used as a measure of CYP2C19 activity[39].

The test in adults involves the administration of 100mg mephenytoin followed by the collection of urine for 8 hours post dose[40,41]. Although mephenytion has been administered to children[42] this test has so far not be applied to a paediatric population possibly because of worries over toxicity[42,43]. Otherwise the test is relatively non-invasive. Stability issues have been raised regarding the assessment of CYP2C19 activity based on the SOHP : PEH ratio[44].

Dextromethorphan

Dextromethorphan is metabolised by CYP2D6 to dextrorphan and by CYP3A to 3-methoxy morphinan[45,46]. Dextromethorphan O-demethylation is well established as a measure of CYP2D6 *in vivo* and is highly correlated with CYP2D6 expression in human liver microsomes[47]. The urinary ratio of dextromethorphan : dextrophan is most commonly used as an index of CYP2D6 activity and previous studies have shown a good correlation with other CYP2D6 probe substrates such as desipramine[48] and debrisoquine[49]. In adults the test involves the administration of 25 to 30 mg of dextromethorphan and the collection of urine samples for 8 to 12 hours post dose[41,50,51].

The dextromethorphan test is relatively non-invasive, involves a relatively innocuous substrate and has been used to determine CYP2D6 polymorphism in children. Evans *et al.*[27] administered a dose of 30 mg to 26 children aged 3 to 21 years and collected urine for 4 hours post dose to determine debrisoquine oxidation phenotype.

The test has been applied to determining dextromethorphan phenotypes in paediatric patients with autoimmune hepatitis[26]. In a recent study 300 mcg/kg dextromethorphan was administered to a group of neonates through the first year of life[25]. Urine was collected into a nappy overnight. CYP2D6 phenotype consistent with genotype was achieved by 14.5 days postnatal age. The maturation of the CYP3A pathway (N-demethylation) was found to be delayed relative to CYP2D6. The use of this substrate in children is potentially problematic especially in neonates and infants because dextrorphan formation as a measure of CYP2D6 is dependent on renal function[52] which changes with developmental age. The ratio of dextromethorphan to dextrorphan also depends on urinary pH.

Erythromycin breath test (ERBT)

The ERBT is one of the most extensively validated measures of *in vivo* CYP3A4 activity in the liver in adults and was considered by some to be the gold standard[53,54]. To date the test has not been applied to the paediatric population. The test is based on the CYP3A4 mediated N-demethylation of erythromycin producing formaldehyde which subsequently appears in the breath as CO_2 [55]. The test involves the intravenous administration of a small quantity of ^{14}C labelled erythromycin, thereafter at timed intervals the subject blows into scintillation vials containing a CO_2 binding agent. The rate of production of radiolabel is then measured and expressed as the percentage of administered radiolabel exhaled during the first hour after injection[55].

The ERBT has been shown to predict the steady state trough blood levels of cyclosporin[56] and to predict its oral clearance[57]. Lown *et al.*[54] showed the positive correlation of the ERBT with the weight adjusted clearance of midazolam (another *in vivo* probe of CYP3A) and McCrea *et al.*[58] have suggested the use of oral midazolam and the ERBT as a combined probe for hepatic and enterocytic CYP3A activity.

More recently doubt has been expressed as to whether the ERBT is a useful predictor of the clearance of some CYP3A drug substrates. In contrast to Lown *et al.*[54], Kinirons *et al.*[59] have failed to show correlation between midazolam clearance and the ERBT, likewise a lack of correlation between alfentanyl clearance and the ERBT and has been observed by Krivoruk *et al.*[60]. A possible reason for the discrepancy may be the fact that erythromycin[61] but not midazolam or alfentanyl[62] is also a substrate for P-glycoprotein. This would result in lower intrahepatic concentrations of radiolabelled erythromycin and ultimately lower $^{14}CO_2$ exhalation[63]. Another reason why the ERBT may not predict midazolam clearance is that erythromycin is a substrate for CYP3A4 but not CYP3A5[64] whereas midazolam is a substrate for both[65]. Hence the ERBT will underestimate overall CYP3A activity in the 20 to 30% of patients with significant CYP3A5 hepatic levels[66].

Other disadvantages of the ERBT include: (i) the use of an intravenous radiolabelled substrate which will preclude its use especially in the paediatric population, (ii) the test does not measure intestinal CYP3A4 activity, (iii) only the rate of demethylation during the first hour is measured, interindividual differences in protein binding or volume of distribution could significantly affect the test result. Overall the ERBT may have limited application in the prediction of the CYP3A mediated metabolism of another substrate but may be useful for exploring the time course of induction or inhibition associated with

drug interactions[67]. There has been some recent debate as to whether the CYP3A N-demethylation of erythromycin is the rate-limiting step in $^{14}CO_2$ production[68].

Midazolam

Midazolam is chiefly metabolised to 1′ and 4′-hydroxymidazolam by CYP3A4 and CYP3A5[65]. To perform the test an intravenous injection of 15 mcg/kg midazolam is administered and multiple blood samples collected up to 6 hours post dose. Drug and metabolites are measured by GC-MS or HPLC-MS.

Midazolam appears to have some advantages over the ERBT: it is metabolised by both CYP3A4 and CYP3A5 and is not a P-gp substrate. Furthermore administration of the drug intravenously and then orally may facilitate an overall assessment of intestinal and hepatic CYP3A[69]. Further studies are required on the optimal use of midazolam as an oral CYP3A probe. The main disadvantages of the test are the administration of a clinically sedating dose of midazolam and the need for multiple blood samples. Rather than formally applying this test to children, studies built around the clinical use of midazolam have been applied to assessing the development of CYP3A activity.

The weight corrected plasma clearance of iv midazolam was significantly reduced in neonates compared to adults, 110–130 ml/h/kg and 380–660 ml/h/kg respectively[70,71] and was even lower in preterm infants younger than 39 weeks gestation, 72–96 ml/h/kg[70,72]. Because midazolam is only metabolised by CYP3A7 to a small extent[65], reduced clearance may be explained by low CYP3A4 activity following birth. The surge in CYP3A4 expression after birth is not mirrored by a large increase in midazolam clearance at this stage[73,74]. Another study by Hughes *et al.*[75] showed lower weight corrected clearance in younger children up to 2 years of age (140–180 ml/h/kg) compared to children aged 3–13 years (780 ml/h/kg) who in turn had higher weight corrected clearance compared to adults (380 to 660 ml/h/kg). In a recent study both the i.v and oral clearance of midazolam were found to be increased (3,000 and 680 ml/h/kg respectively) in children age 6 months to <2 years compared to children age 2 to <12 (2,500 and 600 ml/h/kg respectively) and adolescents 12 to <16 (1500 and 560 L/h/kg respectively)[28].

Urinary 6β-hydroxycortisol

This is a simple non-invasive test that is easily performed even in a neonatal population. The ratio of 6β hydroxycortisol to free cortisol is measured in a spot urine sample.

This ratio has been proposed as a measure of hepatic mixed function oxidase activity[76] and subsequently CYP3A activity[77]. However, there is little evidence to support this. There is lack of correlation between the ERBT and 6βOHC:C ratio[78,79]. Cortisol is also a substrate for P-gp which may in addition to the influence of renal CYP3A5[80] explain the lack of correlation between the 6βOHC:C urinary ratio and midazolam clearance. At best the 6βOHC:C ratio may be useful for looking at enzyme induction[81-83]. A number of studies have applied this test to children. The 6βOHC:C ratio is higher in term compared to premature neonates[30]. In infants 1 to 12 months of age the mean 6βOHC:C ratio was lower compared with that of neonates and adults[29].

Further studies are required on the use of probe substrates in determining *in vivo* CYP3A activity in man, especially with respect to their oral administration and with regard to the influence of P-gp. The tests require further validation for use in children.

Drugs in use

Phase 1

The pharmacokinetics of a number of drugs in routine clinical use have been applied to assessing the *in vivo* development of drug metabolism in children. This method is useful where the drug in question is predominantly metabolised by a single CYP enzyme. Ethical approval for such studies is generally easier to obtain compared to administering a probe substrate especially where iv access is already available and the number of blood samples to be collected is small. A number of approaches have been used to minimise the number of blood samples needed for such studies. These include sparse data pharmacokinetics, (small number of blood samples from a larger group of patients)[84,85] and the use of steady state pharmacokinetics[75]. A disadvantage with this approach is that it is opportunistic and thus more limited in its application.

A number of drugs routinely used in paediatric clinical practice and adopted as probes for specific CYP enzymes have already been mentioned including midazolam, diclofenac and omeprazole. Other drugs used in routine clinical practice that have been used to assess age-related changes in CYP activity include: for CYP3A: cyclosporin, tacrolimus, carbamazepine (also CYP2C8), etoposide, nifedipine, cisapride and for CYP2C9: phenytoin.

Phase 2

Morphine is largely metabolised by UGT2B7 to morphine-6-glucuronide and morphine-3-glucuronide[9] and has been suggested as probe substrate for this UGT isoform[86]. A number of *in vivo* studies have already been described on the development changes in morphine clearance in children[87-90].

Paracetamol is mainly metabolised by UGT1A6 (and to a lesser extent by UGT1A9). The rate of paracetamol glucuronidation is negligible in the fetus[91], low after birth and does not reach adult values before 10 years of age. Paracetamol is not the ideal probe for assessing UGT1A6 activity in children because of the compensatory higher sulphotransferase activity of the younger ages[92].

Propofol has been proposed as an alternative probe for UGT1A9 activity[86]. However, propofol is a high extraction drug with clearance being dependent mainly on blood flow rather than enzyme activity, thus, limiting its application as a probe substrate[93].

2. Human *in vitro* approaches

Several *in vitro* liver preparations are used in studying hepatic drug metabolism. These include liver slices, intact hepatocytes, S9 fractions and microsomes.

Liver slices and hepatocytes are useful for studying sequential oxidative and conjugative biotransformation in the same system as well as xenobiotic mediated induction[94]. Many *in vitro* studies are done using human liver microsomes. Microsomes are closed vesicles of fragments of the endoplasmic reticular membrane and contain several drug metabolising enzymes including CYP, FMO and UDP-glucuronyl-transferase.

A number of investigators have used microsomes prepared from the livers of children at various developmental ages and then measured the expression and or the activity of specific CYP and other enzymes involved in drug metabolism.

The collection of liver biopsies is considered high risk and not justified for research purposes alone but may be acceptable when the primary purpose is for diagnosis or treatment[95]. In practice, paediatric liver biopsy material is not only difficult to obtain for research purposes but when available is likely to have been collected from a patient with liver disease. Thus, many of the livers used to prepare microsomes have been obtained from aborted fetuses and post mortems in the first few hours after death[96,97]. A recent study of the developmental expression and activity of intestinal CYP3A4 was performed using duodenal and jejunal biopsies harvested at the same time as those to be used for clinical and diagnostic purposes[98]. All human tissue used for *in vitro* studies should be characterised histologically for the presence of disease. Post mortem enzyme degradation may occur and tissue should be frozen at –80°C as soon as possible.

The expression of genes coding for specific CYP enzymes is often detected in fresh or frozen tissue by amplifying the gene in question using a reverse transcriptase polymerase chain reaction (RT-PCR) with appropriate primers.

Enzyme expression is usually measured by a technique such as Western blotting which requires separation of proteins by electrophoresis and the detection of the enzyme using specific antibodies.

Enzyme activity is determined by measuring the rate of metabolite appearance from a specific probe substrate. A number of model *in vitro* substrates have been defined for probing specific CYP enzyme activity (Table 4). Preferably more than one substrate should be used to investigate the ontogeny of CYP3A because of potential differences in the enzyme-substrate interaction[99,100].

To illustrate the application of *in vitro* techniques to investigate the ontogeny of specific CYP enzymes in the human liver, Sonnier and Cresteil[34] utilized liver tissue from stillborn or aborted fetuses (14–40 weeks), children aged 1 day to 9 years and from adult donors for kidney transplantation. Microsomes were prepared and CYP1A2 protein was measured by western blotting using a rat polyclonal CYP1A1 antibody. Enzyme activity was determined by co-incubating the microsomes with methoxyresorufin. CYP1A2 expression and activity was absent in microsomes prepared from fetal and neonatal livers and increased in infants aged 1–3 months to attain 50% of the adult value at one year.

Problems with the human *in vitro* approach include the availability of suitable tissue samples, ethical constraints, especially since the Redfern report[101], (The Report of The Royal Liverpool Children's Inquiry) the availability of isoform specific antibodies to assess expression by immunoblotting, size of the tissue samples available, the non-physiological nature of the preparations used and the availability of sufficiently sensitive assay systems to measure metabolite formation. The main advantage of this approach is that changes in true human enzymology with age can be ascertained.

Table 4. Recommended in vitro probe substrates[31]		
CYP	**Substrates**	
	Preferred	**Acceptable**
1A2	Ethoxyresorufin Phenacetic	Caffeine Theophylline
2A6	Coumarin	
2B6	S-Mephenytoin	Bupropion (availability of metabolite standards?)
2C8	Paclitaxel	
2C9	Diclofenac S-Warfarin	Tolbutamide (low turnover)
2C19	Omeprazole S-Mephenytoin	
2D6	Bufuralol Dextromethorphan Metoprolol	Codeine Debrisoquine
2E1	Chlorzoxazone	4-nitrophenol Lauric acid
3A4	Midazolam Testosterone	Cyclosporin Erythromycin *

*Strongly recommend using at least two structurally unrelated substrates.

Developmental factors may influence the suitability of an *in vitro* probe substrate. For instance, caffeine is metabolised by CYP3A in fetal liver[36], whereas in adults it is predominantly metabolised by CYP1A2[102].

Studies on the developmental expression of polymorphic enzymes such as CYP2D6 and CYP2C19 need to account for the numbers of poor metabolisers within each age group. This is itself potentially difficult as there is little information about the appearance of the different phenotypes during development[103]. Peng *et al.*[104] studied the *in vitro* acetylation of 7-amino-clonazepam in human fetal and adult liver preparations. The adults but not the fetuses could be classified into fast and slow acetylators.

In general, enzyme expression and activity correlate closely in adult humans[105]. However, during development this is not always the case. Strassberg *et al.*[106] have demonstrated an early phase in UGT development with the appearance of gene transcripts by 6 months followed by a later phase characterised by up regulation of UGT protein expression to levels found in adults by 2 years. Even by 2 years the activity of many UGT enzymes was significantly lower than in adults. The different stages of ontogeny are characterised by dynamic changes in gene expression. Thus, *in vitro* studies drawing conclusions based on a small number of tissue samples, representing a narrow time window must be viewed with caution[107].

Investigations utilising microsomal and other subcellular liver and intestinal fractions in the study of the development of the major enzymes involved in drug metabolism are shown in Tables 5 and 6. Two reviews are available describing the *in vitro* ontogeny of human phase 1[107] and phase 2[108] drug metabolising enzymes.

3. Animal models

The relative ease of obtaining animals, usually rats, at the relevant stage of development and then harvesting the organs of interest, make the use of animal models attractive for studying the ontogeny of drug metabolism systems.

The development of rat hepatic CYP3A has been shown to be both age and sex dependent[130-131]. Enzyme expression and activity increases up to day 25 and then continues to rise in male but falls away in female animals. The expression and inducibility of P450 enzymes during liver ontogeny has been reviewed by Rich and Boobis[132]. In contrast to rat hepatic CYP3A, small bowel CYP3A expression and activity is virtually absent until weaning (day 20) and then surges, reaching a plateau by 40 days sustained into adulthood[131].

Table 5. In vitro study of developing phase 1 enzymes in humans

Tissue and source	*In vitro* preparation	Enzyme CYP	Probe Substrate	Other methods	Relative % expression / activity			Reference
					Adult	Child (age)	Fetus	
L, AF, SS	Microsomes	1A		Western blotting	Detected (+++)		Not detected	109
AF, SS	Microsomes	1A1	Ethoxyresorufin –o-demethylase		0		100 (7–9wk)	110
L, AF, OD, B	MRNA and cDNA extract	1A1		RT-PCR	Detected (++)		Not detected	111
L, AF, SS,OD	Microsomes	1A2	Methoxyresorufin demethylation	Western blotting	100	50 (1 yr)	0	34
L, AF, OD, B	MRNA and cDNA extract	1A2		RT-PCR	Detected (+++)		Not detected	111
L, AF, PM, SS	Microsomes / RNA/section	1A2		Immunostaining, Western blotting, Northern blotting	100	53 (5yr)	Not detected	112
L, SS, B	Microsomes	2A6		Western blotting	100	<100 (1yr)		113
L, SS, B	Microsomes	2B6		Western blotting	100	10 (1yr)	–	113
L, AF, OD, B	MRNA and cDNA extract	2B6		RT-PCR	Detected (+++)		Not detected	111
L, AF, PM, SS	Microsomes / RNA/section	2C		Immunostaining, Western blotting, Northern blotting	100	47 (5d) 91 (5yr)	< 10	112
L, OD, AF, PM	Microsomes	2C 2C8 2C9 2C10 2C18	Tolbutamide	RT-PCR	100 100 100 100 100	5 (1 d) 20 (1 mo) 65 (7d) 6 (7d) 25 (1mo) 85 (1d) 120 (1yr)	1–2 50 0** 40	114, 115
L, AF, SS	Microsomes	2C		Western blotting	Detected (+++)		Detected (+)	109
L, AF, OD, B	MRNA and cDNA extract	2C8-19*		RT-PCR	Detected (++++)		Detected (++)	111

Tissue and source	*In vitro* preparation	Enzyme CYP	Probe Substrate	Other methods	Relative % expression / activity			Reference
					Adult	Child (age)	Fetus	
L, AF, OD, B	MRNA and cDNA extract.	2D6*		RT-PCR	Detected (+++)		Detected (+)	111
L, AF, OD	Microsomes	2D6*	Dextromethorphan		100	Detected RNA(++)	0	116
L, AF, OD	Microsomes	2D6*	Dextromethorphan	Western blotting, RNA analysis	100	15 (7 d) 50 (1 m)	2–5	117
L, AF, OD	Microsomes	2D6*	Dextromethorphan		100		< 5	118
L, AF, OD, B	MRNA and cDNA extract	2E1		RT-PCR	Detected (++++)		Not detected	111
L, AF, OD, SS	Microsomes	2E1	Ethanol oxidation	Western blotting, RT-PCR	100		12–27	119
L, AF, PM	Microsomes	2E1	Chlorzoxazone hydroxylation	Northern blotting	100	15 (1 mo) 80 (1 yr)	0	120
L, AF		2E1		RT-PCR	100		10–30	121
L, AF, OD, SS and olfactory tissue	Microsomes	2J2		Western blotting	100		Near 100	122
L, AF, PM	Microsomes	3A4/5*	DHEA hydroxylation, Testosterone hydroxylation	Western blotting	100	50 (6 mo) 120 (1y)	<10	96
L, AF, OD, B	MRNA and cDNA extract	3A4/5*		RT-PCR	Detected (++++)		Detected (++)	111
L, AF, PM	Microsomes MRNA and cDNA extract	3A7	DHEA	RT-PCR	<10 Detected (++)	150 (1d) 50 (8d)	100 Detected (++++)	96
Duodenum / Jejunum, B, AF, NS	Microsomes	3A4	Testosterone hydroxylation Dextromethorphan	Western blotting	100	50 (1mo) 75 (2 yr)	5	98
L, AF, PM, SS	Microsomes	3A4	Cisapride		56	100	3.5	123

* Polymorphism

** Approximately 5% detection of CYP2C9 RNA in fetus compared to adult

Tissue sources: AF, Aborted fetus; L, Liver, SS, Surgical section; OD, Organ donor; PM, Post mortem; B, Biopsy.

DHEA, Dehydroepiandrosterone.

Table 6. ***In vitro*** **studies with hepatic microsomes on the development of phase 2 glucuronidation enzymes in humans**

Source	Enzyme UGT	Probe substrate/ Other Method	Relative % expression / activity			Reference
			Adult	Child (age)	Fetus	
L, AF, OD	1A1*	Bilirubin	100	1 (1mo)	0.1	124
L, AF, SS, PM	1A1*	Bilirubin	100		<14	125
L, AF, PM	1A1*	Bilirubin/ Western blotting	100	<14 (term)	<14	126
L, AF, SS, PM	1A3		100		30	14
L, SS	1A4	Amitriptyline	100	5–10 (18mo)	<5	106
L, AF, SS, PM	1A6*	1-naphthol	100		<14	125
L, AF,OD	1A6*	2-naphthol	100		1	127
L, AF, SS, PM	1A9	Phenol	100		<14	125
L, AF, SS, PM	1A9	4-butylphenol	100		5	106
L, AF, SS, PM	2B7*	Androsterone	100		<14	125
L, AF, OD	2B7*	Morphine	100		10–20	128
L, OD,SS	2B7*	Buprenorphine	100		10	106
L, AF, SS, PM	2B17	Testosterone	100	<14		125
L, AF	2B17	Western blotting	Detected (+++)	Detected (+)		129

* Polymorphism
Tissue sources: AF, Aborted fetus; L, Liver; SS, Surgical section; OD, Organ donor; PM, Post mortem.

Although these data are of interest to the toxicologists there are a number of inherent problems in applying it to humans. There are differences in the expressed enzymes. In man the predominant CYP3A enzymes are CYP3A7 (fetal form), CYP3A4 and CYP3A5, whereas in rats they are CYP3A1, CYP3A2, CYP3A9 and CYP3A18[133]. Although they are all involved in the metabolism of certain substrates e.g. testosterone, major differences exist. For instance, mephenytoin is metabolised by CYP3A in the rat but by CYP2C19 in humans[134,135]. Many enzymes in the rodent have a sex specific ontogeny (CYP2C11, CYP2C12, CYP2C13, CYP3A2) whereas this is not the case in humans. Animal models also often ignore wide genetic polymorphisms which occur in humans such as CYP2D6 and CYP2C29[136].

Coupled with the contrasts in enzymology, there are often cross species developmental miss-matches occurring on a more physiological level. This is especially so when investigating the development of drug metabolism in the small bowel. In rodents the bowel continues to develop after birth and only becomes functional by the time of weaning[137]. In contrast, the function of human bowel is ahead of need at birth[138]. Neonates have around 50% of the small intestinal CYP3A expression and activity compared to adults[98]. This contrasts with the newborn rat where the enzyme is absent.

Allometric scaling (AS) using *in vivo* data obtained in animal species has been used to attempt to predict pharmacokinetic (PK) parameters in humans[139]. AS assumes that many physiological processes are a function of the size of the animal and, thus, PK parameters should be a function of total body weight[140]. AS is therefore applied in a retrospective manner to compare the PK of a drug across several species and predict the parameters in humans[136]. There are many problems in using AS to predict PK parameters in adult humans due to its empirical nature[141], including interspecies differences in metabolism. Applying the technique to studying the metabolism of drugs in the developing human from PK data derived from developing animals would introduce yet more confounding factors and further reduce the possibility of useful predictions.

Overall, although animal models are of some relevance to acute toxicity testing of drugs during development, they are generally unreliable at predicting the ontogeny of drug metabolism in humans.

4. *In silico* approaches

Computer simulation is being employed increasingly within the pharmaceutical industry. The prediction of absorption, distribution, metabolism and excretion parameters for a specific drug are becoming possible from its chemical structure alone[142,143]. An area of rapid development is the prediction of *in vivo* pharmacokinetics parameters from *in vitro* data. Such *in vitro-in vivo* extrapolation (IVIVE) depends on the availability of good quality *in vitro* enzyme kinetic data such as maximum enzyme velocity (Vmax), Michaelis-Menten constant (Km) for the drug substrate and inhibitor constant (Ki) and inhibitor concentration (I) for the inhibitor.

Computer simulation using SIMCYP a programme developed at the University of Sheffield and incorporating population variability has been successfully applied in the prediction of the variability of a number of drug-drug interactions including methadone-ritonavir[144], ketoconazole-midazolam[145], fluconazole-midazolam[146] and sildenafil-ritonavir[147].

One future development will be the *in silico* prediction of the likely pharmacokinetic parameters of drugs in children. The *in vitro* developmental patterns of many of the CYP enzymes in children are documented (see earlier) as are other key physiological parameters such as liver size, renal function and intestinal development. The first stage will be to simulate the changes and variability in clearance of a drug such as midazolam with age, and compare the results with those available in the literature. Additional *in vitro* data will be required to build a truly paediatric version of SIMCYP but once validated it will form a valuable tool both for the pharmaceutical industry and in the clinical setting.

Conclusions

An array of approaches exist for studying the development of drug metabolism in children each with their own advantages and disadvantages. Future studies are required not only to extend the current data on the development of specific enzyme systems with age but also to further validate methods for use in children. Drug metabolism is an important determinant of both drug dose and frequency of administration. Thus, it is important that information on the changes in drug metabolism with age is accounted for in the clinical use of medicines for children. To this end, the development of validated *in silico* approaches may facilitate the reliable prediction of pharmacokinetic parameters for drugs to be used in children. This in turn may lead to a more scientific approach to dosage recommendation and the design of clinical studies in children.

An area that is currently generating research interest is the relationship between the ontogeny of drug metabolising enzymes and the pathogenesis of adverse drug reactions (ADRs) in children. Delayed maturation of enzymes systems over the first 2–4 weeks of life may contribute to the development of type A (dose dependent) ADRs. Conversely it is postulated that the increased metabolism and thus circulating metabolites of possibly reactive metabolites in young children may predispose to certain type B (Idiosyncratic, non-dose dependent) ADRs. Future research in this area must not only focus on the ontogeny of the drug metabolising enzymes and transporter proteins, but also other systems involved in the generation of ADRs such as the development of the immune system. Only by understanding the underlying mechanisms of ADRs in children can we ultimately begin to prevent them from occurring.

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